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DIVERSITY OF SEED COAT ULTRASTRUCTURE AND SOLUBLE CARBOHYDRATE CONTENT IN *LUPINUS*

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Seed sculpture, seed coat thickness and soluble carbohydrate profiles were compared in three lupin species, each represented by three different genetic lines, from sections *Pilosus* (*L. pilosus*) and *Atlanticus* (*L. atlanticus*, *L. cosentinii*). Seed coat thickness varied most in the three lines of *L. pilosus* (330–1000 μm), while in the other species it varied little from 400 μm thickness. The studied seeds accumulated approximately 100 mg/g d.m. soluble carbohydrates. The obtained results together with published data suggest that the soluble carbohydrate composition of seeds is clearly section-specific in *Lupinus* sections *Pilosus* and *Atlanticus*. Sect. *Pilosus* was characterized by a high level of galactosyl cyclitols, while seeds of sect. *Atlanticus* contained much higher amounts of the raffinose-family oligosaccharides. In all species, however, the dominating soluble carbohydrate was stachyose. The results on the biochemical parameters analyzed in this paper confirm that *L. atlanticus* and *L. cosentinii* are more closely related to each other than to *L. pilosus*. The seed coat roughness/smoothness criterion seems inadequate for building a classification of lupin species, as one of the analyzed lines of *L. pilosus*, formally ascribed to the rough-seeded lupins, produces smooth seeds.

Key words: Lupin seeds, seed sculpture, seed coat thickness, soluble carbohydrates, galactosyl cyclitols.

INTRODUCTION

The classification of the genus *Lupinus* remains controversial, particularly with respect to the American species (Käss and Wink, 1994; Sholars, 1999). So far, approximately 300 annual and perennial lupin species have been described, along with several woody species, both trees and shrubs. Over 2000 names and synonyms have been used in the literature to denote these taxa (Gladstones, 1998). The inconsistency and incoherence of *Lupinus* systematics is due to the vast diversity within this genus and the lack of generally accepted diagnostic criteria (Ainouche and Bayer, 1999).

One of the diagnostic traits applied in Old World lupin classification is the roughness/smoothness of the seed coat. *Lupinus pilosus, L. cosentinii, L. digitatus, L. atlanticus, L. princei* and *L. somaliensis* are described as rough-seeded species, while *L. albus, L. angustifolius, L. hispanicus, L. luteus, L. micranthus* and *L. mutabilis* are considered smooth-seeded (Gladstones, 1998). Can this dichotomy be confirmed by analyses more objective and sensitive than the tactile sense, such as scanning electron microscopy?

The complexity of *Lupinus* may also be reflected in the biochemical composition of seeds. Even in comparison to other legumes, lupin seeds are characterized by high protein and rather low carbohydrate content: 35–45% d.m. and 30–40% d.m., respectively, as opposed to 26–40% d.m. and 55–60% d.m. reported for pea (Hedley, 2000; Atta et al., 2004). Starch content in lupin seeds is fairly low, ranging from 0.4% to 1.5%, depending on seed mass; in other legumes it can reach 45% (Hedley, 2000). On the other hand, lupin seeds contain high amounts (3–12%) of raffinose-family oligosaccharides (RFOs) and galactosyl cyclitols (Gal-C)

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TABLE 1. Origin of studied seeds

Species	Line number	Number of collection	Section	Main area of occurrence
Lupinus atlanticus Glads.	1	USDA – PI 384612	*Atlanticus*	Marocco (Atlas Mountains)
	2	USDA – PI 384613		
	3	USDA – PI 386413		
Lupinus cosentinii Guss.	1	USDA – PI 32530	*Atlanticus*	Mediterranean
	2	USDA – PI 32533		
	3	USDA – PI 32534		
Lupinus pilosus Murr.	1	USDA – PI 11424	*Pilosus*	Turkey
	2	USDA – PI 491183		
	3	USDA – PI 249758		

TABLE 2. Morphological characteristics of *Lupinus* seeds (means ± SD of 15 replicates)

Species	Line number	Fresh mass (mg x seed^{-1})	% mass of seed		
			testa	axis	cotyledon
L. atlanticus	1	290 ± 12	31.6	1.2	67.2
	2	272 ± 16	29.7	1.4	68.9
	3	233 ± 18	30.6	1.4	68
L. cosentinii	1	123[b] ± 10	30.5	1.6	67.9
	2	143[b] ± 11	34.6	1.5	63.9
	3	189[b] ± 12	28.7	1.4	69.9
L. pilosus	1	516[c] ± 21	27.7	2.4	69.9
	2	464[c] ± 23	26.6	2.5	70.9
	3	588[c] ± 37	36.0	2.5	61.5

[a]result significantly different from corresponding values for each of the other lines of the same species; [b]result for one of the species of *Lupinus* section *Atlanticus* significantly different from corresponding values for the other species of this section; [c]result for *Lupinus pilosus* significantly different from corresponding values for both species of section *Atlanticus.*

(0.05–2%) (Piotrowicz-Cieślak et al., 1999). The above data come from studies focussed mainly on cultivated lupin species. So far there have been very few data for wild species (Piotrowicz-Cieślak et al., 2003), and the systematics of wild lupin species is less established. Based on DNA sequence data (internal transcribed spacer of nuclear ribosomal DNA), Ainouche and Bayer (1999) found that rough-seeded species are a monophyletic, well defined group, but the phylogenetic relationships between them remain to be elucidated. These remarks make it clear that *Lupinus* systematics requires a broader factual basis. The purpose of this paper was to compare three wild *Lupinus* species: *L. atlanticus* (sect. *Atlanticus*), *L. cosentinii* (sect. *Atlanticus*) and *L. pilosus* (sect. *Pilosus*) in terms of seed coat ultrastructure (an objective trait plausibly related to the sensation of seed smoothness/roughness) and seed metabolic parameters very characteristic of lupins: content of RFOs, Gal-C and cyclitols.

MATERIALS AND METHODS

Seeds of nine lines of three wild lupin species (three lines per species) obtained from the Western Regional Plant Introduction Station, Washington State University (Pullman, Washington) were used in the experiments. The lines are identified in Table 1.

SEED MORPHOLOGY

In all seeds the following structural traits were scored: weight, color, shape, and percentage mass ratio of seed coat/seed total.

For scanning electron microscope (SEM) observations the seeds were coated with gold using a JEOL JFC 1200 ion coater and observed in a JEOL JSM–5310LV scanning electron microscope at 20 kV.

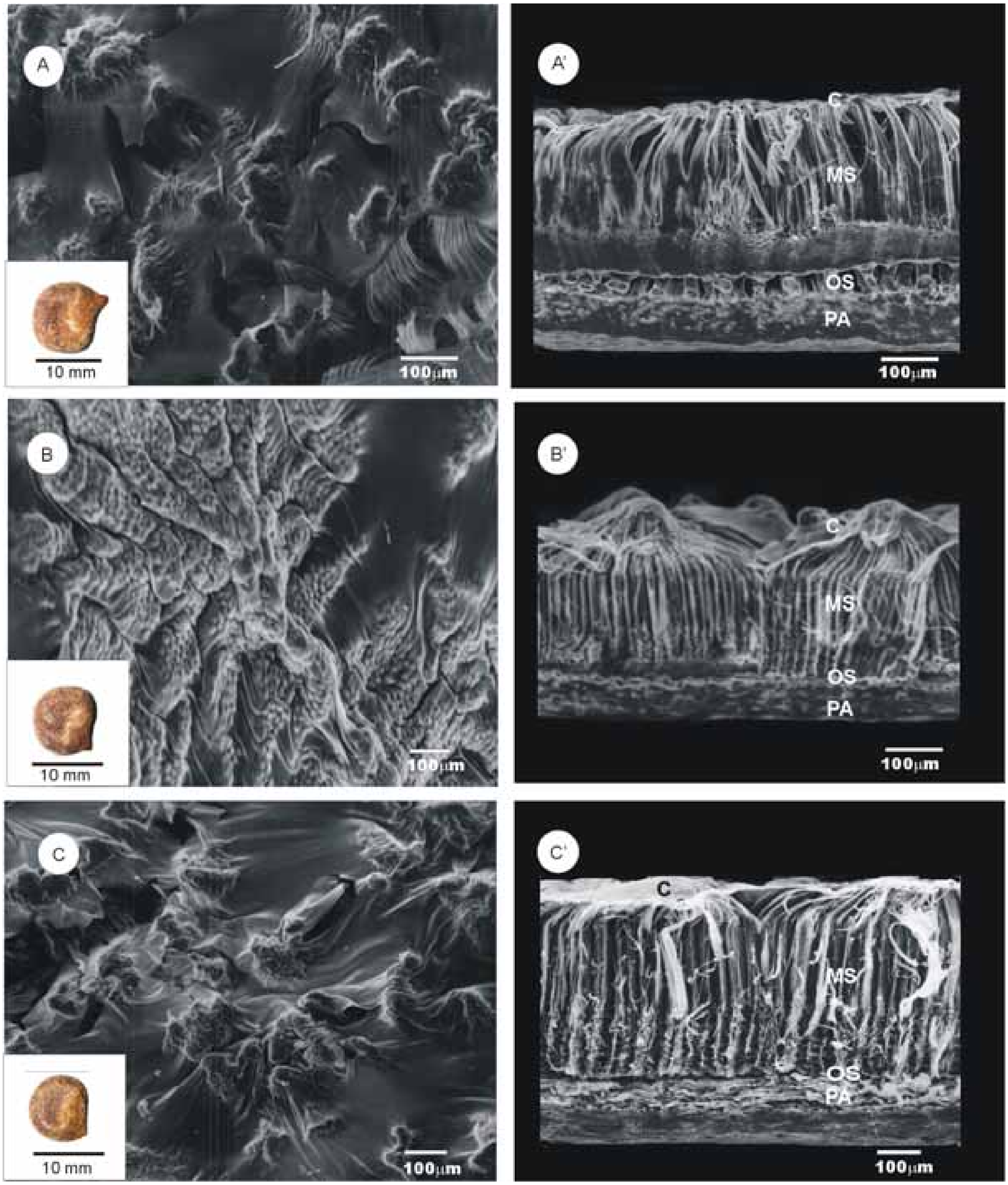

Fig. 1. *Lupinus atlanticus* seed sculpture. SEM images of seed surface (**a, b, c,** general view of seeds in inserts) and cross sections of seed coat (**a', b', c'**). C – cuticle; MS – macrosclereid layer; OS – osteosclereid layer; PA – parenchyma cells. a,a' – line 1; b,b'- line 2; c,c' – line 3.

SOLUBLE CARBOHYDRATES

Content of cyclitols, galactosyl cyclitols and soluble carbohydrates were analyzed according to Piotrowicz-Cieślak et al. (2003); 30 mg dry tissue was homogenized in ethanol:water (1:1, v/v) containing 100 μg phenyl-α-D-glucose as internal standard. Soluble carbohydrates were identified with internal standards when available, and as calculated from the peak area ratios of each known carbohydrate to the internal standard.

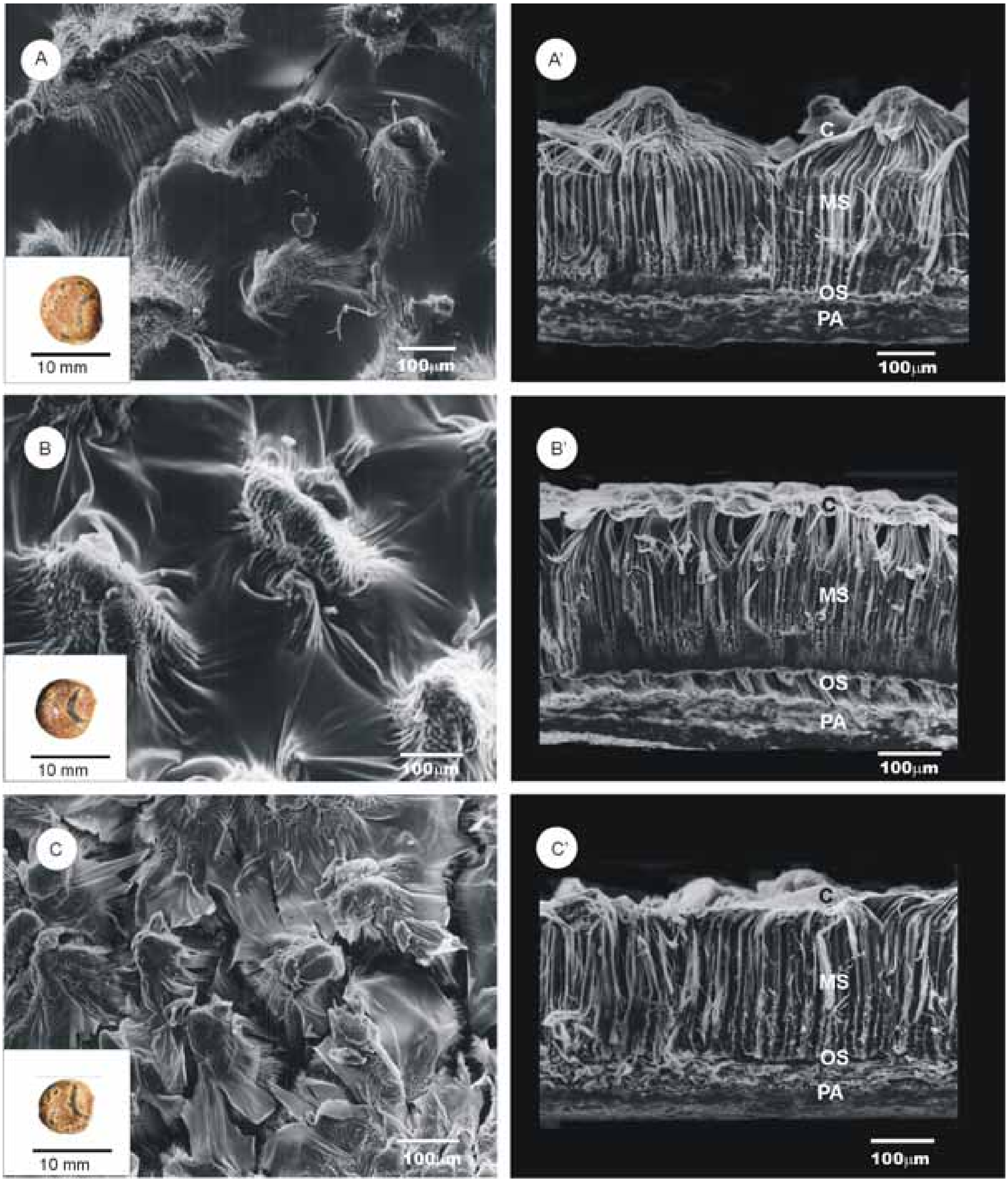

Fig. 2. *Lupinus cosentinii* seed sculpture. SEM images of seed surface (**a, b, c,** general view of seeds in inserts) and cross sections of seed coat (**a', b', c'**). C – cuticle; MS – macrosclereid layer; OS – osteosclereid layer; PA – parenchyma cells. a,a' – line 1; b,b'- line 2; c,c' – line 3.

STATISTICAL ANALYSES

Biochemical assays were done in 4 replicates, and morphological observations in 15 replicates. The results are given as means ± SD. The significance of differences was checked by the Newman-Keuls test at p = 0.05, using STATISTICA 6.0.

RESULTS

SEED MORPHOLOGY

The studied seeds of nine lupin lines differed appreciably in size, coloration, and most evidently in seed coat relief. The seeds were 9–12 mm in

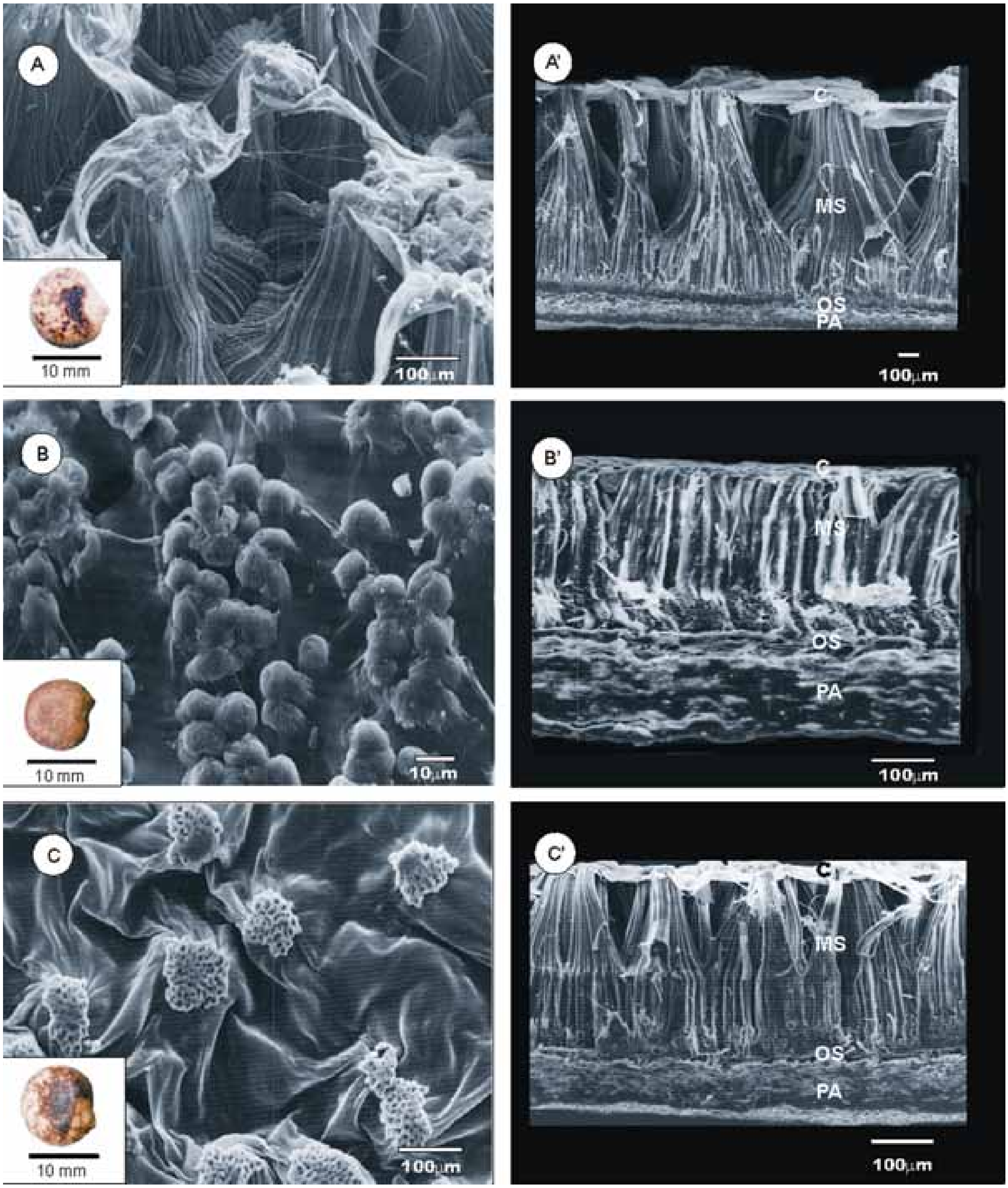

Fig. 3. *Lupinus pilosus* seed sculpture. SEM images of seed surface (**a, b, c**, general view of seeds in inserts) and cross sections of seed coat (**a', b', c'**). C – cuticle; MS – macrosclereid layer; OS – osteosclereid layer; PA – parenchyma cells; a,a' – line 1; b,b' – line 2; c,c' – line 3.

size, nearly circular, and flattened (Figs. 1, 2, 3a'–c'). The seed coat was yellow, in some cases with dark brown areas. Seed fresh mass ranged from 123 mg (±10 mg) in *L. cosentinii* line 1 to 588 mg (±37 mg) in *L. pilosus* line 3 (Tab. 2). The share of the seed coat in total seed fresh mass ranged from 26.6% to 36% (both extreme values noted in *L. pilosus*, lines 2 and 3, respectively; Tab. 2). Seed coat thickness was highest (1000 µm) in *L. pilosus* line 1 (Fig. 3), and in all the other lines of the analyzed species it averaged ~400 µm (330–530 µm). The seed coat surface was cristate-

TABLE 3. Soluble carbohydrate composition of *Lupinus atlanticus* seeds (means ± SD of 4 replicates; mg/g d.m.)

Soluble carbohydrate	line 1	line 2	line 3
	Monosaccharides and sucrose		
fructose	1.99 ± 0.02	2.07 ± 0.20	2.99 ± 0.02
glucose	0.15 ± 0.00	0.12 ± 0.02	0.26[ab] ± 0.00
galactose	0	0	0
sucrose	18.86 ± 1.99	17.97 ± 1.98	16.86 ± 1.99
	Cyclitols		
D-pinitol	0.34 ± 0.05	0.33 ± 0.04	0.34 ± 0.05
myo-inositol	0.74 ± 0.09	0.59 ± 0.17	0.74 ± 0.04
D-*chiro*-inositol	0.12 ± 0.02	0.17[ab] ± 0.02	0.12 ± 0.02
	Galactosyl cyclitols		
myo-Inositol series:			
galactinol	0.98 ± 0.11	0.87 ± 0.24	0.98 ± 0.11
digalacto-*myo*-inositol	0.23 ± 0.04	0.17 ± 0.05	0.23 ± 0.04
Pinitol A series:			
galactopinitol A	0.17 ± 0.02	0.33[a] ± 0.09	0.17 ± 0.02
ciceritol	1.57 ± 0.25	2.11 ± 0.44	2.17 ± 0.15
trigalactopinitol A	0	0	0
Pinitol B series:			
galactopinitol B	0.13 ± 0.04	0.19 ± 0.02	0.13 ± 0.04
digalactopinitol B	0	0	0
	Raffinose-family oligosaccharides		
raffinose	17.75 ± 1.44	15.99 ± 1.98	17.10 ± 2.34
stachyose	51.54 ± 2.33	52.25 ± 4.45	53.38 ± 5.47
verbascose	5.35 ± 0.69	4.98 ± 1.44	5.05 ± 0.89
Sum of sugars	100	98	101
Sum of RFOs	74.6	73.2	75.5
Sucrose/raffinose	1.06	1.12	0.98
Sucrose/RFOs ratio	0.25	0.24	0.22
Sum of galactosyl cyclitols	3.08	3.67	3.68

[a]result significantly different from corresponding values for each of the other lines of the same species; [b]result for one of the species of *Lupinus* section *Atlanticus* significantly different from corresponding values for the other species of this section; [c]result for *Lupinus pilosus* significantly different from corresponding values for both species of section *Atlanticus*.

papillate, formed by elongated polygonal cells (Figs. 1–3). The following tissues were clearly distinguished in the seed coat microphotographs: cuticle, macrosclereid layer, osteosclereid layer, and parenchyma cells. The macrosclereid layer was thickest (200–850 μm) in all seeds, and the layer of osteosclereids was thinnest (≥ 30 μm). SEM images showed high variation of seed sculpture, with depressions of different sizes in the periclinal part of the macrosclereid layer, various patterns of cuticle cracks, and deposits of cuticle remnants. Unlike all the other lines of the analyzed species, *L. pilosus* line 2 had few depressions in the macrosclereid layer, and the external periclinal walls of these cells were rounded and even. A network of pits on the outer surface of the macrosclereid layer could be seen only at very high magnification (800×; Fig. 3b).

SOLUBLE CARBOHYDRATES

The seeds analyzed accumulated RFOs, Gal-C, cyclitols, sucrose and monosaccharides. The average total content of soluble carbohydrates in seeds was 100 mg/g d.m. (± 1.5 mg).

The amount of RFOs within the pool of soluble carbohydrates ranged from 46% to 76% (Tabs. 3–5).

TABLE 4. Soluble carbohydrate composition of *Lupinus cosentinii* seeds (means ± SD of 4 replicates; mg/g d.m.)

Soluble carbohydrate	line 1	line 2	line 3
		Monosaccharides and sucrose	
fructose	1.78 ± 0.06	1.70 ± 0.07	1.65 ± 0.02
glucose	0.11 ± 0.01	0.13 ± 0.02	0.12 ± 0.00
galactose	0	0	0
sucrose	19.04 ± 1.64	18.74 ± 1.33	17.35 ± 2.22
		Cyclitols	
D-pinitol	0.33 ± 0.01	0.30 ± 0.02	0.34 ± 0.05
myo-inositol	0.70 ± 0.08	0.74 ± 0.05	0.57 ± 0.09
D-*chiro*-inositol	0.11 ± 0.01	0.10 ± 0.01	0.11 ± 0.02
		Galactosyl cyclitols	
myo-Inositol series:			
galactinol	1.02 ± 0.14	0.97 ± 0.05	0.99 ± 0.11
digalacto-*myo*-inositol	0.22 ± 0.03	0.20 ± 0.01	0.23 ± 0.04
Pinitol A series:			
galactopinitol A	0.16 ± 0.02	0.17 ± 0.02	0.17 ± 0.02
ciceritol	1.60 ± 0.05	1.58 ± 0.25	1.57 ± 0.47
trigalactopinitol A	0	0	0
Pinitol B series:			
galactopinitol B	0.11 ± 0.03	0.13 ± 0.02	0.13 ± 0.04
digalactopinitol B	0	0	0
		Raffinose-family oligosaccharides	
raffinose	16.97 ± 1.34	17.67 ± 1.27	17.25 ± 2.33
stachyose	52.37 ± 2.98	51.98 ± 3.67	53.94 ± 3.13
verbascose	5.54 ± 0.68	5.66 ± 0.94	5.35 ± 1.23
Sum of sugars	100	100	100
Sucrose/raffinose	1.12	1.06	1.0
Sum of RFOs	74.9	75.3	76.5
Sucrose/RFOs ratio	0.25	0.25	0.22
Sum of galactosyl cyclitols	3.11	3.05	3.09

[a]result significantly different from corresponding values for each of the other lines of the same species; [b]result for one of the species of *Lupinus* section *Atlanticus* significantly different from corresponding values for the other species of this section; [c]result for *Lupinus pilosus* significantly different from corresponding values for both species of section *Atlanticus.*

Stachyose predominated among the RFOs, and its content averaged 43.2 mg/g d.m. (± 14.01 mg). *Lupinus cosentinii* line 3 seeds had the highest level of stachyose (53.9 mg/g d.m., ± 3.1 mg), and *Lupinus pilosus* line 3 seeds the lowest (23.5 mg/g d.m. ± 2.3 mg). The average raffinose content in seeds of *L. atlanticus* and *L. cosentinii* was 17.1 mg/g d.m.(± 0.6 mg), while verbascose content in these seeds was a third that of *L. pilosus* seeds.

The lupin seeds also contained Gal-C. The average content of these metabolites across the three species analyzed was 16.76 mg/g d.m.(± 16 mg). The highest Gal-C content was found in the lines of *Lupinus pilosus*, average 34.6 mg/g d.m. (± 1.18 mg), which was ten times higher than in *L. cosentinii* and *L. atlanticus*. Seven Gal-Cs were found in *L. pilosus*. The predominant members of this metabolite class were trigalactopinitol A, ciceritol and galactopinitol B. Much smaller amounts of galactopinitol A, galactopinitol B, galactinol and digalacto-*myo*-inositol were also detected. *L. cosentinii* and *L. atlanticus* did not contain any trigalactopinitol A or digalactopinitol B.

The cyclitols were represented by D-pinitol, D-*chiro*-inositol and *myo*-inositol in the studied seeds. The highest level of cyclitols was found in seeds of

TABLE 5. Soluble carbohydrate composition of *Lupinus pilosus* seeds (means ± SD of 4 replicates; mg/g d.m.)

Soluble carbohydrate	line 1	line 2	line 3
		Monosaccharides and sucrose	
fructose	2.27 ± 0.23	2.27 ± 0.24	2.99 ± 0.02
glucose	0.11 ± 0.01	0.12 ± 0.02	0.25[a] ± 0.00
galactose	0.12[ac] ± 0.02	0.12[ac] ± 0.12	0
sucrose	15.24 ± 2.35	14.97 ± 1.98	16.86 ± 1.99
		Cyclitols	
D-pinitol	0.28 ± 0.07	0.33 ± 0.04	0.34 ± 0.05
myo-inositol	1.56[c] ± 0.24	1.59[c] ± 0.37	1.74[c] ± 0.09
D-*chiro*-inositol	0.21[c] ± 0.03	0.27[c] ± 0.08	0.22[c] ± 0.02
		Galactosyl cyclitols	
myo-Inositol series:			
galactinol	0.63 ± 0.05	0.87 ± 0.24	0.98 ± 0.11
digalacto-*myo*-inositol	4.12[c] ± 0.64	4.67[c] ± 0.98	0.23[ac] ± 0.04
Pinitol A series:			
galactopinitol A	0.28 ± 0.04	0.33 ± 0.09	0.17 ± 0.02
ciceritol	5.67[c] ± 0.37	6.11[c] ± 0.27	6.57 ± 0.25
trigalactopinitol A	15.24[c] ± 2.08	16.21[c] ± 2.57	16.27[c] ± 2.57
Pinitol B series:			
galactopinitol B	0.21 ± 0.04	0.19 ± 0.02	0.13 ± 0.04
digalactopinitol B	7.08[c] ± 0.72	6.98[c] ± 0.91	8.54[c] ± 1.24
		Raffinose-family oligosaccharides	
raffinose	5.46[c] ± 0.57	5.87[c] ± 0.98	7.75[c] ± 0.44
stachyose	24.59 ± 1.14	25.67 ± 1.37	23.54 ± 2.33
verbascose	15.64[c] ± 1.64	16.24[c] ± 2.27	15.35[c] ± 0.69
Sum of sugars	99	103	102
Sucrose/raffinose	2.79	2.55	2.17
Sum of RFOs	45.7	47.8	46.7
Sucrose/RFOs ratio	0.33	0.31	0.36
Sum of galactosyl cyclitols	33.23	35.36	35.19

[a] result significantly different from corresponding values for each of the other lines of the same species; [b] result for one of the species of *Lupinus* section *Atlanticus* significantly different from corresponding values for the other species of this section; [c] result for *Lupinus pilosus* significantly different from corresponding values for both species of section *Atlanticus*.

Lupinus pilosus (2.1 mg/g d.m.; Tab. 5), but the content of cyclitols varied little across seed lines and species.

All seeds studied accumulated similar amounts of sucrose: 14.9–19.04 mg/g d.m., or ~17% of total soluble carbohydrates.

Monosaccharides (fructose, glucose, galactose) occurred in very low amounts in the studied seeds, not more than 0.3% d.m. (Tabs. 3–5). Predominant among these metabolites was fructose, with average content of 2.19 mg/g d.m. (± 0.5 mg). D-Galactose could not be detected in seeds of *L. cosentinii* and *L. atlanticus*, but a small amount of it, 0.08 mg/g d.m. (± 0.06 mg), was found in *L. pilosus*, although not in its third line.

DISCUSSION

The systematics of the Mediterranean lupins is believed to be fairly settled, but as shown in this paper, one of the diagnostic criteria commonly applied for classification of this genus (or more specifically its Old World species) proved inadequate. In the literature, *Lupinus atlanticus*, *L. cosentinii* and *L. pilosus* should be classified as rough-seeded

(*Scabrispermae*), as opposed to smooth-seeded species (*Malacospermae*) (Naganowska et al., 2003). The 'rough seed coat' characteristic turned out to be less than consistent; in studying three different lines within each of the three species, we found that one of them, *L. pilosus* line 2, produced seeds with a seed coat that was evidently smooth to the touch. Detailed SEM analyses of the testae should explain the background of this deviation. High variability of seed surface sculpture was observed in all species analyzed (Figs. 1–3). The seed surface in *L. pilosus* line 2 differed from all the other lines of analyzed lupin species, resembling the seed coat sculpture of the typically smooth-seeded *L. luteus* and *L. hispanicus* (cf. Piotrowicz-Cieślak, 2005). The occasional occurrence of smooth-seeded plants resembling *L. pilosus* has also been reported by other authors, and the classification of these plants has raised some controversy (Gladstones, 1998).

The biochemical characters analyzed in this study strongly confirm that *L. pilosus* line 2 is properly assigned its species name, that is, it clearly resembles the other two lines of this species. Moreover, soluble carbohydrates showed a variability pattern that corresponded well to the accepted division of *Lupinus* into *Pilosus* and *Atlanticus* sections. This paper gives soluble carbohydrate profiles of the three wild lupin species (*L. atlanticus, L. cosentinii* and *L. pilosus*) for the first time. Taking together these data and previously published findings on the biochemical composition of other members of sections *Atlanticus* and *Pilosus*, we see an emerging pattern: seeds of both *L. palaestinus* and *L. pilosus*, ascribed to the section *Pilosus*, are characterized by a high level of galactosyl cyclitols (~33% dry mass; Piotrowicz-Cieślak, 2005), which has never been observed in *L. atlanticus*, *L. cosentinii* or any other lupin species outside sect. *Pilosus* (Piotrowicz-Cieślak, 2005).

Studies of soluble carbohydrates in lupin can provide data supporting the systematics of this genus, and they seem important for understanding lupin seed physiology and potential applications. In many plants, soluble carbohydrates are the transported form of assimilates (Haritatos et al., 2000); they can alleviate the effects of environmental stresses such as temperature extremes or dehydration (Bachmann et al., 1994; Taji et al., 2002; Obendorf, 1997; Górecki et al., 1997). All lupin species are characterized by the presence of RFOs, with stachyose as their chief member (Horbowicz and Obendorf, 1994). These substances are accumulated in embryo tissues but not in the seed coat, so the relative thickness of the seed coat must be taken into account when analyzing total soluble carbohydrate content. Seeds of sections *Atlanticus* and *Pilosus* have a high level of soluble carbohydrates, comparable to that of *L. luteus* but much higher than in *L. angustifolius* or especially *L. albus*.

The sucrose/raffinose ratio calls for special attention. Accumulation of RFOs correlates positively with seed longevity (Bernal-Lugo and Leopold, 1995) and sucrose/RFO ratio values below 1 suggest a viability period of over 10 years (Horbowicz and Obendorf, 1994). The longevity of the seeds analyzed in this paper has not been studied so far, but the biochemical data presented here (Tabs. 3–5) suggest that the seeds of *L. atlanticus, L. cosentinii* and *L. pilosus* probably are not very prone to ageing.

So far, lupins have been used very rarely for human nutrition, but it is believed that lupin seeds, with their RFOs and Gal-C, could have health-promoting effects, for example stimulating the gut microflora and exerting chemopreventive action against cancers of the digestive tract (Ito et al., 1993). As shown in this paper, these substances occur in even higher quantities in wild lupin species.

L. atlanticus, L. cosentinii and *L. pilosus* may one day be used as a source of genes encoding high levels of seed RFOs and, in the case of *L. pilosus*, also Gal-C, for improvement of cultivated lupin species.

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