COMPOSITION OF SEED SOLUBLE CARBOHYDRATES AND ULTRASTRUCTURAL DIVERSITY OF TESTA IN LUPINS FROM THE MEDITERRANEAN REGION

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ABSTRACT

This article present a comparison of soluble sugar levels in seeds of *Lupinus atlanticus*, *Lupinus cosentinii*, *Lupinus palaestinus* and *Lupinus pilosus*, *Lupinus hispanicus* subsp. *hispanicus* and *Lupinus luteus* of Juno variety. Considering that sugars are accumulated in embryos, only the embryonic tissues were used for biochemical analyses. Additionally, the share of testa and embryo in seed tissues was evaluated. The seed-coat thickness was measured using scanning electron microscopy. The seed coat had the largest share in seeds of *Lupinus pilosus* and *Lupinus palaestinus*, and the least share in seeds of *Lupinus hispanicus* subsp. *hispanicus* and *Lupinus luteus* of Juno variety. In the seed of *Lupinus pilosus* the thickness of the seed coat was 1100 µm, while in *Lupinus luteus* it was 300 µm. The analysed *Lupinus* seeds accumulated from 73 mg/g d.m. (dry matter of seed embryo) to 155 mg of soluble sugars/g d.m. The highest quantity of oligosaccharides of the raffinose series was found in seeds of *Lupinus luteus*, while the lowest amount in seed of *Lupinus palaestinus*. Galactosyl cyclitols appeared in largest amount in seeds of *Lupinus palaestinus* and *Lupinus pilosus*, appropriately 41.93 and 33.75 mg/g dm. The lowest amount of galactosyl cyclitols appeared in *Lupinus atlanticus*, *Lupinus cosentinii* and *Lupinus hispanicus*.

KEY WORDS: *Lupinus* seeds, seed coat, cyclitols, galactosyl cyclitols, raffinose family oligosaccharides, gas chromatography, scanning electron microscopy.

INTRODUCTION

Lupinus L. (Fabaceae) is a large and diverse genus comprising 200-500 annual and perennial herbaceous species, as well as a few soft-woody shrubs and small trees (Turner 1995) which occur in a wide range of ecogeographical conditions in both the New and the Old World. Only 12-13 species are native to the Mediterranean region and Africa, with some populations extending to highlands of East African tropical areas (Gladstones 1998). Old World lupins are all annual, herbaceous, and predominantly autogamous. Their fruits and seeds are generally large, and their leaves are always digitate. Two distinct groups are recognized primarily on the basis of the seed coat texture: the smooth-seeded and the rough-seeded species (Heyn and Herrnstadt 1977). The smooth-seeded group comprises five species usually treated as members of four sections, *Albi*, *Micranthi*, *Angustifoli*, and *Lutei* (Gladstones 1998). This group is generally typified by *L. pilosus* Murr. and often designated at sectional rank. The rough-seeded species are mainly distributed in North Africa and in the eastern part of the Mediterranean region.

Apart from the characteristic structure of seed coat, the seeds of lupin are distinguished by presence of oligosaccharides of the raffinose family (RFO). Raffinose, stachyose and verbascose are members of this group. For years these compounds were considered undesirable in human diet. However, nowadays they undergo a reappraisal. They are accumulated in the process of seed maturation and are used during germination. Recent development in nutritional sciences are changing the opinion on their actual role (Alles et al. 1999). The digestive tract of humans and monogastric animals lacks the enzymes breaking down α (1$\rightarrow$6)-galactosyl linkage. It is hydrolysed however, in the large intestine by bacterial galactosidase. The effect (Tomomatsu 1994; Hamilton-Miller 2000) of discomfort after consumption of α-D-galactosides is caused by enterobacteriae of the digestive tract, which produce hydrogen, methane and carbon dioxide. RFO supplied in larger quantities with food stimulates the bacterial flora of the digesti-

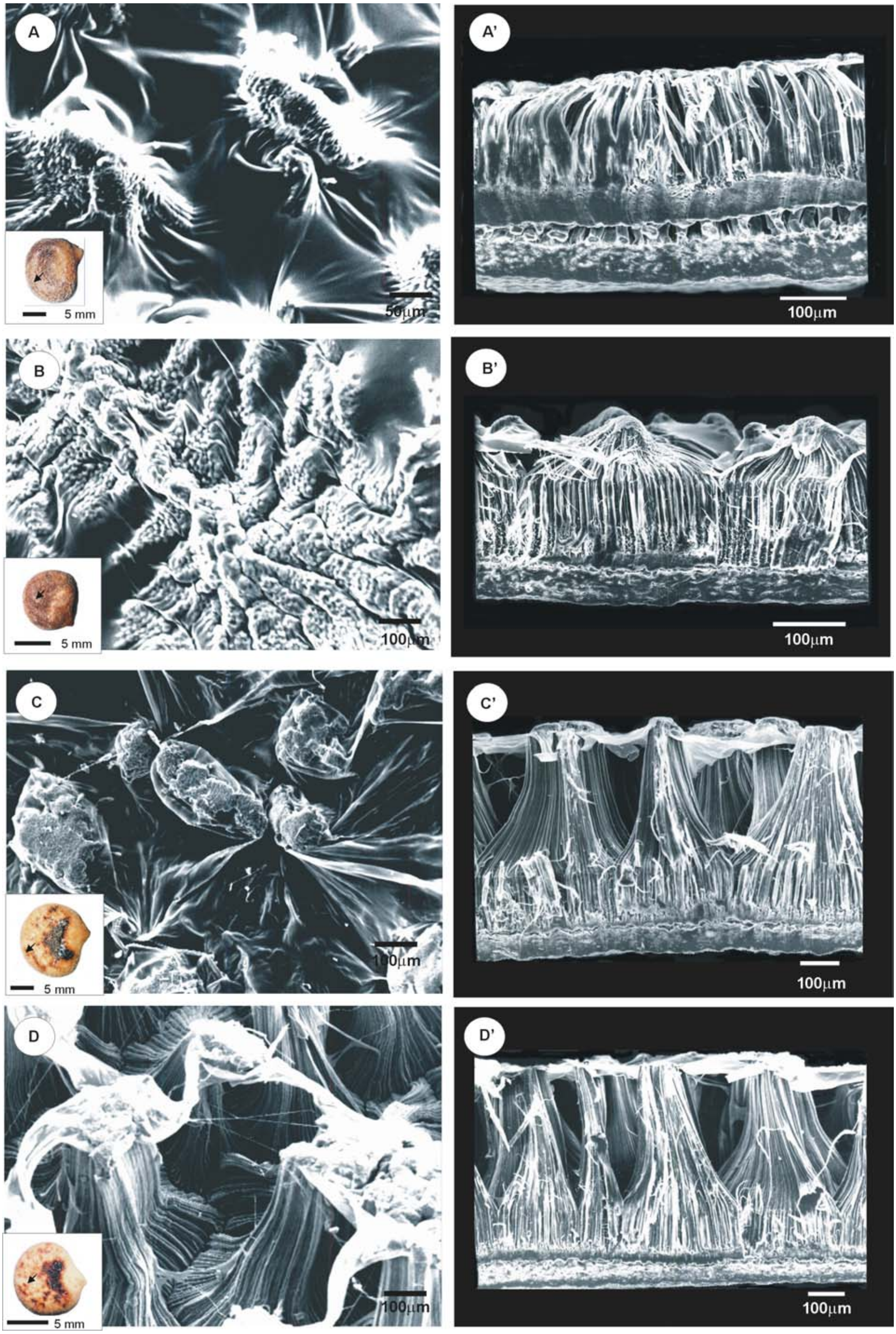

Fig. 1. Scanning electron micrographs of selected seeds of *Lupinus*. Each figure is divided into an A, B, C, D panel coat surface of seed (showing the entire seed) and A', B', C', D' panel transverse section coat of seeds.
A, A' – *Lupinus atlanticus* B, B' – *Lupinus cosentinii*, C, C' – *Lupinus palaestinus*, D, D' – *Lupinus pilosus*

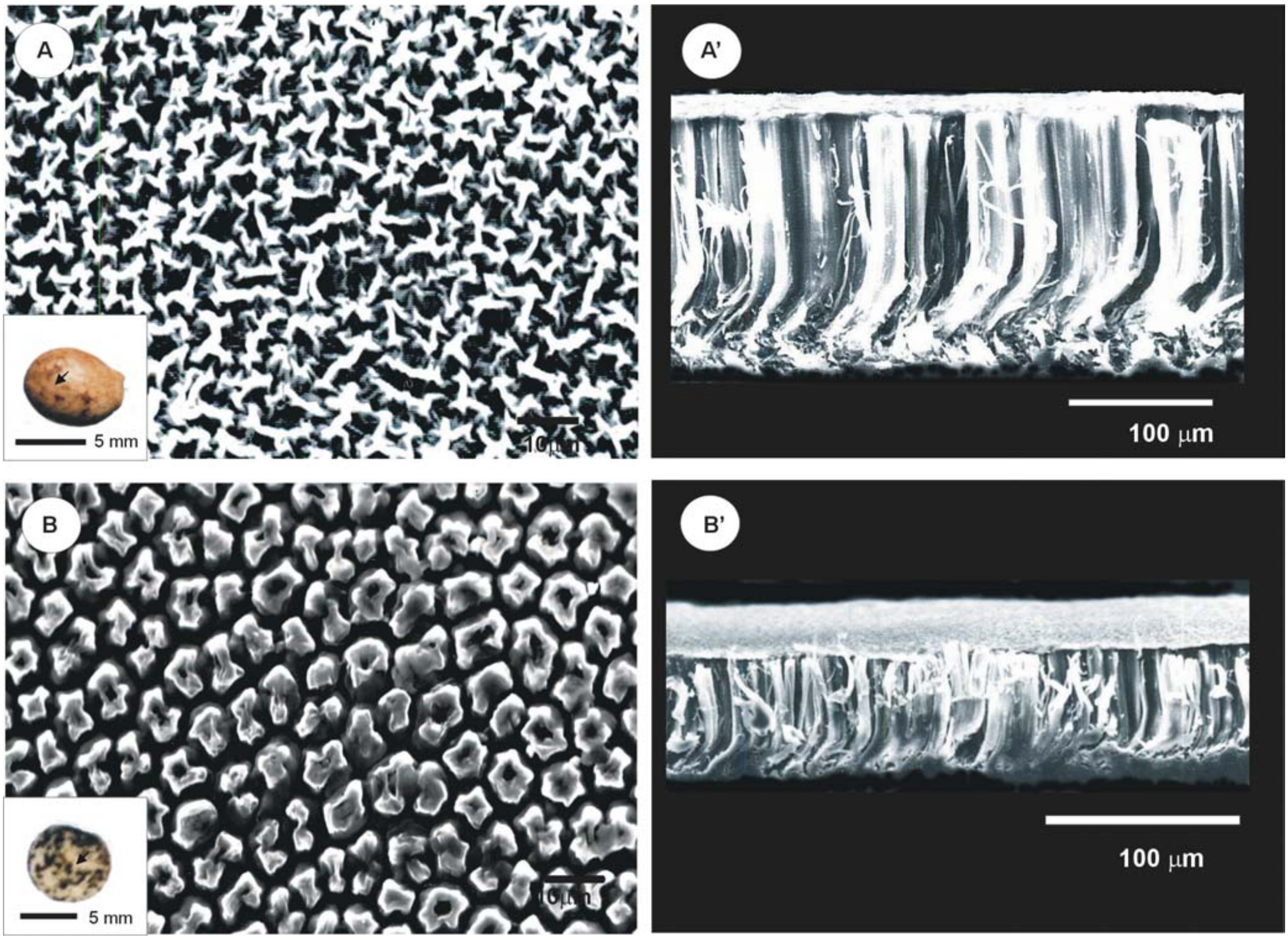

Fig. 2. Scanning electron micrographs of selected seeds of *Lupinus*. Each figure is divided into an A, B panel coat surface of seed (which showing the entire seed) and A', B' panel ransverse section coat of seeds.
A, A' – *Lupinus hispanicus* subsp. *hispanicus* B, B' – *Lupinus luteus* cv. Juno

ve tract. As a result, bifidobacteria dominate the intestinal microflora. They appear beneficial for the organism through stimulating the immune system. Consumption of α-D--galactosides diminishes the risk of large-intestine tumours (Gibson and Roberfroid 1995).

Lupins are especially rich in oligosaccharides of the raffinose series (Piotrowicz-Cieślak et al. 1999). These compounds appear in the axes in quantities two or three times bigger than in cotyledons (Górecki et al. 1997). In seed coats these oligosaccharides do not appear. It seems that in terms of the contents of RFO a thin seed coat and a big embryo would be most satisfactory. This paper's objective was to compare the carbohydrate composition and the share of seed testa in seeds of wild growing lupin and cultivated yellow lupin of Juno variety.

MATERIAL AND METHODS

MATERIAL

Seeds of six lupins from Mediterranean species were used in the experiments: four species: *Lupinus atlanticus*, *Lupinus cosentinii*, *Lupinus palaestinus* and *Lupinus pilosus* were rough-seed species, and *Lupinus hispanicus* subsp. *hispanicus* and *Lupinus luteus* cv. Juno were smooth-seeded (Table 1).

METHODS

Scanning electron microscopy

For observation in the scanning electron microscope (SEM) the seeds were coated with gold using a JEOL JFC

TABLE 1. List and origin of species of seeds from genus Lupinus used for investigations.

No.	Species	Section	Main areas of occurrence
	The Mediterranean rough-seeded species		
1	*Lupinus pilosus* Murr.	Pilosus	Turkey
2	*Lupinus palaestinus* Boiss.	Pilosus	Israel
3	*Lupinus atlanticus* Glads.	Atlanticus	Marocco
4	*Lupinus cosentinii* Guss.	Atlanticus	Spain
	The Mediterranean smooth-seeded species		
5	*Lupinus hispanicus* Boiss. et Reut. subsp. *hispanicus*	Luteus	Spain
6	*Lupinus luteus* L. cv. Juno	Luteus	Poland

TABLE 2. The morphological characteristic of *Lupinus* genus seeds (values are mean ±SD of the mean for fifteen replicate samples).

Species genus of seeds	Weight [mg × seeds^{-1}]	Anatomical part of seeds [%]		
		cotyledon	axis	testa
Lupinus atlanticus	207.4±6.5	66.8±2.7	2.7±0.4	30.5±1.8
Lupinus cosentinii	213.9±8.3	69.4±4.8	5.2±0.3	25.4±2.0
Lupinus palaestinus	592.4±14.8	66.5±3.4	1.3±0.2	32.1±1.5
Lupinus pilosus	548.7±15.2	64.4±2.4	1.8±0.2	33.8±1.3
Lupinus hispanicus subsp. *hispanicus*	136.7±6.7	72.0±4.7	4.9±0.4	23.1±1.4
Lupinus luteus cv. Juno	141.7±4.3	72.3±3.2	3.1±0.2	24.6±1.1

1200 ion coater and observed in a JEOL JSM-5310LV scanning electron microscope under a 20 kV.

Soluble sugars

Cyclitols, galactosyl cyclitols and soluble sugars content were analyzed according to Piotrowicz-Cieślak et al. (2003). Dry and fresh tissue mass were also examined. 30 mg of dry milled embryo tissue were homogenized in ethanol: water, 1:1 (v/v) containing 100 µg phenyl-α-D-glucose as internal standard (Horbowicz and Obendorf 1994). The homogenate and the rinse were combined in a 1.5-ml microfuge tube, heated at 75°C. for 30 min to inactivate endogenous enzymes and centrifuged in a centrifuge MPW-365 (Poland) at 15 000 g for 20 min. The supernatant was passed through a 10 000 MW cut-off filter (Lida, Kenosha, USA). Aliquots of 0.5 ml filtrate were transferred to silylation vials and evaporated to dryness under a nitrogen stream. Residues were kept overnight in a desiccator, over phosphorus pentoxide. Dry residues were derived with 300 µl of silylation mixture (trimethylsilylimidazole: pyridyne, 1:1, v/v) in silylation vials (Supelco) at 70°C for 30 min and then cooled at room temperature. One µl of dried soluble carbohydrates was injected into a split-mode injector of a Shimadzu GC-14A gas chromatograf equipped with flame ionization detector and Shimadzu C-R6A integrator. Soluble carbohydrate were analysed on a DB-1 capillary column (15 m lengh, 0.25 mm ID, 0.25 µm film thickness, Varian) operated with a programmed initial temperature of 150°C, adjusted to 200°C at 3°C/min, adjusted 325°C at 7°C/min, and held 325°C for 40 min, the injector port was at 335°C and the detector at 350°C. The carrier gas was helium at 3 ml/min, split 1:50 and the detector gas were hydrogen at 30 ml/min and air at 300 ml/min. Soluble carbohydrates were identified with intermediate standards as available and calculated from the ratios of area of peaks for each known carbohydrate to the area of peak for the internal standards.

RESULTS

Morphological analysis

The analysed seeds of *Lupinus* were distinguished by considerable morphological variation. It was related to seed colour, shape and size. The biggest were seeds of *Lupinus palaestinus* (∅14 mm) and *L. pilosus* (∅12 mm), the smallest were *L. hispanicus* and *L. luteus* (Figs 1 and 2). The same regularities were observed in the mass of the seeds, which was highest in *L. palaestinus* and *L. pilosus*, while the lowest in *L. hispanicus* and *L. luteus* (Table 2). The share of the seed coat in various anatomical parts of the seeds ranged from 33.8 to 23.1%, for *L. pilosus* and *L. hispanicus* subsp. *hispanicus*, respectively. The square shaped seeds (*L. palaestinus* and *L. pilosus*) had the highest share of seed coats, while the ball shaped seeds had the smallest one. Electron microscopy showed a high variety of surface relief and seed coat thickness. The thickness ranged from 100 µm to 1100 µm, in *L. luteus* and *L. pilosus*, respectively. The seeds with the thickest coats featured the biggest epidermal sclereids. Subepidermal sclereids were biggest in *Lupinus atlanticus* (ca. 30 µm). In other lupin species the size of subepidermal sclereids varied from 1 to 10 µm. The seeds having thin coats – *L. hispanicus* and *L. luteus* – had a thick and continuous cuticle. The thickest cuticle appeared in the seeds of *L. luteus* (30 µm). The seeds of *L. atlanticus*, *cosentinii*, *palaestinus* and *pilosus* had thin and shattered cuticles (Figs 1 and 2).

Soluble sugar composition of lupin seeds

The research comparing *Lupinus atlanticus*, *cosentinii*, *palaestinus*, *pilosus*, *hispanicus* and *luteus*, showed a high variation in quantity and quality of soluble carbohydrates (Table 3). The seeds accumulated monosaccharides, sucrose, cyclitols, galactosyl cyclitols and oligosaccharides of the raffinose series. The content of soluble sugars in the analysed seeds of all varieties averaged 114 mg/g dry matter of embryo (d.m.). The highest content of soluble sugars was found in *L. pilosus* (155.51 mg/g d.m.), the lowest in *L. cosentinii* (73 mg/g d.m.). The seeds of *L. hispanicus* and *L. luteus* accumulated more than 100 mg of soluble carbohydrates per 1 g of embryo dry matter (Table 3). A not much lower level of soluble sugars was found in *L. palaestinus* and *L. atlanticus*, and it was 99.92 and 96.24 mg/g d.m., respectively. Regardless of the quantity of synthesised soluble sugars, the RFO contributed substantially (63%) to this group of compounds. They included raffinose, stachyose and verbascose. The share of the oligosaccharides of the raffinose series ranged from 45 to 76% in total soluble sugars. Among these oligosaccharides stachyose dominated. It appeared in the highest amount in the seeds of *Lupinus luteus* of Juno variety (59.2 mg/g d.m.), and in the lowest amount in the seeds of *Lupinus palaestinus* (21.82 mg/g d.m.). From the remaining oligosaccharides of the raffinose series, verbascose reached higher concentration than raffinose. The highest level of raffinose appeared in *L. hispanicus*, the lowest in *L. cosentinii*. The highest concentration of verbascose was found in *L. luteus* (Table 3) and the lowest in *L. atlanticus*.

Apart from the high level of RFO, the seeds of lupin contained considerable amounts of galactosyl cyclitols, represented by the products of *myo*-inositol, pinitol A and B. The *Lupinus* seeds contained from 5 to 8 galactosyl cyclitols. In all the analysed seeds there was galactinol, digalac-

TABLE 3. Composition of soluble carbohydrates (mg per g dry matter) in lupin seeds from the Mediterranean Sea (values are mean ± SD of the mean for four replicate samples).

Soluble carbohydrates [mg/g]	Rough-seeded				Smooth-seeded	
	L. atlanticus	*L. cosentinii*	*L. palaestinus*	*L. pilosus*	*L. hispanicus* subsp. *hispanicus*	*L. luteus* cv. Juno
Monosaccharides and sucrose						
fructose	1.99±0.02	1.78±0.06	2.80±0.22	6.26±0.17	1.33±0.22	1.33±0.31
glucose	0.15±0.000	0.11±0.01	0.11±0.06	0.10±0.01	0.07±0.01	0.89±0.16
galactose	0	0	0.08±0.08	0.12±0.12	0	0.85±0.07
sucrose	18.86±1.99	19.04±1.64	15.6±1.23	32.65±1.56	15.54±1.35	27.9±1.47
Cyclitols						
D-pinitol	0.34±0.05	0.33±0.01	0.14±0.02	0.08±0.02	2.95±0.44	0.66±0.07
myo-inositol	0.74±0.09	0.70±0.08	0.25±0.08	1.24±0.34	1.19±0.09	0.18±0.01
D-*chiro*-inositol	0.12±0.02	0.11±0.01	0.11±0.04	0.23±0.04	0.23±0.03	2.07±1.47
Galactosyl cyclitols						
***myo*-inositol serie:**						
galactinol	0.98±0.11	1.02±0.14	0.17±0.02	0.73±0.03	1.84±0.17	1.02±0.14
digalacto-*myo*-inositol	0.23±0.04	0.22±0.03	1.96±0.03	4.54±0.67	0.74±0.08	2.86±2.86
Pinitol A serie:						
galactopinitol A	0.17±0.02	0.16±0.02	0.24±0.02	0.29±0.02	0.24±0.01	0.24±0.07
ciceritol	1.57±0.25	1.60±0.05	3.13±0.08	5.64±0.67	0.41±0.06	1.27±0.18
trigalactopinitol A	0	0	12.36±1.22	21.24±0.06	0	2.31±0.32
Pinitol B serie:						
galactopinitol B	0.13±0.04	0.11±0.03	0.01±0.01	0.13±0.04	0.15±0.01	0.24±0.04
digalactopinitol B	0	0	6.66±0.02	1.23±0.21	0	0.64±0.11
trigalactopinitol A	0	0	9.22±1.07	8.13±0.64		0.86±0.08
Raffinose family oligosaccharides						
raffinose	17.75±0.57	5.87±0.98	6.83±0.89	6.75±0.84	17.84±1.22	8.88±0.83
stachyose	51.54±1.14	25.67±1.37	21.82±0.57	43.17±2.27	55.66±2.68	59.2±5.49
verbascose	5.35±1.64	16.24±2.27	14.75±2.57	22.98±1.26	6.04±0.52	42.0±2.44
Sum of sugars	100	73	96	155	104	153
Sum of RFO	74.6	47.8	43.4	72.9	79.5	110.0
Ratio sucrose to RFO	0.25	0.40	0.36	0.45	0.20	0.25
Sum of galactosyl cyclitols	3.08	3.11	33.75	41.93	3.38	9.44

to-*myo*-inositol, galactopinitol A, ciceritol and galactopinitol B. The greatest amount of galactosyl cyclitols appeared in the seeds of *Lupinus pilosus* and *Lupinus palaestinus*, 41.9 and 33.75 mg/g d.m., respectively.

Sucrose appeared in biggest amounts in *Lupinus pilosus* and *Lupinus luteus*, 32.65 and 27.9 mg/g d.m., respectively. The smallest amounts of sucrose were found in the seeds of *Lupinus palaestinus* and *Lupinus hispanicus*.

Monosaccharides appeared in minute amounts in the analysed seeds and were represented by fructose, glucose and galactose. Their amounts ranged from 0.01% to 0.6% in dry matter. Also cyclitols, D-pinitol (3-*O*-methyl D-*chiro*-inositol), D-*chiro*-inositol and *myo*-inositol, appeared in small amounts. These quantities approached the level of monosaccharides and ranged from 0.05% to 0.7% in dry matter.

DISCUSSION

Most species of the Fabaceae have seeds with a high proportion of physical exogenous dormancy caused by the testa (outer seed coat), which prevents them from imbibing water even under favourable environmental conditions (Morrison et al. 1998). All legume have the same characteristic type of testa. The seeds dry during their desiccation (with the hilum or pleurogram acting as a hygroscopic valve), and the coat hardens and becomes impermeable as the seeds moisture content falls below of 20±25%, reaching full impermeability at 10±15% (van Staden et al. 1989). The seed coat functions as an important mechanical barrier, however it does not contain any soluble sugars.

In the Mediterranean-type ecosystem the major natural mechanism causing dormancy breakage is the disruption of testas in legume soil seed bank (Auld and O'Connell 1991). The thick seed coat is considered unfavourable in the cultivated varieties.

The oligosaccharides of the raffinose series are the main storage material for the seeds. Their amounts vary from a few percent (peas, beans, some lupin species) to ca. 11% percent in dry matter (lupin) (Cerning-Beroard and Filiatre 1976). The content of RFO differs according to the species, variety, degree of maturity, way of storage (McCleary and Matheson 1974; Frias et al. 1999), temperature of maturation (Piotrowicz-Cieślak 2002) and other environmental factors (Trugo et al. 1988).

Galactosyl cyclitols, similarly to RFO, are a vast group occurring in many species (Horbowicz and Obendorf 1994). The seeds of buckwheat, castor-oil plant and lentils are especially rich in galactosyl cyclitols. The seeds of cultivated lupin contain up to 2% galactosyl cyclitols in dry matter (Piotrowicz-Cieślak et al. 1999). In seeds of yellow lupin of the Juno variety the amount of galactosyl cyclitols was about 1% d.m. The greatest amount of galactosyl cyclitols was found in *L. pilosus* and *L. palaestinus*, 4.1 and 3.3% d.m., respectively. The amount of galactosyl cyclitols depends on the presence of free cyclitols in the seeds, since they function as substrates for galactosyl cyclitols biosynthesis (Piotrowicz-Cieślak et al. 2004). In the analysed seeds, however, no clear correlation between the presence of galactosyl cyclitols and cyclitols could be found. In mature seeds of *L. palaestinus* the amount of cyclitols is the lowest among all the analysed seeds, it is 0.5 mg/g d.m. The ability to link galactose and cyclitols is undoubtedly determined genetically.

A decrease in the share of seed testa in the seeds would cause an increase in the amounts of RFO and galactosyl cyclitols, without any necessity to husk the seeds. The amount of soluble sugars in whole seeds of *L. pilosus* is lower than 115.8 mg/g d.m., while in seeds devoid of testas it is 155 mg/g d.m. The thin testas cause the seeds to germinate faster, which is quite favourable with respect to the cultivated forms, while not always important with respect to the wild-growing ones. Changing the share of testa in seed tissues from 33.8 to 23%, with unaffected soluble sugar level in the embryo would raise the soluble sugar level in whole seed by nearly 10% (8.2%).

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