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Characterization of Lupin Cultivars Based on Phenotypical, Molecular and Metabolomic Analyses

Athanasios Mavromatis [1], Irini Nianiou-Obeidat [1], Alexios Polidoros [1], Zoi Parissi [2], Eleni Tani [3,*], Maria Irakli [4], Konstantinos A. Aliferis [5,6], Ioannis Zafeiriou [1], Photini V. Mylona [4], Efi Sarri [3], Evgenia-Anna Papadopoulou [5], Rafail Tagiakas [1], Leonidas Kougiteas [3], Stavroula Kostoula [1] and Eleni M. Abraham [2,*]

[1] Laboratory of Genetics & Plant Breeding, Faculty of Agriculture, Aristotle University of Thessaloniki, 54124 Thessaloniki, Greece
[2] Laboratory of Range Science, Department of Forestry & Natural Environment, Aristotle University of Thessaloniki, 54124 Thessaloniki, Greece
[3] Laboratory of Plant Breeding & Biometry, Department of Crop Science, Agricultural University of Athens, 11855 Athens, Greece
[4] Institute of Plant Breeding and Genetic Resources, Hellenic Agricultural Organization—DEMETER, 57001 Thermi, Greece
[5] Laboratory of Pesticide Science, Department of Crop Science, Agricultural University of Athens, 11855 Athens, Greece
[6] Department of Plant Science, McGill University, Macdonald Campus, Ste-Anne-de-Bellevue, Montreal, QC H3A 0G4, Canada
* Correspondence: etani@aua.gr (E.T.); eabraham@for.auth.gr (E.M.A.)

Abstract: Lupins are an important source of protein that could replace soybeans in the diet of ruminants and monogastrics, without reducing their performance. *Lupinus albus* (*L. albus*) is the main species of the genus *Lupinus* that is cultivated in the Mediterranean region. The aim of the present research was to study commercial cultivars and advanced breeding lines of *L. albus* by using phenotypical, molecular and biochemical data, in order to be used in breeding projects. Seven commercial cultivars (Estoril, Fas Sweet, Multitalia, Magnus, Orus, Ulysse Sulimo and Figaro) and three advanced lines from the company AGROLAND (LKML, LKAP and LKAU) were used. Eleven morphological traits were described using UPOV Guidelines (International Union for the Protection of New Varieties of Plants). Additionally, agronomical traits and yield components were measured. Regarding the nutritional value, grain samples were analyzed for N and the crude protein (CP), neutral detergent fiber (NDF), acid detergent fiber (ADF), acid detergent lignin (ADL), total alkaloids (TA), total phenolic content (TP), total tannins content (TT) and condensed tannins (CT) were calculated. Genetic diversity among genetic materials was assessed by SSRs molecular markers. The metabolomic analysis for four selected cultivars (Figaro, Magnus, Multitalia and Sulimo) was performed on the seeds with the GC/EI/MS technique. According to the results, the advanced lines were most productive but also with higher content of total alkaloids than the commercial cultivars. The only exception was the cultivar Multitalia that was characterized by a high content of alkaloids. Based on the SSRs, the cultivars Magnus, Orus and Estoril were grouped together while the breeding lines LKAP, LICML and LKAU were grouped with Multitalia. Regarding the metabolomic profile, the cultivars Multitalia and Magnus were together, while Sulimo was grouped with Figaro. Finally, the content of several beneficial metabolites for human and animal nutrition was significantly increased in Sulimo and Figaro, compared to Magnus and Multitalia. Both commercial varieties and lines have characteristics that can be exploited and used in breeding programs.

Keywords: *Lupinus albus*; phenotypical traits; physicochemical analysis; molecular analysis; alkaloids; metabolomics

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1. Introduction

Lupin belongs to the very diverse and widespread genus *Lupinus* of the family Fabaceae (*Leguminosae*). This genus includes numerous herbaceous annuals, perennials and shrub species [1]. Lupins have been cultivated since ancient times for green manure, cosmetic and medicine use, but mainly for animal and human nutrition [2–4]. Despite the numerous species of the genus, only the following are used in agriculture nowadays: *L. angustifolius* (commonly known as blue lupin or as narrow-leafed lupin) and *L. luteus* (yellow lupin) are cultivated in Baltic countries, *L. albus* (white lupin) in Mediterranean countries, and *L. mutabilis* (Andean lupin) in the Andean region [2–4]. Although lupin cultivation is limited worldwide, there is a growing interest for its expansion due to its high nutritional value and high adaptability to extreme environmental conditions [5]. Particularly for Europe, this trend is supported by the EU to foster European protein crop production [6].

Since lupin is an excellent source of protein, its seeds can replace soybean in livestock diets without loss in quality and quantity of animal products [7,8]. Lupin seeds contain high levels of protein compared to soybean, and with strikingly higher levels of essential amino acids than soybean [9]. Although their oil content is quite low compared to oilseeds, they are characterized by a high concentration of polyunsaturated fatty acids [10], which is more important than the quantity from the nutritional point of view [11]. These nutritional properties of lupin render it ideal for use in animal feed but also for human consumption [12].

On the other hand, the content of anti-nutritional factors such as alkaloids, and the specific carbohydrate composition in lupin seeds, reduces its nutritive value and its use in animal feeding. Among the main anti-nutritional factors are the quinolizidine alkaloids, which account for the bitter taste of the seed [2]. Another anti-nutritional factor that affects the feed intake and digestibility mainly in monogastrics is the specific carbohydrate composition [8] of lupin seeds with a low level of starch, high level of non-starch polysaccharides (NSP) [13,14] and high level of raffinose oligosaccharides [15]. An additional limiting factor for use in the monogastrics' diet is their lower content in nutritionally essential sulfur-containing amino acids than soybean [16].

The aforementioned factors that limit the use of lupin in animal nutrition are breeding targets. The major objectives of recent breeding programs comprise yield stabilization, seed quality traits, resistance to biotic and abiotic stresses and late maturation [17–19]. In this respect, modern varieties have improved agronomical and seed quality traits such as low alkaloid content [20], which does not affect intake. Nevertheless, the breeding efforts for lupins are recent, and thus, there is room for further improvement.

Characterization of the germplasm resources using phenotypic, molecular and biochemical markers is an essential step of any breeding program. The genetic diversity of *L. albus*'s germplasm including commercial cultivars and landraces has been studied by phenotype [21,22] and by molecular markers [23,24]. However, to meet the demands of healthier diets in line with a more sustainable farming system, there is an emerging need to utilize high-throughput technologies such as metabolomics to better characterize and manage the wealth of legumes' genetic resources [25]. Research thus far on lupin has mainly focused on the study of specific metabolites, with special attention on anti-nutritious compounds [23,26,27]. Only the work by Farag et al. [28] has presented a total metabolomic comparative profile of lupin and lens seed accessions to highlight their health benefits.

The objectives of this work were: (i) the study of the genetic variation among white lupin cultivars and advanced breeding lines using phenotypic and molecular data, (ii) the evaluation of quality traits of seeds from lupin genotypes and cultivars and (iii) the selection of the most promising genotypes with high yield potential and high nutritive value.

2. Materials and Methods

2.1. Plant Material

A group of white lupin (*L. albus*) commercial cultivars including Estoril, Fas Sweet, Multitalia, Magnus, Orus, Ulysse Sulimo and Figaro from different sources along with three advanced breeding lines (LKML, LKAP and LKAU) obtained from AGROLAND SA (Table 1), were used in this work.

Table 1. Lupin cultivars used in this research.

Code	Genetic Material	Origin	Genotype/Type of Cultivar	Species
L1	Estoril	Portugal	Commercial cultivar	*Lupinus albus*
L2	Fas Sweet	Serbia	Commercial cultivar	*Lupinus albus*
L3	Figaro	France	Commercial cultivar	*Lupinus albus*
L4	Magnus	France	Commercial cultivar	*Lupinus albus*
L5	Multitalia	Italy	Commercial cultivar	*Lupinus albus*
L6	Orus	France	Commercial cultivar	*Lupinus albus*
L7	Sulimo	France	Commercial cultivar	*Lupinus albus*
L8	Ulysse	France	Commercial cultivar	*Lupinus albus*
L9	LKML	Greece	Advanced breeding line	*Lupinus albus*
L10	LKAP	Greece	Advanced breeding line	*Lupinus albus*
L11	LKAU	Greece	Advanced breeding line	*Lupinus albus*

Seeds of each genotype were planted into pots and cultivated in the greenhouse of the Laboratory of Genetics and Plant Breeding, Department of Agriculture, A.U.Th. The experimental design followed was randomized (complete block with 3 replications). Each replication consisted of 5 pots, with one plant per pot (3 R × 5 pots × 11 genotypes). The volume of pots was 5 L, which contained peat moss (pH: 5.4) and perlite in a ratio of 3:1. The pots were irrigated systemically with 350 mL per week using a drip irrigation system.

2.2. Phenotypic Data

Morphological and Agronomic Traits

Morphological and agronomic traits were measured on 15 individual plants per genotype. Eleven morphological traits were described using UPOV Guidelines (International Union for the Protection of New Varieties of Plants) (Upov.org) (Table 2). Additionally, the following agronomic traits and yield components were measured: (1) number of pods per plant, (2) weight of pods per plant (g), (3) number of seeds per pod, (4) weight of 100 seeds (g) and (5) chlorophyll content (SPAD) measured onto 3 upper leaflets per plant at the full anthesis stage (Figure 1a–c).

Table 2. Morphological traits estimated according to UPOV guidelines.

A/A	Morphological Traits
1	Seed size (coDES)
2	Color of leaf: intensity of green color prior to bud emergence
3	Shape of leaflets
4	Type of inflorescence
5	Date of anthesis and flowering period
6	Plant height at the beginning of anthesis
7	Color of flower wings
8	Color of the flower keel
9	Type of development
10	Pod maturation time
11	Seed decoration



(**a**) (**b**) (**c**)

Figure 1. (**a**) Size of pods and number of seeds per pod; (**b**) number and weight of 100 seeds and (**c**) suitable stage of chlorophyll content (SPAD) measurement.

2.3. Nutritional Traits

The grains of *Lupinus* varieties and advanced lines were oven-dried at 50 °C for 48 h, ground through a 1 mm screen, and analyzed for N using a Kjeldahl procedure [29]. After that, crude protein (CP) was calculated by multiplying the N content by 6.25. Moreover, samples were analyzed for neutral detergent fiber (NDF), and sequential acid detergent fiber (ADF) concentrations using the ANKOM fiber analyzer and the fiber bag technique developed by ANKOM (ANKOM Technology, Macedon, NY, USA). Heat-stable α-amylase and sodium sulfite were added for the NDF determination, as recommended with the ANKOM system. The acid detergent lignin (ADL) was determined according to the methods of Goering and Van Soest [30].

2.4. Antinutritional Traits

2.4.1. Determination of Total Alkaloids

Alkaloid extraction was performed as described by Buzuk et al. [31] with modifications. Lupin seeds were finely ground, and 1 g of each sample was mixed with 25 mL chloroform containing 1 mL 25% ammonia. After stirring for 16 h at room temperature, the homogenate was centrifuged for 10 min at 4 °C at 4000 rpm. The supernatant was evaporated in a rotary evaporator and the oily residue was reconstituted with 500 µL chloroform and 15 mL 2% (*w*/*v*) acetic acid in water. After centrifugation at 4000 rpm for 10 min, 1 mL of supernatant was transferred to a separating funnel containing 5 mL of acetate buffer and 5 mL of bromocresol green solution. Then, the mixture was twice extracted with chloroform until the final volume of 10 mL and centrifuged for 10 min at 2000 rpm. The absorption of the supernatant was measured at 430 nm against a blank reagent. The total content of alkaloids was expressed in terms of atropine equivalent, as measured by the calibration curve for atropine standard solution.

2.4.2. Extraction of Phenolic Compounds

For the extraction of phenolic compounds, 0.4 g of ground lupin seeds was mixed with 5 mL of the extraction solvent (acetone/water, 70:30, *v*/*v*), vortexed for 30 s and then sonicated for 15 min at room temperature. After centrifugation at 2200 rpm for 10 min, the supernatant was collected, and the extraction was repeated twice. The obtained phenolic extracts were stored at −25 °C until further analysis.

2.4.3. Determination of Total Phenols

The content of total phenols was estimated by the Folin–Ciocalteu method [32]. In short, 200 µL of appropriately diluted phenolic extracts were mixed with 800 µL of Folin-Ciocalteu reagent. Following neutralization with 2 mL of saturated sodium carbonate

(75 g/L), the absorption was measured at 765 nm after 60 min of reaction in the dark. Total phenolic content was expressed as mg of gallic acid equivalents per 100 g of dry weight (mg GAE/100 g).

2.4.4. Determination of Hydrolyzed Tannins

The content of hydrolyzed tannins was determined as the PVPP-bound phenolics, according to Makkar et al. [33]. Briefly, 400 µL of phenolic extracts were mixed with 400 mg PVPP and then centrifuged at 10,000 rpm at 4 °C for 20 min. The non-phenolic content was determined in the obtained supernatants as above by the Folin-Ciocalteu method. Total hydrolyzed tannins were calculated by subtracting non-tannin phenolics from total phenolics and were expressed as mg of gallic acid equivalents per 100 g of dry weight (mg GAE/100 g).

2.4.5. Determination of Condensed Tannins

The condensed tannins were measured with the butanol-acid assay [34] with some modifications. Appropriately diluted extracts (0.5 mL) were mixed with 3 mL of the reagent n-butanol/HCl (95:5, *v*/*v*), followed by 100 µL ferric ammonium sulfate (20 g/L) in 2N HCl. Following boiling for 60 min in a water bath, the absorbance was measured at 550 nm. The absorbance of the untreated tubes was considered as blank. The content of total condensed tannins was expressed as mg of procyanidin B2 equivalents per 100 g dry weight (mg PCNE/100 g).

2.5. Statistical Analysis

Data were subjected to one-way analysis of the variance (ANOVA). All means for agronomical characteristics and yield components were separated using Duncan's multiple range test ($p \leq 5\%$). The Pearson correlation analysis between the agronomic parameters was also carried out. The Tukey test at a = 0.05 was used to compare means for nutritional and anti-nutritional traits. Statistical analysis was performed by the statistical package SPSS v.15.0 (SPSS Inc. Chicago, IL, USA).

2.6. Molecular Analysis

2.6.1. SSR-HRM Genotyping

Eight individuals of every cultivar were subjected to SSR genotyping. Genomic DNA was extracted from seeds, using the QIAGEN DNeasy Plant Pro Kit (Qiagen, Hilden, Germany). The yield of the extracted DNA was estimated with a Qbit 4 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) and normalized to 5 ng/µL for downstream applications. Genotypic analysis was performed using 6 polymorphic microsatellite markers (Table 3), based on reports by Nelson et al. [35]. PCR amplification reactions and high-resolution melting analysis were carried out on a LightCycler® 96 (Roche Diagnostics Gmbh, Mannheim, Germany), using the KAPA HRM FAST PCR Kit (KAPA Biosystems, Wilmington, MA, USA), in a total reaction volume of 12 µL, containing 5 ng genomic DNA template, 1X KAPA HRM FAST Master Mix, 0.2 µM of each primer, 2.5 mM $MgCl_2$, and PCR-grade H_2O. The PCR amplification program constitutes an initial denaturation step at 94 °C/4 min, followed by 35 cycles of denaturation at 94 °C/30 s, primer annealing at 58 °C, and extension at 72 °C/40 s. The HRM step includes denaturation at 95 °C/60 s, annealing at 40 °C/60 s, gradual denaturation from 65 °C to 97 °C by 0.05 °C/s, and fluorescence detection 20 times per °C.

2.6.2. Analysis of Molecular Genetic Diversity

The HRM semi-autonomous data analysis was performed with LightCycler® 96 SW 1.1 software (Roche Diagnostics, Germany). The samples were assigned to groups, according to the amplicon melting temperature (Tm), the shape of the normalized melting curves, and the difference plots, generated by the HRM analysis, with different groups denoting distinct genetic profiles, and a binary matrix was formed, where "1" indicates the presence

of a specific allelic pattern, and "0" indicates its absence, for further analyses. For all 11 cultivars, the genetic diversity indices and the polymorphic loci content were calculated. The genetic distances of the accessions were estimated based on Nei's genetic distance [36] and were used for tree construction under the neighbor-joining method. The polymorphic information content of the SSR markers was calculated according to Smith et al. [37]. Additionally, principal coordinates analysis (PCoA) was carried out using R software [38].

Table 3. Primer sequences and annealing temperature of the SSRs.

Marker	Primers	Ta (°C)
LSSR06a	F: 5′-GTTGTTTTGGGACAACACCC-3′	54
	R: 5′-AAAACCCGAACCTGTGTAGC-3′	
LSSR07	F; 5′-GGATCAGGTGCTTTCAGTCTTG-3′	56
	R: 5′-AACCTCATCGAGTGTGAGACTGTAC-3′	
LSSR10	F: 5′-CGTCATCCATCAATGTTTGG-3′	54
	R: 5′-TTAAGGAAACACTGGCCCAT-3′	
LSSR11	F: 5′-TCCCTTGCTCTTTTCCTCAC-3′	53
	R: 5′-CCGTTTAGCACATTGGCAC-3′	
LSSR14	F: 5′-GGTGACCCTCACCAGAACAT-3′	54
	R: 5′-GGTCCTTTGATGATGGTGCT-3′	
LSSR41	F: 5′-TCAAGGGTGCTCAGTATGGG-3′	56
	R: 5′-TCATCCTTTCCCTCCAAAGA-3′	

2.7. Metabolomic Analysis

Metabolomic Profiling of Lupin Seeds

Four cultivars (Magnus, Multitalia, Sulimo and Figaro) were used for the metabolomic analysis. These varieties were selected based on their alkaloid content (low vs. high, i.e., Multitalia-high/Magnus, Sulimo, Figaro-low) as well as their good combining ability in crosses (Magnus, Sulimo and Figaro). All chemicals and reagents that were used in lupin seed extraction and extracts' derivatization were of the highest commercially available purity [39,40]. Ribitol, which was used as the internal standard, was purchased from Sigma-Aldrich Ltd. (Steinheim, Germany). Lupin seeds from 4 cultivars were grinded in a grinding mill, and 100 mg of the resulting fine powder was added into Eppendorf tubes (1.5 mL). In total, six biological replications were performed per cultivar. The extraction of seeds' metabolites was performed following a previously described protocol [39,40], using 1.0 mL of a methanol-ethyl acetate mixture (1:1 v/v). The resulting extracts were filtered and finally evaporated to dryness. The dried extracts were derivatized in a two-step process using methoxylamine hydrochloride in pyridine and N-methyl-N-(trimethyl-silyl) trifluoroacetamide (MSTFA, Macherey-Nagel, Düren, Germany). The polar metabolome of seeds was recorded employing an Agilent 6890 MS (Agilent Technologies Inc., Santa Clara, CA, USA) platform that was equipped with a 5973 inert mass selective detector (MSD). Previously described analytical conditions were used [39,40]. The analyzer was controlled using the MSD ChemStation E.02.01.1177 software (Agilent Technologies Inc., Santa Clara, CA, USA). Metabolomics data pre-processing and their analyses for the detection of trends and the corresponding biomarkers was based on a pipeline that we have previously described [39,40]. The former was performed using the software AMDIS v.2.66, the mass spectra library of NIST 08 (National Institute of Standards and Technology library, NIST, Gaithersburg, MD, USA) and MS DIAL v.4.90 [41]. The latter was based on multivariate analysis performed using the software SIMCA-P+ v.16.0. (Umetrics, Sartorius Stedim Data Analytics AB, Umeå, Sweden).

3. Results

3.1. Phenotypical Analysis: Morphological and Agronomical Traits

Lupin cultivars tested in this experiment originated from different sources and could be discriminated into two groups (commercial cultivars and advanced breeding lines). Most of the cultivars were characterized by high homogeneity in some phenotypic traits such as the type of plant development and type of inflorescences, color of flowers in central inflorescence and color of the flower keel (Table 4). Specifically, all genotypes have an intermediate type of development, raceme type of inflorescences, and white color of flowers, except cv. Estoril and cv. Multitalia, with black and white and blue-black color for the flower keel (Table 4). Significant morphological differentiations were detected in the shape and color of leaves, type of flowers, and also color of flower wings, between the two groups of commercial cultivars and advanced breeding lines (Table 4).

Table 4. Phenotypic data referring to the type of development and related traits.

Lupin Cultivar Genotype	Flower Color	Leaf: Intensity of Green Color	Central Leaflet: Width	Type of Inflorescence	Color of the Flower Keel	Color of Flower Wings	Type of Development
Estoril	White	medium	medium	raceme	blue-black	white–violet	Indeterminate
FAS Sweet	White	dark	medium	raceme	blue-black	white	Indeterminate
Figaro	White	medium	medium	raceme	blue-black	white	Indeterminate
Magnus	White	dark	medium	raceme	blue-black	white	Indeterminate
Multitalia	White	medium	medium	raceme	blue-black	white-violet	Indeterminate
Orus	White	dark	medium	raceme	blue-black	white	Indeterminate
Sulimo	White	dark	medium	raceme	blue-black	white	Indeterminate
Ulysse	White	dark	medium	raceme	blue-black	white–violet	Indeterminate
LKML	White	medium	board	raceme	blue-black	blue–white	Indeterminate
LKAP	White	medium	board	raceme	blue-black	violet	Indeterminate
LKAU	White	light	board	raceme	blue-black	white	Indeterminate

According to the yield and its components, the advanced lines (LKAM, LKAU and LKAP) were more productive than the commercial cultivars as the latter were improved for high yield, unless characterized by a high concentration of alkaloids, as presented later in the physicochemical analysis (Tables 5 and 6). Among commercial cultivars, cv. Sulimo had the highest yield and is statistically differentiated from all other lines used into this experiment (Table 5). The advanced breeding line LKAP was the best, yielding 356.5 g, and presented statistically significant differences from all other cultivars and advanced lines tested (Table 5). The mean yield was 179.26 g, having a range from 66.9 to 356.5 g. Among all yield components studied in our experiments, the size and weight of pods are the main characteristics which most influence the total yield in lupin (Table 5). As presented in this table, the advanced lines (LKAP, LKML and LKAU) were characterized by high yield, indicative of the large size and weight of pods. The number of seeds per pod was related to the size of pods and may impact the total yield as confirmed from our data (Table 5).

Table 5. Yield components of Lupin cultivars.

Code	Lupin Cultivar	Yield (gr)	Chlorophyll Content at Stage of Anthesis	Weight of 100 Pods (gr)	Weight of 100 Seeds (gr)	Size of 10 Random Pods (cm)	Number of Seeds/Pod
L1	Estoril	163.6 c*	31.12 b	172 c	42.6 a	8.95 b	4
L2	Fas Sweet	89.9 de	42.50 a	185 c	28.8 c	8.15 b	4
L3	Figaro	111.4 d	39.94 ab	180 c	35.4 b	6.7 d	3
L4	Magnus	80.4 de	34.20 ab	178 c	35.3 b	7.85 c	4
L5	Multitalia	185.6 c	30.6 b	180 c	26.8 c	7.45 c	4
L6	Orus	66.9 e	33.80 b	160 d	24.2 c	7.75 c	4

Table 5. *Cont.*

Code	Lupin Cultivar	Yield (gr)	Chlorophyll Content at Stage of Anthesis	Weight of 100 Pods (gr)	Weight of 100 Seeds (gr)	Size of 10 Random Pods (cm)	Number of Seeds/Pod
L7	Sulimo	248.5 b	31.85 b	204 b	37.6 b	7.3 c	4
L8	Ulysse	120.9 cd	32.40 b	157 d	34.6 b	7.35 c	3
L9	LKML	287.7 b	18.54 c	170 cd	36.9 b	9.25 a	5
L10	LKAP	356.5 a	19.22 c	193 bc	33.7 b	8.55 cd	5
L11	LKAU	266.8 b	18.04 c	243 a	34.3 b	9.25 a	5
	Mean	179.26	30.161	184.2	34.34	8.11	4.1

* values followed by different letters within a column indicate significant differences according to the Duncan test ($p \leq 0.05$).

Table 6. Antinutritional traits of lupin cultivars and advanced lines.

Code	Lupin Cultivar	Total Alkaloids (%)	Total Phenols (mg GAE/100 g)	Total Hydrolyzed Tannins (mg GAE/100 g)	Total Condensed Tannins (mg PCNB/100 g)
L1	Estoril	0.025 ef *	80.11 efg	36.82 d	13.93 g
L2	Fas Sweet	0.035 de	93.75 d	24.09 f	22.01 b–e
L3	Figaro	0.009 f	71.93 g	35.46 de	20.91 b–f
L4	Magnus	0.015 ef	88.30 de	27.27 def	23.45 bcd
L5	Multitalia	0.23 c	377.05 a	298.18 b	18.40 ef
L6	Orus	0.020 ef	77.73 fg	25.91 ef	24.75 b
L7	Sulimo	0.054 d	60.34 h	30.91 def	41.05 a
L8	Ulysse	0.004 f	83.86 ef	35.46 de	23.60 bc
L9	LKML	0.312 a	366.82 b	309.54 a	19.27 c–f
L10	LKAP	0.260 b	322.16 c	272.27 c	17.15 fg
L11	LKAU	0.233 c	326.59 c	280.91 c	19.12 def

* values followed by different letters within a column indicate significant differences according to the Tukey test ($p \leq 0.05$).

Chlorophyll content at the stage of anthesis had no correlation to the total yield but could be related to the weight of seeds and size of pods. Fast Sweet, Figaro and Magnus were characterized by high chlorophyll content at the stage of anthesis, as opposed to the advanced breeding lines LKAU, LKML and LKAP with the lower content, unless proven as the most dynamic for their yield potential (Table 5).

The weight of 100 pods ranged from 157 to 243 g, with significant differences among tested lupin cultivars. The advanced line LKAU presented the highest value (Table 5).

The weight of 100 seeds ranged from 28.8 to 37.6 g and differentiated significantly among the cultivars tested. Cultivar Estoril had the highest, and Fas Sweet had the lowest weight of seeds. All lupin cultivars (commercial and advanced lines) have a quite similar weight of 100 seeds, which is very close to the mean weight (34.34 g). Some differentiations were also observed in the size of pods, mainly among lupin cultivars with the advanced lines, and two commercial cultivars (Estoril and Fas Sweet) having the higher values (Table 5).

3.2. Nutritional Traits and Antinutritional Traits

Significant differences among the cultivars and advanced lines were detected for all the studied antinutritional and nutritional traits (Tables 6 and 7). The total alkaloid content was significantly lower in cultivars compared to the advanced lines, except for cultivar Multitalia. The same trend was also observed for total phenols and total hydrolyzed tannins. On the other hand, the advanced lines tend to have a significantly lower content of total condensed tannins compared to cultivars, except for cultivar Estoril, which had the lowest content (Table 6).

Table 7. Nutritional traits of lupin cultivars and advanced lines.

Code	Lupin Cultivar	CP (g/kg DM)	NDF (g/kg DM)	ADF (g/kg DM)	ADL (g/kg DM)
L1	Estoril	321.83 ef *	216.93 c	140.22 cd	9.49 ab
L2	Fas Sweet	387.26 c	204.22 de	135.04 cd	8.86 ab
L3	Figaro	459.83 b	208.69 cde	134.09 cd	8.70 ab
L4	Magnus	310.14 f	256.63 a	184.99 a	11.73 a
L5	Multitalia	364.45 cde	199.89 e	133.46 d	6.18 b
L6	Orus	339.85 def	209.17 cde	143.13 c	7.69 ab
L7	Sulimo	383.97 c	215.65 c	138.21 cd	9.00 ab
L8	Ulysse	334.01 def	232.70 b	166.7 b	8.74 ab
L9	LKML	527.66 a	208.87 cde	140.09 cd	8.79 ab
L10	LKAP	308.68 f	212.74 cd	141.78 cd	9.49 ab
L11	LKAU	373.23 cd	206.67 cde	141.29 cd	9.15 ab

* values followed by different letters within a row indicate significant differences according to the Tukey test ($p \leq 0.05$).

The higher CP content was recorded in the advanced line LKML followed by cultivar Figaro. Although cultivar Multitalia was characterized by high content of total alkaloids, it had the lowest content in NDF, ADF and ADL. Inversely, cultivar Magnus, with low content of total alkaloids, had the highest content of NDF, ADF and ADL (Table 7).

Seeds of *L. albus* have been utilized for both animal and human nutrition [42]. It is well known that *L. albus* seeds are a high protein source as soybean seeds [7,43]. The advanced line LKML had the higher CP content in comparison to the others. In general, the CP and fiber content were comparatively high and low, respectively, and inside the ranges that were mentioned in a previous study for white lupin [44,45].

The total alkaloids were significantly lower in the commercial cultivars compared to advanced lines, except cultivar Multitalia. It is noteworthy that Multitalia is one of the most commonly used cultivars in Greece. Similar results have been found by Musco et al. [44] for the historical Italian variety. It is remarkable that genotypes with the lowest total alkaloid content have been found in either varieties or advanced lines. Kroc et al. [46] observed a very wide variation of total alkaloids (0.016 to 12.73%), analyzing 367 samples of white lupin that included natural populations, and improved series and varieties. The utilization of these results could lead in the enlargement of the Lupinus breeding gene pool.

In general, all the cultivars had lower concentrations of total phenols than the advanced lines, with the exception of the Multitalia cultivar, which has the same trend in total phenols as in alkaloids. Variations of lupin species in phenolic contents have been observed among different cultivars and growth locations [47]. Concerning the condensed tannins, the advanced lines tend to have a significantly lower content of total condensed tannins compared to cultivars, with the only exception of cultivar Sulimo, which had the higher content. However, the concentration of CT of all tested varieties and advanced lines was <50 g/kg DM, which means that the voluntary feed intake of the ruminants could not be reduced. It is known that the consumption of forage with a concentration of CT < 50 g/kg DM may lead to a greater availability of amino acid absorption and perhaps improve animal performance under grazing, as reviewed by Min et al. [48] and Besharati et al. [49].

3.3. Molecular Analysis

The SSR-HRM analysis of 10 commercial varieties with six polymorphic microsatellites elucidated 94 distinct allelic patterns, which were assumed to be different genotypes. For each marker, the number of alleles ranged from 13 (LSSR14) to 19 (LSSR41). The marker PIC values ranged from 0.8 (LSSR14) to 0.96 (LSSR41), with an average of 0.86, indicating their functionality in population studies (Figure 2b). The genetic polymorphism indices are presented in detail in Table 8. The polymorphic content of the cultivars ranged from 25.5% (Magnus, Orus and Ulysse) to 35.1% (Figaro), with an average of 29.8%. Generally, white lupin is considered self-pollinating; however, high levels of cross-pollination have been previously reported in white lupin flowers due to tripping, mediated by *A. mellifera*,

Bombus spp., *Andrena ovulata*, *Andrena labialis*, *Osmia* sp. and *Eucera* sp. [50–52]. The PCoA, where the first two axes explain 71.3% of the observed variability, distributes the cultivars into two groups, as shown in Figure 2a. The cultivars Ulysse, FAS, Sulimo and Figaro and the breeding materials LKAP, LKML and LKAU are grouped together, separate from the cultivars Magnus, Orus and Estoril. This was confirmed with genetic distance analysis, depicted in the dendrogram in Figure 2d(i). In a previous study, we examined a set of commercial varieties along with local landraces and determined genetic relationships using the same molecular markers and bulked seed sampling [23]. We re-analyzed data of this study, only including the set of genotypes examined here, and the genetic distance is depicted in a dendrogram (Figure 2d(ii)). The neighbor joining analysis resulted in the formation of a two-clustered dendrogram, where cv. Multitalia and the breeding lines LKAP, LICML and LKAU are grouped together, separate from the rest of the cultivars. This is indicative of the genetic proximity of those breeding lines with cv. Multitalia, suggesting that they might share a common origin (Figure 1d). Since Multitalia was not included in the genetic analysis of the present study, we assume that its genetic characteristics are relative to the breeding lines.

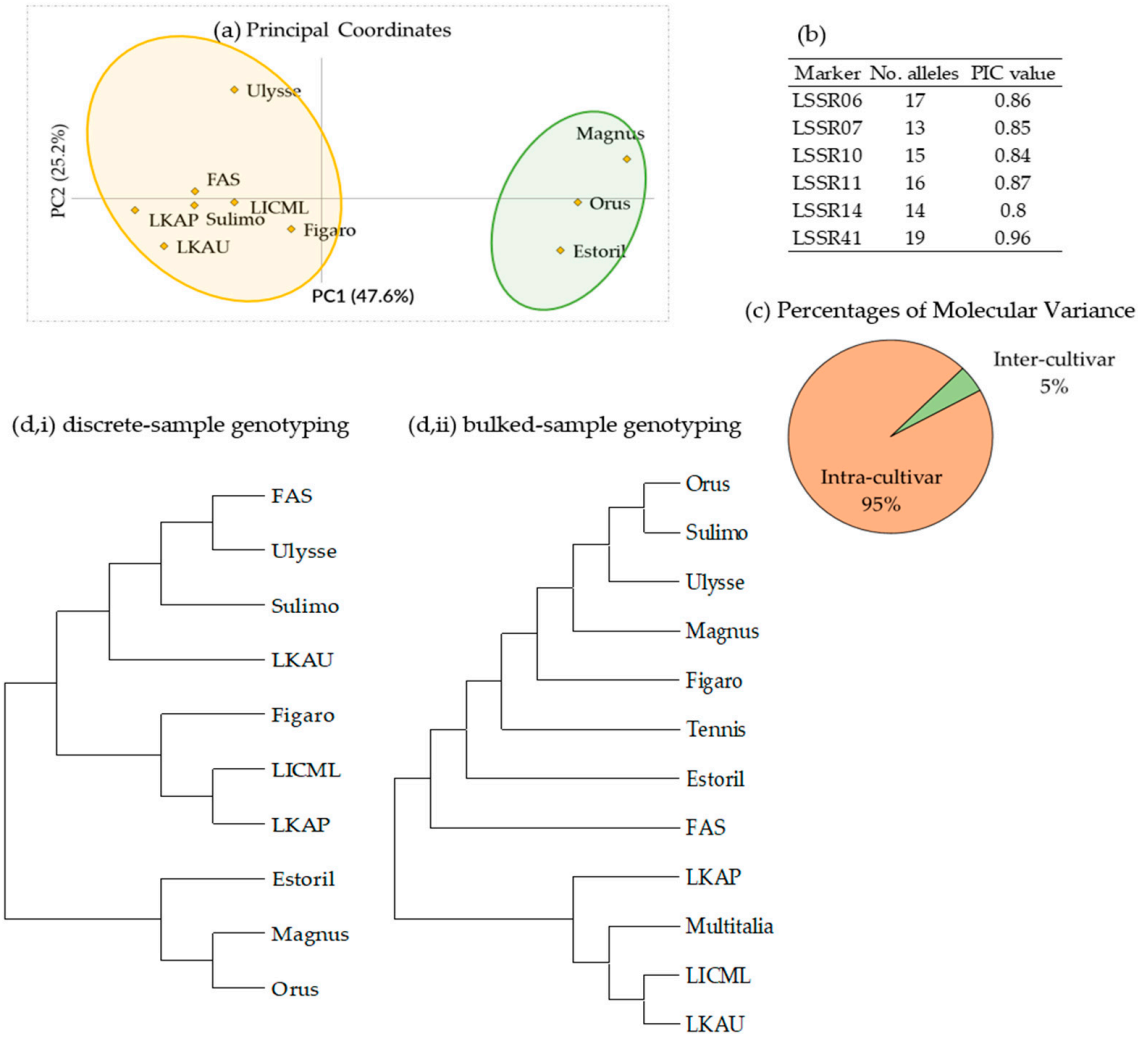


Figure 2. (**a**) Principal coordinates analysis of the molecular genetic diversity observed among 10 *L. albus* cultivars, where the first two coordinates explain 72.8% of the total variability; (**b**) polymorphic information content of the 6 SSRs used in the study; (**c**) AMOVA pie plot where 95% of the observed variability is assigned within each cultivar ($p < 0.001$); (**d**) comparison of the genetic relationships of the accessions, based on genotyping of (**i**) individual and (**ii**) re-analyzed bulked samples.

Table 8. Genetic polymorphism indices of the 10 lupin cultivars.

Lupin Cultivar	Na	Ne	I	h	uh	P%	No. Alleles	No. Private Alleles
Estoril	0.6 ± 0.1	1.14 ± 0.03	0.14 ± 0.02	0.09 ± 0.02	0.10 ± 0.02	29.8%	28	4
FAS	0.6 ± 0.1	1.15 ± 0.03	0.14 ± 0.02	0.09 ± 0.02	0.11 ± 0.02	29.8%	28	3
Figaro	0.7 ± 0.1	1.14 ± 0.03	0.15 ± 0.02	0.10 ± 0.02	0.11 ± 0.02	35.1%	33	3
Magnus	0.5 ± 0.1	1.13 ± 0.03	0.13 ± 0.02	0.08 ± 0.02	0.09 ± 0.02	25.5%	24	3
Orus	0.6 ± 0.1	1.15 ± 0.03	0.15 ± 0.02	0.09 ± 0.02	0.11 ± 0.02	25.5%	28	2
Sulimo	0.6 ± 0.1	1.15 ± 0.03	0.15 ± 0.02	0.09 ± 0.02	0.11 ± 0.02	31.1%	30	5
Ulysse	0.5 ± 0.1	1.13 ± 0.03	0.12 ± 0.02	0.08 ± 0.02	0.09 ± 0.02	25.5%	24	2
LKML	0.6 ± 0.1	1.14 ± 0.03	0.14 ± 0.02	0.09 ± 0.01	0.11 ± 0.02	30.9%	29	3
LKAP	0.6 ± 0.1	1.14 ± 0.03	0.14 ± 0.02	0.09 ± 0.02	0.11 ± 0.02	29.8%	28	5
LKAU	0.6 ± 0.1	1.13 ± 0.03	0.14 ± 0.02	0.09 ± 0.02	0.11 ± 0.02	29.8%	28	5
Mean	0.6 ± 0.03	1.14 ± 0.01	0.14 ± 0.01	0.09 ± 0.01	0.1 ± 0.01	29.8%	28	3.5

* Na = number of different alleles, Ne = number of effective alleles, I = Shannon's information index, h = diversity, uh = unbiased diversity, P% = percentage of polymorphic loci.

Re-analysis of the previously examined dataset, and comparison to genotyping of individual samples of each cultivar reveal similar genetic relationships for some accessions, although the two dendrograms do not match perfectly. It has been shown that bulking might affect the discrimination efficacy of SSR-HRM genotyping [53], suggesting that analysis of individual genotypes is a more comprehensive approach.

3.4. Metabolomics Analysis

A targeted GC/EI/MS method was used to obtain a seed-specific metabolite profile, in four *Lupinus albus* cultivars. The OPLS-DA (Figure 3) separated samples into two clusters, with L7 (*L. albus* cv. Sulimo) and L8 (*L. albus* cv. Figaro) defined as one cluster, and the other cluster defined by L3 (*L. albus* cv. Multitalia) and L4 (*L. albus* cv. Magnus). OPLS-DA was applied to discriminate between cultivars and extract potential signatory metabolites corresponding to each cultivar. The OPLS-DA model had a goodness-of-fit (R^2Y) of 0.978 with a predictability (Q^2Y) of 0.974, where PC1 and PC2 explained 33.3% and 32.9% of the variation, respectively, separating the samples into distinct groups. By applying a hierarchical cluster analysis using the Ward's method, the same two main groups were evident (Figure 4). The first group consisted of L3 and L4, while the second group consisted of L7 and L8. Selected metabolites responsible for the differences between group 1 and group 2 are presented in Figure 5. The importance and significance of the selected metabolites were determined by analyzing the VIP plot with jack-knifing uncertainty bars. Discriminant features identified by OPLS-DA (Figure 3) were selected based on the variable importance in projection (VIP) scores (Figure 5). In Figures 5 and 6, the annotated discriminant metabolites obtained from multivariate analyses with the highest leverage to the observed separation among cultivars, are displayed.

Cultivars Multitalia and Magnus accumulated several metabolites known for their role against abiotic stress tolerance (i.e., pipecolic acid, GABA, citric acid, etc.) [54–56]. On the other hand, the content in several beneficial metabolites for human and animal nutrition was profoundly increased in cv. Sulimo and cv. Figaro compared to cv. Magnus and cv. Multitalia. The most substantial differences were monitored in α,α-trehalose and γ-tocopherol contents, metabolites known for their importance as antioxidants as well as oil stability factors [57–60]. Recently, attention has been drawn into unraveling lupin genetic material with high seed oil quantity and quality [61]. In our study, fatty acid content, mainly the oleic acid and linoleic acid content, were noticeably higher in cv. Sulimo and cv. Figaro. However, their content in free amino acids was lower compared to the other two cultivars. This is in accordance with previous studies in soybean, where it was indicated that oil content was negatively correlated to protein content [62]. Finally, the content of 13-hydroxy-lupanine, one of the main anti-nutritional factors found in lupin

seeds, was substantially lower in cv. Sulimo and cv. Figaro, similar to the results previously presented by Zafeiriou et al. [23].

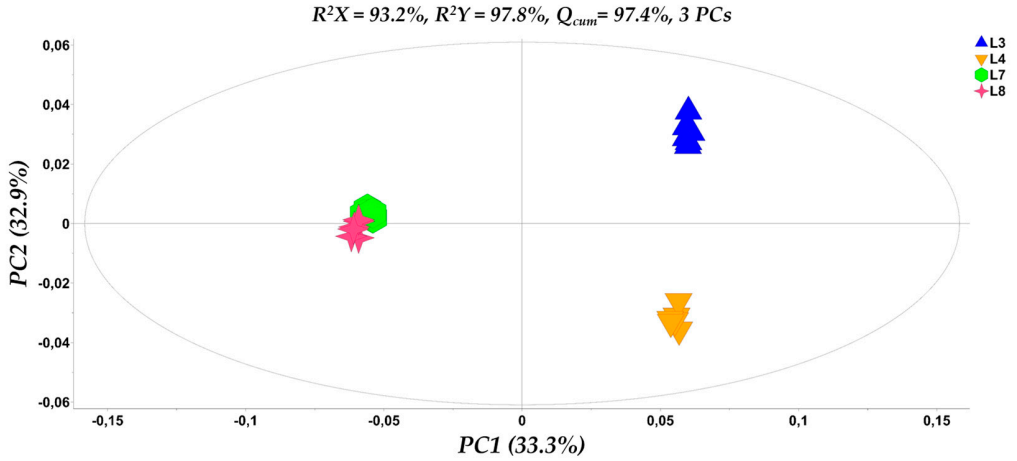


Figure 3. Orthogonal partial least squares discriminant analysis (OPLS-DA) PC1/PC2 score plot for the analyzed GC/EI/MS metabolite profiles of *Lupinus albus* cultivars. The ellipse represents the Hotelling's T^2 [R^2X and R^2Y; fraction of the sum of squares of X's and Y's explained by the components, $Q^2_{(cum)}$; cumulative fraction of the total X's variation that can be predicted, PC; principal component].

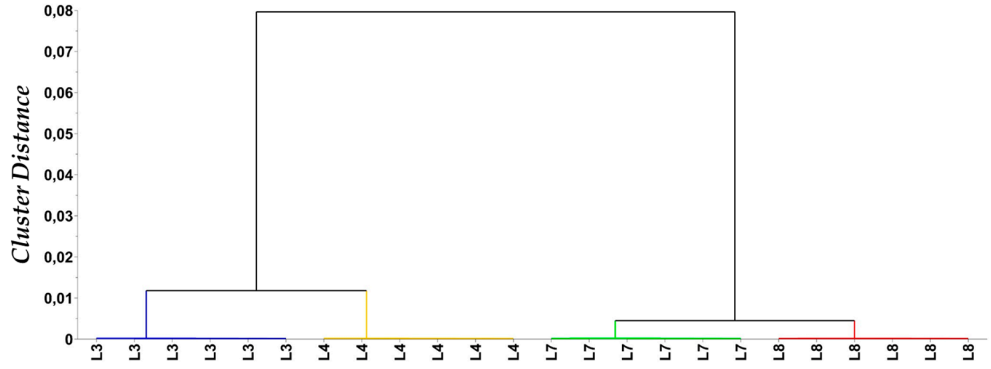


Figure 4. Hierarchical tree diagram of the GC/EI/MS metabolite profiles of *Lupinus albus* cultivars using the Ward's method.

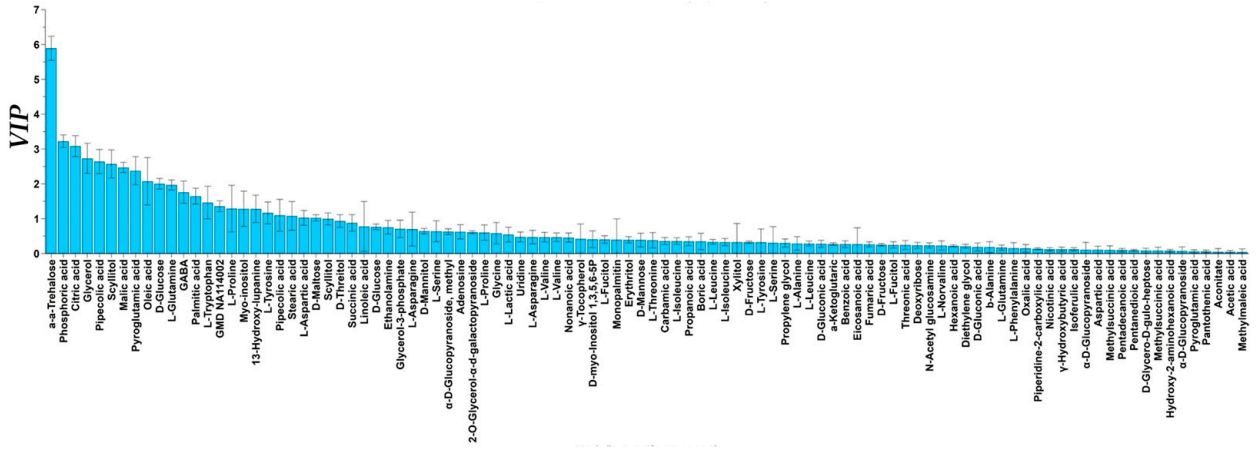


Figure 5. Variable influence on projection (VIP) plot, displaying the VIP values of the annotated *Lupinus albus* cultivar seeds' metabolites and the corresponding jack-knife uncertainty bars. High VIP values correspond to metabolites with high leverage to the observed separation among cultivars.

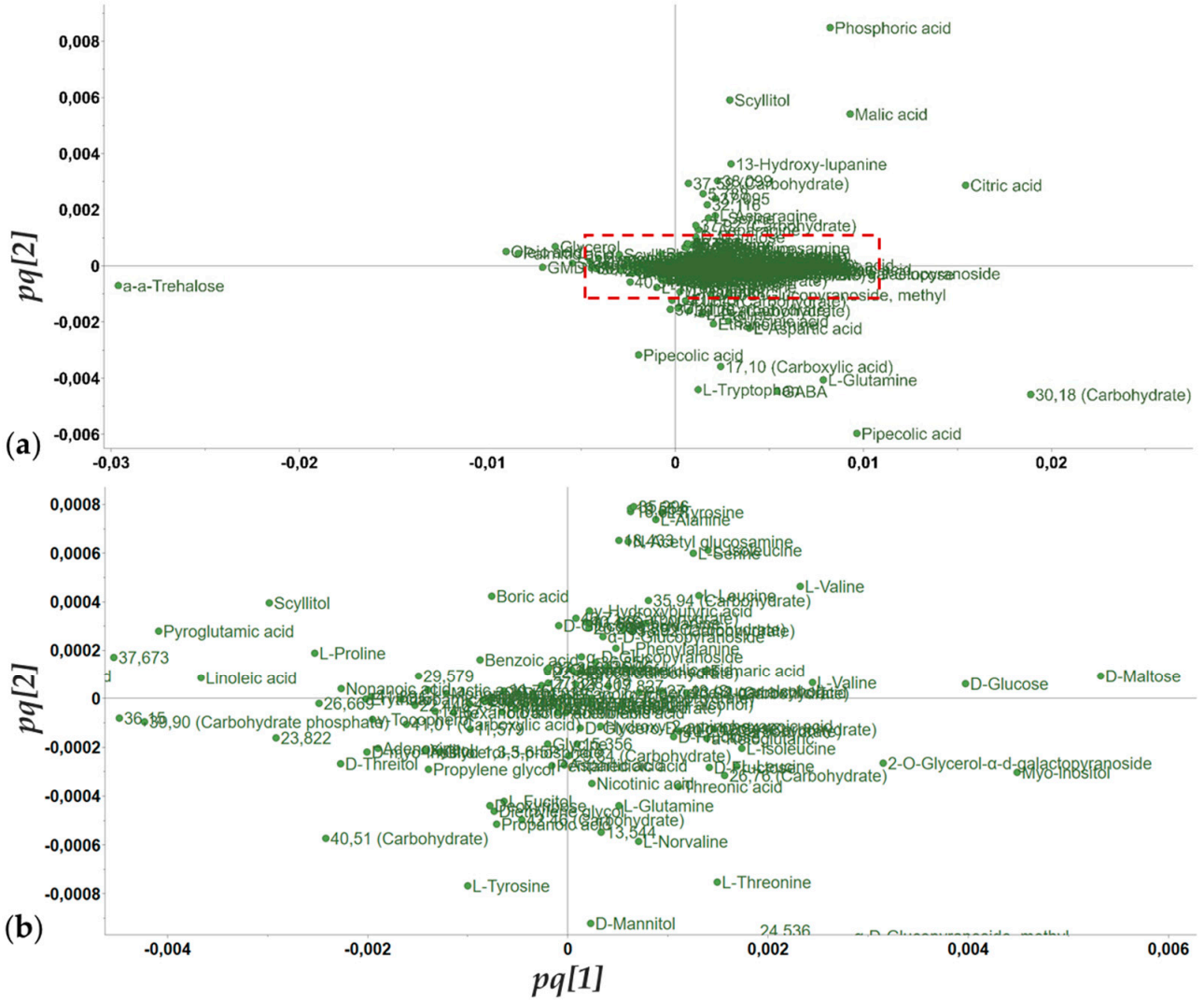


Figure 6. (**a**) OPLS-DA predictive component loading scatter plot for the discrimination among the analyzed *Lupinus albus* cultivar seeds' metabolites; (**b**) magnification of the red dashed area of the upper panel.

4. Conclusions

The phenotypical analysis revealed that both the studied cultivars and the advanced breeding lines were characterized by high homogeneity in traits that were related to inflorescences and flower color. On the other hand, they are differentiated in traits that are related to shape and color of the leaves. It is noteworthy that the advanced breeding lines presented higher seed production compared to the commercial cultivars. Regarding the nutritive value, all the studied genetic material was characterized by a high content of proteins. All the studied anti-nutritional factors, except the total alkaloids, were within the limits that do not affect the intake and animal's performance. All commercial cultivars assessed, with the exception to Multitalia, possessed lower alkaloid content compared to the advanced breeding lines.

According to the genetic analysis based on the SSR markers, it seems that Multitalia and the advanced breeding lines might share a common ancestor. Among the examined cultivars, Magnus, Orus and Estoril were grouped together, indicating genetic proximity. Nevertheless, according to the metabolomics analysis, Magnus was grouped with Multitalia, while Figaro was grouped with Sulimo. The metabolite profile of the latter was particularly promising, mainly in terms of its content in beneficial metabolites for human and animal nutrition.

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Data Availability Statement: The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflicts of Interest: The authors declare no conflict of interest.

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