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Changes of Isoflavones during the Growth Cycle of *Lupinus albus*

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The objective of this study was to monitor the changes in isoflavone content in different plant organs (leaves, stems, roots) during the crop growth stage of three cultivars of *Lupinus albus* (white lupin) under field conditions, taking into account sowing time effects (autumn and early spring) and cultivar effects. Three sampling dates (from late vegetative to late grain growth stages) were evaluated. Seven isoflavones and four flavonoids were identified by LC-ESI-MS analysis. The isoflavone content was higher in leaves than in stems, and it was highest before flowering, whereas it decreased during maturity. Autumn-sown plants showed higher isoflavone content than early spring-sown plants, especially in late vegetative and early reproductive stages. Genistein 7-*O*-glucoside was the main isoflavone of leaves and stems in the late vegetative stages of early spring sowing, whereas genistein was the main isoflavone under autumn sowing. Variation among cultivars affected only marginally the total isoflavone content. No isoflavones were detected in seeds.

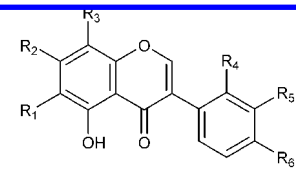
KEYWORDS: White lupin; genetic variation; LC-ESI-MS; hydroxyl isoflavone; prenyl isoflavone

INTRODUCTION

Isoflavones are an important group of secondary metabolites synthesized by plants belonging to the Fabaceae family. They exhibit a wide spectrum of biological activities. In particular, they play a key role as signaling molecules during the interaction of the legumes with nitrogen-fixing bacteria (*1, 2*) and are synthesized and accumulated as phytoalexins in response to pathogen attacks; isoflavones are also constitutively expressed and serve in antifungal defense (*3*).

Due to their structural similarity to mammalian β-estradiol, isoflavones are known to possess estrogenic activity (*4*). These compounds appear to influence the cardiovascular system and the production, metabolism, and biological activity of sex hormones, as well as malignant cell proliferation, differentiation, and angiogenesis (*4, 5*). Recently, skepticism has developed concerning the true potential of phytoestrogens to beneficially modify these processes. Furthermore, contrasting data have been reported on the potential of phytoestrogens to prevent hormone-dependent cancers (e.g., breast and prostate) and to successfully treat postmenopausal complaints, an indication for which they are widely used (*6*). These potentially negative findings have led health authorities in several countries to suggest maximum daily intake levels for phytoestrogens (*7*).

Every legume plant has a specific isoflavone pattern. In *Lupinus albus* L. (white lupin), it consists of 3 simple isoflavones (aglycones), 5 mono- and 3 diprenylated derivatives, 33 complex isoflavones, and 11 simple or complex isoflavone glycosides (*8, 9*). Many other minor isoflavonoids with different patterns of prenylation on the isoflavone skeleton or with additional pyran or furan rings have also been identified (*8*). The main isoflavones of white lupin are listed in **Figure 1**. The prenylated isoflavones,



Compound	R_1	R_2	R_3	R_4	R_5	R_6
2'-IIydroxygenistein 7,4'-*O*-diglucoside	II	OGlc	II	OII	II	OGlc
2'-Hydroxygenistein 7-*O*-glucoside	H	OGlc	H	OH	H	OH
2'-Hydroxygenistein 4'-*O*-glucoside	H	OH	H	OH	H	OGlc
Genistein 7-*O*-glucoside	H	OGlc	H	H	H	OH
2'-Hydroxygenistein	H	OH	H	OH	H	OH
Genistein	H	OH	H	H	H	OH
Luteone	Pre	OH	H	OH	H	OH
Wighteone	Pre	OH	H	H	H	OH

Figure 1. General structure of the isoflavones and their conjugates identified in extracts from different organs of *Lupinus albus*.

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Table 1. Onset of Flowering and Maturity Dates of Three Cultivars of *L. albus* under Autumn and Early Spring Sowing[a]

		onset of flowering		maturity	
cultivar	type	autumn	early spring	autumn	early spring
Luxe	dwarf, sweet-seeded	May 7, 2005	May 21, 2005	July 4, 2005	July 10, 2005
Molise ecotype	tall, bitter-seeded	April 29, 2005	May 15, 2005	July 3, 2005	July 8, 2005
Multitalia	tall, sweet-seeded	April 25, 2005	May 11, 2005	July 1, 2005	July 7, 2005

[a] Sowing dates: Nov 15, 2004, for autumn sowing; Feb 15, 2005, for early spring sowing. Sampling dates: April 14, 2005; May 19, 2005; and June 23, 2005.

wighteone and luteone, have been isolated as typical prohibitins (preinfectional fungitoxins) on the surface of the leaves and have been confirmed as phytoalexins, being inducibly biosynthesized when lupin plants are treated with biotic or abiotic elicitors (*10, 11*).

Some literature data indicate that in lupin plants the content of isoflavones is affected by the development stage, being negligible in seeds and high in leaves and roots (*11, 12*). The characterization of isoflavone pattern has been carried out mainly on young lupin plants grown in laboratory conditions (*11–14*), with the exception of two recent studies relative to open-field experiments (*15, 16*).

The objective of this study was to monitor the changes of isoflavone composition and content in different plant organs (leaves, stems, and roots) during different growth stages under field conditions. Possible genetic differences were evaluated by testing three cultivars of different types and origins. Environmental effects related to sowing time (autumn or early spring) were also investigated.

MATERIALS AND METHODS

Materials. Four commercial isoflavone standards were used for identification and quantification: genistein (>98% purity, Fluka, Milan, Italy), genistein 7-*O*-glucoside (>99% purity, Fluka), and 2′-hydroxygenistein and luteone (>99% purity, Apin, Abingdon, Oxon, U.K.). HPLC-grade acetonitrile, methanol, and acetic acid were purchased from Baker (Deventer, The Netherlands); HPLC-grade water was prepared with a Milli-Q water purification system (Millipore, Billerica, MA). HPLC eluents were filtered through disposable nylon filters (0.45 μm) (Alltech, Milan, Italy); samples were filtered through HPLC syringe filters (0.45 μm) (Alltech).

Plant Material. Three *L. albus* cultivars were investigated: Luxe and Multitalia, two sweet varieties of French and Italian origin, respectively, and one Italian alkaloid-rich ecotype collected in Molise (Molise ecotype). The genetic diversity of Luxe and Multitalia is also revealed by their different plant architectures (dwarf and tall, respectively) and adaptation pattern across Italian environments, whereas both of these cultivars differ markedly from the Molise ecotype also in terms of seed weight (below 0.45 g, relative to >0.85 g for Molise ecotype) (Annicchiarico, unpublished results).

Experimental Design and Field Conditions. The experiment design was a split-plot with three replications holding sowing dates on main plots and cultivars on subplots. The cultivars were grown under rainfed conditions in Lodi (Lombardy, northern Italy), a location characterized by a subcontinental climate with relatively cold winters and favorable soil conditions for lupin growth (i.e., subacid pH and low content of $CaCO_3$). The 2004−2005 cropping season was characterized by the following climatic features: lowest temperature, −9 °C; number of frost days, 78; spring (from March 1 to June 15, 2005) mean temperature, 14.2 °C; spring rainfall, 182 mm. Lupin seeds were treated with active ingredients iprodione (0.95 g/kg of seed) and carbendazim (0.45 g/kg of seed) and were inoculated with NPPL HiStick of Becker Underwood prior to sowing. The fertilization rate was 20 kg/ha of N, 48 kg/ha of P_2O_5, and 112 kg/ha of K_2O. The seed rate was 45 germinating seeds/m^2, and the sowing depth was 3 cm. The plot size was 9 m^2.

Sampling. Two sowing times were assessed: late autumn (sowing A), sowing date November 15, 2004; and early spring (sowing S), sowing date February 15, 2005. Three sampling dates at 35-day intervals were considered: April 14, May 19, and June 23, 2005. Considering the flowering and maturity dates of each cultivar reported in **Table 1**, the first sampling took place from 1.5 to 3 weeks before the onset of flowering in autumn-sown material and from 3 to 5 weeks before the onset of flowering in early spring-sown plants; the second sampling occurred from 2 to 3.5 weeks after the onset of flowering in autumn-sown material and around the onset of flowering in early spring-sown plants; the third sampling took place during late grain filling in all cases. Each sample (per replication and sampling date) was represented by eight random plants collected from each plot.

Isoflavone Extraction. The harvested samples were freeze-dried and then ground to a fine powder. Each sample (1 g) was suspended in 5 mL of acetonitrile, 2.5 mL of water, and 1 mL of 0.1 N HCl. The suspension was sonicated for 15 min, then stirred at room temperature for 2 h and filtered through a glass filter. The solvent was evaporated to dryness under vacuum, keeping the temperature below 30 °C. The solid residue was dissolved in 1 mL of 80% aqueous methanol for the HPLC analyses (*17, 18*). The procedure was further scaled down to analyze samples of 500, 200, and 100 mg, changing the dissolving volume to keep the weight/volume ratio of 1.

LC-UV Analysis. The HPLC analyses of isoflavones were performed on an Agilent HP-1100 quaternary pump fitted with a Rheodyne injector (5 μL, loop) and equipped with a HP-1050 diode array detector (DAD). Data were processed with a HP Chemstation (for LC 3D, Agilent Technologies, Palo Alto, CA). The analyses were carried out on a 150 mm × 2.1 mm i.d., 3 μm, Alltima C18 LC-MS column (Alltech). The injection volume was 5 μL and the flow rate, 0.2 mL/min; chromatograms were recorded at 254 nm, and spectra data were registered in the range of 190−600 nm. Mobile phase A was water/acetonitrile/acetic acid (95:4.5:0.5, v/v/v), whereas mobile phase B was acetonitrile/water/acetic acid (95:4.5:0.5, v/v/v); after 5 min at 10%, solvent B was increased to 30% at 40 min and then to 100% in 8 min and held at 100% for 60 min.

HPLC-ESI-MS. HPLC-ESI-MS/MS analyses were performed on a HP-1100-MSD ion trap system SL version (Agilent Technologies). HPLC conditions were the same as described for LC-UV analyses; the column eluent was split 1:1 before entering the electrospray interface. The LC-MS analysis conditions were as follows: capillary voltage, 4000 V; drying gas flow, 11 L/min; temperature, 350 °C; nebulizer gas pressure, 60 psi. Spectra were acquired in negative ion mode; data were acquired either in full scan or in MS/MS mode (*17*).

Qualitative and Quantitative Analysis. All samples were analyzed by HPLC-ESI-MS to identify and characterize each isoflavone; peaks were attributed by taking into account the MS data and by comparing their retention times with those of authentic standards. The quantitation was done on the basis of UV analysis, taking into account the area of each identified peak. Isoflavone stock solutions were prepared by dissolving pure standards in methanol to give a 1 mg/mL concentration for all isoflavones except for 2′-hydroxygenistein, which was 0.1 mg/mL. Each stock solution was then diluted to achieve a range of concentration between 0.25 and 0.005 mg/mL for genistein and genistein 7-*O*-glucoside and between 0.125 and 0.0025 mg/mL for all other isoflavones. The linearity of each calibration curve was assured by an R^2 of at least 0.99. The limit of quantification (LOQ) and the limit of detection (LOD) were 0.5 and 0.25 μg/mL, respectively, for all isoflavones (S/N > 3) except in the case of genistein, for which the same limits were 0.1 and 0.05 μg/mL (S/N > 3). The precision of the method was estimated by analyzing genistein solutions within the same day (intraday with $n = 10$) and in different days (interday with $n = 5$), obtaining relative

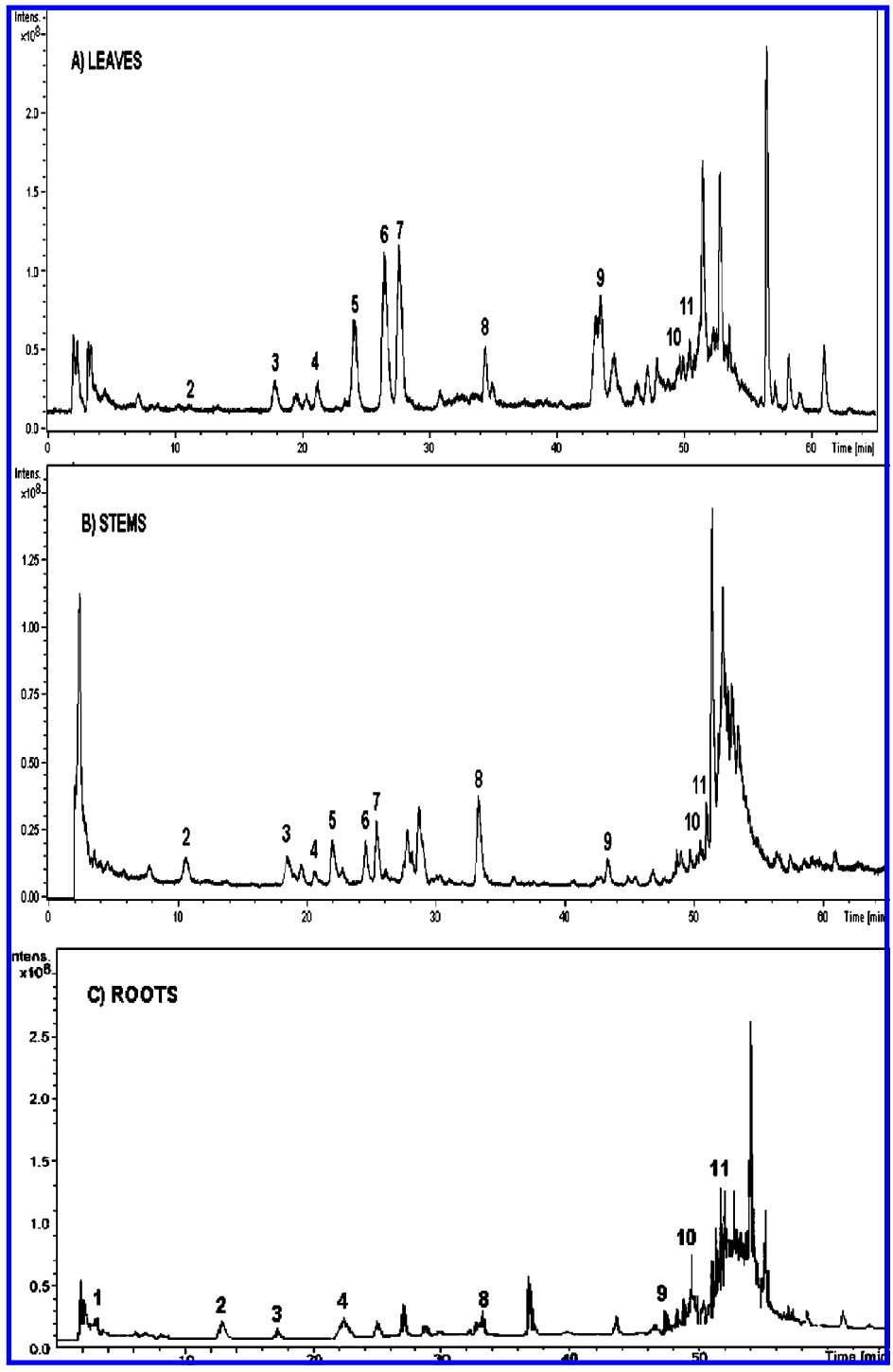

Figure 2. LC-ESI-MS chromatograms of (**A**) leaves, (**B**) stems, and (**C**) roots of *L. albus* cv. Luxe under early spring sowing: (**A**, **B**) second sampling date; (**C**) third sampling date. Peaks: 1, 2′-hydroxygenistein 7,4′-*O*-diglucoside; 2, 2′-hydroxygenistein 7-*O*-glucoside; 3, 2′-hydroxygenistein 4′-*O*-glucoside; 4, genistein 7-*O*-glucoside; 5, kaempferol 3-*O*-glucoside; 6, kaempferol 3-*O*-galactoside + isorhamnetin 3-*O*-glucoside; 7, isorhamnetin 3-*O*-galactoside; 8, 2′-hydroxygenistein; 9, genistein; 10, luteone; 11, wighteone.

standard deviations (RSD%) of 2−3 and 5−6%, respectively. The recovery, evaluated by spiking the seeds of *L. albus* (Molise ecotype) with known amounts of genistein, was always >95%.

Statistical Analysis. All analyses were run in triplicate. Statistical analysis, including linear regression and ANOVA test, was performed using the software Statgraphics Plus (2.1 for Windows) (Statgraphics Centurion, Herndon, VA).

RESULTS

Isoflavone Identification. In the literature, the MS analyses of isoflavones were mostly performed in positive ion mode (*11, 13–15, 19, 20*). Nevertheless, we have set up a different MS method using negative ion mode, which works quite well on different matrix samples (*17, 21*). In our analyses, seven isoflavones and four flavonoids were identified in leaf and stem samples (**Figure 2**). Their spectrometric data are listed in **Table 2**. In LC-ESI-MS/MS analyses, the peak with retention time (t_R) of 11.3 min showed two ions at *m*/*z* 447 and 284.9, corresponding to the molecular ion of 2′-hydroxygenistein 7-*O*-glucoside and a fragment deriving from the loss of glucose. Peak 3 was the structural isomer 2′-hydroxygenistein 4′-*O*-

Table 2. Identified Isoflavones and Flavonoids in Leaves, Stems, and Roots of *L. albus* by HPLC-ESI-MS Analysis and Their Spectrometric Data

peak	compound	t_R (min)	MS data	leaves	stems	roots
1	2′-hydroxygenistein 7,4′-*O*-diglucoside	3.5	447 [(M − H) − Glc]$^-$; 284.9 [(M − H) − Glc]$^-$	−	−	+
2	2′-hydroxygenistein 7-*O*-glucoside	11.3	447 [M − H]$^-$; 284.9 [(M − H) − Glc]$^-$	+	+	+
3	2′-hydroxygenistein 4′-*O*-glucoside	19.4	447 [M − H]$^-$; 284.8 [(M − H) − Glc]$^-$	+	+	+
4	genistein 7-*O*-glucoside	21.1	431.1 [M − H]$^-$; 268.9 [(M − H) − Glc]$^-$; 491.0 [(M − H) + CH_3COO^-]$^-$	+	+	+
5	kaempferol 3-*O*-glucoside	24.0	447.0 [M − H]$^-$; 284.9 [(M − H) − Glc]$^-$	+	+	−
6	kaempferol 3-*O*-galactoside isorhamnetin 3-*O*-glucoside	26.4	447.0 [M − H]$^-$; 284.9 [(M − H) − Glc]$^-$ 477 [M − H]$^-$; 314.9 [(M − H) − Glc]$^-$	+	+	−
7	isorhamnetin 3-*O*-galactoside	27.6	477 [M − H]$^-$; 314.9 [(M − H) − Glc]$^-$	+	+	−
8	2′-hydroxygenistein	34.0	284.9 [M − H]$^-$; 240.9 [(M − H) − CO_2]$^-$	+	+	+
9	genistein	42.9	268.9 [M − H]$^-$	+	+	+
10	luteone	50.8	353 [M − H]$^-$; 285 [(M − H) − prenyl]$^-$	+	+	+
11	wighteone	52.0	337 [M − H]$^-$	+	+	+

glucoside. Because they have the same pattern of fragmentation, the definitive identification of the two isomers was done by comparing their relative $t_R$with literature data (*12*). The peak at 21.1 min matched with genistein 7-*O*-glucoside, with the MS spectra showing two peaks at *m*/*z* 431.1 and 268.9, corresponding to the [M − H]$^-$ ion and the fragment deriving from glucose loss. Moreover, an ion at *m*/*z* 491.0 was detected, corresponding to the adduct with the acetic acid present in the mobile phase. The presence of these adducts was often registered in ESI analyses, and it could be useful for identification purposes. Peaks 5−7 agreed with flavonoid derivatives, and, in particular, peak 5 was kaempferol 3-*O*-glucoside. Peak 6 showed two [M − H]$^-$ ions at *m*/*z* 447 and 477, corresponding to the coelution of kaempferol 3-*O*-galactoside and isorhamnetin 3-*O*-glucoside, and peak 7 was assigned to isorhamnetin 3-*O*-galactoside. The flavonoid detection and their coelution was confirmed by literature data (*11, 12*). The peak at 34.0 min corresponded to the 2′-hydroxygenistein; its spectrum showed the [M − H]$^-$ ion at *m*/*z* 284.9 and the fragment obtained from the neutral loss of CO_2 at *m*/*z* 240.9. The MS spectrum of peak 9 showed a single ion at *m*/*z* 268.9, corresponding to the [M − H]$^-$ ion of genistein, and no fragmentation was detected, even in the MS/MS experiment. This was in agreement with previous work (*21*). Peak 10 was assigned to luteone because of the presence of the molecular ion at *m*/*z* 353 and a fragment at *m*/*z* 285 due to the loss of the prenyl group. The other prenylated isoflavone, wighteone, was identified as the chromatographic peak at t_R 52.0 min because of the presence of the [M − H]$^-$ ion at *m*/*z* 335. Similarly to genistein, this compound also did not show any fragmentation in an MS/MS experiment. The assignments of 2′-hydroxygenistein, genistein 7-*O*-glucoside, genistein, and luteone were confirmed by comparison with t_R and MS data of authentic standards.

In roots, the pattern of isoflavones was quite similar to those in leaves and stems. The main differences were the detection of 2′-hydroxygenistein 7,4′-*O*-diglucoside and the complete absence of flavonoids (**Table 2**). Both immature and mature seeds were analyzed, but no isoflavones were detected, in agreement with literature data (*22*).

Changes of Isoflavones in Different Stages of Organ Growth. The content across sampling dates of the main isoflavones, that is, 2′-hydroxygenistin, genistein 7-*O*-glucoside, 2′-hydroxygenistein, genistein, and luteone, is provided in **Table 3** for leaves, in **Table 4** for stems, and in **Table 5** for roots.

In leaves of the autumn-sown plants, four isoflavones were quantified in the following order of abundance: genistein > genistein 7-*O*-glucoside > 2′-hydroxygenistein > 2′-hydroxygenistein 7-*O*-glucoside (**Table 3**). The total amount of isoflavones ranged from 25.20 mg/g in the Molise ecotype to 34.48 mg/g in Multitalia on the first sampling date (a few weeks before the onset of flowering) and from 1.85 mg/g (Molise ecotype) to 8.89 mg/g (Multitalia) on the last sampling date (late grain-filling stage). The decreases in total isoflavone content of leaves between these sampling dates were 74% for Multitalia, 89% for Luxe, and 93% for the Molise ecotype. The most abundant isoflavone was genistein, which represented 61−81% of the total isoflavone content of leaves depending on the cultivar and the sampling date (**Table 3**). Multitalia had the greatest content of isoflavones on the first and last sampling dates (**Table 3**).

The isoflavone content of stems from autumn-sown plots showed a fairly similar pattern and changes across sampling dates, that is, a sharp reduction from the first to the third sampling date (90% for the Molise ecotype, 91% for Multitalia, and 93% for Luxe) and the overwhelming relative presence of genistein (**Table 4**). Overall stems tended to contain less isoflavone than leaves. Luteone, however, which was usually present only in traces in the leaves (**Table 3**), was consistently detected in the stems on the last sampling date (**Table 4**). The trend toward greater total isoflavone content in Multitalia relative to the other cultivars was less pronounced in stems (**Table 4**) than in leaves (**Table 3**).

On average, in early spring sowing, the total isoflavone content was lower than in autumn-sown plants (**Tables 3** and **4**), both in leaves (decrease of 29% on the first sampling date, 65% on the second, and 64% on the third) and in stems (decrease of 51% on the first sampling date, 73% on the second, and 52% on the third). The second difference was the pattern of isoflavones. Indeed, five isoflavones were detected in lupin fresh organs: 2′-hydroxygenistein 7-*O*-glucoside, genistein 7-*O*-glucoside, 2′-hydroxygenistein, genistein, and luteone (quantified only in the leaves of the second sampling date of sweet cultivars). In Luxe and Multitalia, on average, the isoflavones ranked in the following order of abundance: genistein 7-*O*-glucoside > 2′-hydroxygenistein 7-*O*-glucoside > genistein > 2′-hydroxygenistein > luteone. On the other hand, in the Molise ecotype the order was genistein 7-*O*-glucoside > genistein > 2′-hydroxygenistein 7-*O*-glucoside > 2′-hydroxygenistein.

Moreover, genistein 7-*O*-glucoside was the main isoflavone of early spring-sown plants on the first sampling date, when it accounted for about 90% of total isoflavones in leaves (**Table 3**) and for 66% (Molise ecotype) to 72% (Multitalia) of total isoflavones in stems (**Table 4**). This contrasts with the predominant content of genistein observed in leaves and stems under autumn sowing (**Tables 3** and **4**). A third difference between sowing times concerned the comparison of the cultivars, for which Multitalia, in early spring-sown leaves, did not confirm the trend toward greater isoflavone content that had emerged after autumn sowing (**Table 3**). One last difference between

Table 3. Isoflavone Content (Milligrams per Gram) of Leaves in Three Cultivars of *L. albus* (Mean ± SD)

cultivar	sampling[a]	2′-hydroxygenistein 7-*O*-glucoside	genistein 7-*O*-glucoside	2′-hydroxygenistein	genistein	luteone	total isoflavones
Molise ecotype	A1	0.16 ± 0.03	3.48 ± 0.08	0.40 ± 0.04	21.13 ± 0.56	traces[b]	25.20 ± 0.70
Molise ecotype	A2	0.13 ± 0.04	1.74 ± 0.41	0.110 ± 0.003	6.50 ± 1.57	traces	8.48 ± 2.03
Molise ecotype	A3	0.023 ± 0.007	0.61 ± 0.06	0.07 ± 0.02	1.14 ± 0.35	traces	1.85 ± 0.44
Molise ecotype	S1	0.89 ± 0.10	19.29 ± 0.94	0.19 ± 0.02	0.98 ± 0.1	traces	21.30 ± 1.16
Molise ecotype	S2	0.18 ± 0.04	3.27 ± 0.58	0.08 ± 0.01	0.66 ± 0.10	traces	4.18 ± 0.73
Molise ecotype	S3	0.41 ± 0.22	2.05 ± 0.02	0.12 ± 0.06	0.22 ± 0.03	traces	2.80 ± 0.32
Luxe	A1	0.86 ± 0.05	6.64 ± 0.73	0.66 ± 0.04	18.58 ± 0.83	traces	26.7 ± 1.65
Luxe	A2	0.17 ± 0.02	0.96 ± 0.17	0.24 ± 0.03	6.76 ± 0.79	traces	9.16 ± 0.45
Luxe	A3	0.14 ± 0.04	0.38 ± 0.03	0.23 ± 0.02	2.13 ± 0.06	traces[b]	2.88 ± 0.03
Luxe	S1	1.40 ± 0.34	19.04 ± 3.30	0.081 ± 0.003	0.38 ± 0.03	traces	20.9 ± 3.54
Luxe	S2	0.059 ± 0.001	0.627 ± 0.004	0.64 ± 0.05	2.12 ± 0.18	0.044 ± 0.005	3.49 ± 0.23
Luxe	S3	0.28 ± 0.03	0.30 ± 0.02	0.51 ± 0.04	0.23 ± 0.05	traces	1.31 ± 0.06
Multitalia	A1	0.75 ± 0.01	8.27 ± 1.62	0.60 ± 0.04	24.88 ± 1.44	traces	34.48 ± 3.11
Multitalia	A2	0.11 ± 0.01	1.69 ± 0.06	0.133 ± 0.004	8.19 ± 0.42	traces	10.12 ± 0.35
Multitalia	A3	0.40 ± 0.024	0.64 ± 0.09	0.38 ± 0.01	7.47 ± 1.03	traces	8.89 ± 0.96
Multitalia	S1	1.18 ± 0.13	15.14 ± 0.82	0.15 ± 0.01	0.73 ± 0.04	traces	17.20 ± 0.90
Multitalia	S2	0.14 ± 0.03	0.80 ± 0.04	0.17 ± 0.01	0.54 ± 0.01	0.06 ± 0.02	1.71 ± 0.11
Multitalia	S3	0.27 ± 0.01	0.47 ± 0.10	0.13 ± 0.03	0.22 ± 0.10	traces	1.09 ± 0.02

[a] A1, A2, A3, autumn sowing, sampling dates 1 (April 14, 2005), 2 (May 19, 2005), and 3 (June 24, 2005), respectively; S1, S2, S3, early spring sowing, sampling dates 1, 2, and 3, respectively. [b] <LOQ.

Table 4. Isoflavone Content (Milligrams per Gram) of Stems in Three Cultivars of *L. albus* (Mean ± SD)

cultivar	sampling[a]	2′-hydroxygenistein 7-*O*-glucoside	genistein 7-*O*-glucoside	2′-hydroxygenistein	genistein	luteone	total isoflavones
Molise ecotype	A1	0.27 ± 0.02	1.63 ± 0.05	0.78 ± 0.02	20.51 ± 1.8	traces[c]	23.18 ± 1.85
Molise ecotype	A2	nd[b]	0.22 ± 0.03	0.021 ± 0.002	2.226 ± 0.125	traces	2.47 ± 0.156
Molise ecotype	A3	0.039 ± 0.006	0.038 ± 0.008	0.633 ± 0.046	1.485 ± 0.396	0.233 ± 0.003	2.43 ± 0.46
Molise ecotype	S1	1.223 ± 0.128	7.145 ± 0.820	0.581 ± 0.066	1.847 ± 0.001	traces	10.80 ± 1.02
Molise ecotype	S2	nd	0.251 ± 0.038	0.023 ± 0.006	0.230 ± 0.037	traces	0.50 ± 0.08
Molise ecotype	S3	0.152 ± 0.023	0.052 ± 0.007	0.586 ± 0.013	0.201 ± 0.007	0.258 ± 0.010	1.25 ± 0.06
Luxe	A1	0.877 ± 0.007	2.980 ± 0.086	0.999 ± 0.009	23.56 ± 0.33	traces	28.41 ± 0.42
Luxe	A2	0.017 ± 0.001	0.104 ± 0.010	0.126 ± 0.002	4.765 ± 0.089	traces	4.99 ± 0.07
Luxe	A3	0.104 ± 0.024	0.022 ± 0.009	0.432 ± 0.036	1.271 ± 0.407	0.198 ± 0.048	2.03 ± 0.41
Luxe	S1	2.216 ± 0.085	8.417 ± 0.179	0.534 ± 0.008	1.086 ± 0.033	traces	12.25 ± 0.30
Luxe	S2	0.350 ± 0.010	1.051 ± 0.070	0.145 ± 0.011	0.539 ± 0.043	traces	2.08 ± 0.12
Luxe	S3	0.096 ± 0.008	0.024 ± 0.003	0.666 ± 0.053	0.205 ± 0.072	0.280 ± 0.066	1.27 ± 0.20
Multitalia	A1	1.091 ± 0.141	2.909 ± 0.343	1.088 ± 0.169	27.44 ± 3.67	traces	32.21 ± 4.32
Multitalia	A2	nd	0.191 ± 0.021	0.041 ± 0.005	2.812 ± 0.285	traces	3.05 ± 0.30
Multitalia	A3	0.136 ± 0.003	0.036 ± 0.009	0.986 ± 0.026	2.275 ± 0.144	0.131 ± 0.006	3.56 ± 0.11
Multitalia	S1	3.073 ± 0.052	12.923 ± 0.001	0.613 ± 0.001	1.386 ± 0.110	traces	17.99 ± 0.06
Multitalia	S2	nd	0.191 ± 0.040	0.057 ± 0.013	0.346 ± 0.087	traces	0.60 ± 0.14
Multitalia	S3	0.092 ± 0.001	0.032 ± 0.001	0.429 ± 0.013	0.247 ± 0.055	0.219 ± 0.003	1.02 ± 0.04

[a] A1, A2, A3, autumn sowing, sampling dates 1 (April 14, 2005), 2 (May 19, 2005), and 3 (June 23, 2005), respectively; S1, S2, S3, early spring sowing, sampling dates 1, 2, and 3, respectively. [b] Not detected, i.e., no UV signal. [c] <LOQ.

Table 5. Isoflavone Content (Milligrams per Gram) of Roots in Three Cultivars of *L. albus* (Mean ± SD)

cultivar	sampling[a]	2′-hydroxygenistein 7,4′-*O*-diglucoside	2′-hydroxygenistein 7-*O*-glucoside	genistein 7-*O*-glucoside	2′-hydroxygenistein	genistein	total isoflavones
Molise ecotype	S1	traces[b]	0.278 ± 0.015	0.728 ± 0.036	0.448 ± 0.077	0.170 ± 0.012	1.62 ± 0.11
Luxe	S2	traces	0.415 ± 0.029	0.912 ± 0.026	1.488 ± 0.238	0.248 ± 0.030	2.88 ± 0.05
Multitalia	S3	traces	0.469 ± 0.003	0.967 ± 0.047	1.128 ± 0.103	0.207 ± 0.007	2.80 ± 0.19

[a] S1, S2, S3, early spring sowing, sampling dates 1 (April 14, 2005), 2 (May 19, 2005), and 3 (June 23, 2005), respectively. [b] <LOQ.

sowing dates was that luteone could be observed in detectable amounts under early spring sowing in the leaves of the sweet cultivars on the second sampling date (**Table 3**) and in the stems of all cultivars on the third sampling date.

Also, early spring-sown plants exhibited a sharp decrease in total isoflavone content between the first and last sampling dates, both in leaves (decreases of 87% in the Molise ecotype and 94% in Luxe and Multitalia) and in stems (decreases of 88% in the Molise ecotype, 90% in Luxe, and 94% in Multitalia). The difference in total isoflavones between leaves and stems was more pronounced under early spring sowing than autumn sowing for the Molise ecotype and Luxe. On the other hand, Multitalia had similar isoflavone contents in the two plant organs (**Tables 3** and **4**). The relative contents of isoflavones in leaves and stems were similar also under early spring sowing.

In the roots of ripening plants of early spring sowing, the total isoflavone content was fairly low but comparable with, or greater than, that observed in leaves and stems of plants in the same sowing time and sampling date (**Table 5**). It ranged from about 2.8 mg/g (in Luxe and Multitalia) to 1.6 mg/g in the Molise ecotype. The main isoflavone was genistein 7-*O*-glucoside in the Molise ecotype and the hydroxylated aglycone in Multitalia and Luxe (**Table 5**).

DISCUSSION

It is well-known that in order to achieve exact results at least a five-year data comparison would be needed. Nevertheless, some preliminary considerations may be stated after a one-year experiment. This study, unlike earlier ones, enabled environmental and cultivar effects on the isoflavone content of lupin leaf, stem, and root, under field conditions, to be assessed simultaneously. On the whole, our qualitative data were in agreement with available literature, in particular regarding stems and leaves (*11–16*). The main differences were due to the absence of the malonyl and acetyl derivatives in root samples. In the literature there are some stability studies on malonyl and acetyl isoflavone derivatives, to verify their likely acid hydrolysis, but they are carried out at high temperature, that is, 80 °C (*23, 24*); no acid hydrolysis occurs with our method, because the extraction was performed at room temperature. Moreover, other papers have demonstrated that acid extraction improves the extraction yield (*17, 18, 21, 25*). Thus, it is possible conclude that our analytical procedure is efficient in extracting and detecting acetyl and malonyl isoflavones (*17, 21*). As a matter of fact, earlier studies showed that malonyl derivatives are abundant only in the early stages of plant development, such as 6-week-old plants cultivated in laboratory conditions (*26*). Garcia-Lopez et al. (*15*) detected only one malonyl isoflavone in leaves of 14-week-old plants, whereas Von Baer et al. (*16*) detected only four isoflavones, that is, genistein 7-*O*-glucoside, 2′-hydroxygenistein, genistein, and licoisoflavone A in flowered and ripe plants of *L. albus* and *L. angustifolius* under field conditions in Chile. The trend toward a greater isoflavone content in leaves than in other plant organs has been reported in other legume species, for example, red clover (*27*), and has been attributed to the greater frequency of pathogen attack to which leaves are subjected (*11*). The greater isoflavone content of late vegetative and early reproductive stages compared to later stages may be explained by the greater impact on plant survival and reproduction of biotic and environmental stresses in younger plants and the consequent need to maximize plant defense (*28, 29*).

The observed total isoflavone content was higher than in earlier studies, but it is known to vary in relation to environmental conditions (temperature, light exposure, and drought stress) and the type or age of the plants (*11, 16, 26, 30*). The greater isoflavone contents detected in earlier stages of autumn-sown plants as compared to early spring-sown ones were probably due to the low-temperature stress that these plants had to face during their vegetative development. Early spring-sown plants essentially escape winter cold stress by the time their emergence is complete. The greater content of aglycone isoflavones at the first sampling date in each autumn-sown plant organ (77% of total isoflavones) relative to early spring-sown ones (4% of total isoflavones) is consistent with the observed increase of these compounds during elicitation experiments (*11*), supporting the hypothesis that autumn-sown plants undergo greater stress. The different development stage of the plants at the first sampling date, however, may have introduced some bias in the comparison of sowing dates. The difference between sowing times at successive development stages became progressively smaller because high temperatures and terminal drought led early spring-sown plants to accelerate their maturation and approach the maturity stage of autumn-sown plants (**Table 1**).

Ripening plants showed isoflavone contents in roots similar to or greater than that in other organs, unlike juvenile plants that exhibited a much greater content of isoflavones in roots (*11, 15, 26*). This difference may be due to the essential role that these compounds play as signaling molecules in nodulation during the very early stage of lupin growth (*31, 32*).

The three cultivars displayed similar changes of isoflavone content during the crop growth stage, showing that cultivar features marginally affect the isoflavone production; ANOVA results showed that in autumn-sown plants only Multitalia was significantly different from Luxe and the Molise ecotype ($P < 0.05$). On the other hand, early spring-sown plants showed no significant differences ($P < 0.05$). This is in good agreement with literature data showing that Multitalia proved to be less winter-hardy than Luxe or the Molise ecotype (*33*). It may be hypothesized that its greater production of isoflavones, as occurred after autumn sowing, was a heightened physiological response to low-temperature stress in this cultivar. As far as seeds were concerned, no isoflavones were detected either in green seeds or in mature seeds, in complete agreement with literature data (*12, 22*).

ABBREVIATIONS USED

EIC, extracted ion chromatogram; ESI, electrospray ionization; LOQ, limit of quantification; LOD, limit of detection; min, minutes; MS/MS, tandem mass spectrometry; t_R, retention time; TIC, total ion chromatogram.

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