EVOLUTION OF ISOFLAVONES DURING THE GROWTH OF PLANTS OF *LUPINUS ALBUS*

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ABSTRACT

The aim of this paper was to monitor the variation of the isoflavone content in different plant organs (leaves, stems and roots) during the crop growth cycle of three cultivars of *Lupinus albus* (white lupin) under field conditions, taking account of environmental effects due to sowing time (autumn and winter) and genetic effects. Three sampling dates were evaluated, considering different growth stages of the plants. Seven isoflavones and four flavonoids were identified. The isoflavone contents were higher in leaves than in stems and before flowering than during maturity. Autumn-sown plants contained more isoflavones than late winter-sown plants, especially at the first sampling date. Genetic features appeared to affect only marginally the isoflavones production.

KEYWORDS

White lupin, isoflavones, LC-ESI-MS, crop cycle

INTRODUCTION

Isoflavones are a biologically important group of secondary metabolites of plants from Fabaceae family. These compounds exhibit a wide spectrum of biological activities, i.e. have a key-role as signalling molecules during the nodulation process of legumes, and are phytoalexins in response to pathogen attacks (Subramanian *et al.* 2007; Katagiri *et al.* 2000). Generally, the isoflavones of *L. albus* consist of three simple isoflavones (aglycones), their glucosides and diprenylated derivatives (Katagiri *et al.* 2000). Available literature indicates that the lupin isoflavone content is affected by the stage of the plant development: in the seeds the isoflavone content is negligible, while it is very high in leaves and roots (Katagiri *et al.* 2000; Bednarek *et al.* 2001; Sirtori *et al.* 2004).

The studies on lupin isoflavones have been carried out mainly on young lupin plants grown in laboratory conditions or in a greenhouse (Katagiri *et al.* 2000; Bednarek *et al.* 2001), with the exception of two recent papers relative to open-field conditions (Garcia-Lopez *et al.* 2006; Von Baer *et al.* 2006). In our work, the changes in isoflavone concentration in different plant organs (leaves, stem and roots) were monitored during different development stages of the growth cycle of plants sowed in open-field conditions. Additional features of this investigation are that our data were collected simultaneously on three cultivars of *L. albus*: Molise Landrace, Multitalia, and Luxe, and two sowing times have been assessed in order to evaluate the influence of environmental stresses.

MATERIALS AND METHODS

The *L. albus* cultivars submitted to this investigation were, Luxe (L) and Multitalia (M), two sweet varieties of French and Italian origin, respectively; and a bitter Italian ecotype from Molise (Molise Landrace, ML). The cultivars were grown in Lodi (Lombardy, Northern Italy) that has optimal soil characteristics for lupin growth. The climatic data for 2004-2005 were: absolute minimum temperature: - 9°C; number of frost days: 78; spring mean temperature: 14.2°C; spring rainfall (from 1st March to 15th June): 182 mm. Two sowing times were assessed: late autumn (experiment A), sowing date (mm/dd/yy) 11/15/04 and late winter (experiment W), sowing date 02/15/05. Three different sampling dates were assessed: first sampling date: 04/14/05; second sampling date: 05/19/05; third sampling date: 06/23/05. Considering the flowering and maturity dates (Table 1), the first sampling took place 1.5-3 weeks before the onset of flowering in A experiment and 3-5 weeks before the onset of flowering in W experiment; the second sampling took place 2-3.5 weeks after the onset of flowering in A experiment and during the onset of flowering in W experiment; the third sampling took place during late grain filling (D'Agostina *et al.* 2008)

For isoflavone extraction, 1 g of freeze-dried and ground fresh tissue was suspended in 5 ml acetonitrile, 2.5 ml of water, and 1 ml HCl 0.1 N. The suspension was sonicated for 15 min, then stirred at room temperature for 2 h and filtered through a glass filter. After solvent evaporation, the solid residue was dissolved in 1 mL of 80% aqueous methanol, for HPLC

IN J.A. Palta and J.B. Berger (eds). 2008. 'Lupins for Health and Wealth' Proceedings of the 12th International Lupin Conference, 14-18 Sept. 2008, Fremantle, Western Australia. International Lupin Association, Canterbury, New Zealand. ISBN 0-86476-153-8.

Table 1. Onset of flowering and maturity dates of three cultivars of L. albus during A and W experiments.

Cultivar	Onset of flowering		Maturity	
	A experiment	W experiment	A experiment	W experiment
Luxe	05/07/05	05/21/05	07/04/05	07/10/05
Molise Landrace	04/29/05	05/15/05	07/03/05	07/08/05
Multitalia	04/25/05	05/11/05	07/01/05	07/07/05

Table 2. Identified flavonoids in lupin fresh tissues by HPLC-ESI-MS and their spectral data.

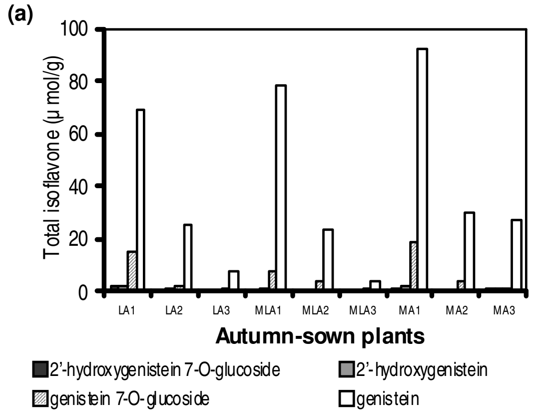
Flavonoid	Rt (min)	MS data	Leaves	Stems	Roots
2'-Hydroxygenistein ,4'-*O*-diglucoside	3.5	447 [(M-H)-Glc]$^-$; 284.9 [(M-H)-Glc]$^-$	-	-	+
2'-Hydroxygenistein 7-*O*-glucoside	11.3	447 [M-H]$^-$; 284.9 [(M-H)-Glc]$^-$	+	+	+
2'-Hydroxygenistein 4'-*O*-glucoside	19.4	447 [M-H]$^-$; 284.8 [(M-H)-Glc]$^-$	+	+	+
Genistein 7-*O*-glucoside	21.1	431.1 [M-H]$^-$; 268.9 [(M-H)-Glc]; 491.0 [(M-H)+CH_3COO^-]$^-$	+	+	+
Kaempferol 3-*O*-glucoside	24.0	447.0 [M-H]$^-$; 284.9 [(M-H)-Glc]$^-$	+	+	-
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]$^-$	+	+	-
Isorhamnetin 3-*O*-galactoside	27.6	477 [M-H]$^-$; 314.9 [(M-H)-Glc]$^-$	+	+	-
2'-Hydroxygenistein	34.0	284.9 [M-H]; 240.9 [(M-H)-CO_2]$^-$	+	+	+
Genistein	42.9	268.9 [M-H]$^-$	+	+	+
Luteone	50.8	353 [M-H]$^-$; 285 [(M-H)-Prenyl]$^-$	+	+	+
Wighteone	52.0	337 [M-H]	+	+	+

Table 3. Main isoflavones detected in different cultivars of L. *albus* stems; values are in mg/g as mean ± S.D.

Sample	2'-Hydroxygenistein 7-*O*-glucoside	Genistein 7-*O*-glucoside	2'-Hydroxygenistein	GENISTEIN	Luteone
MLA1	0.27 ± 0.02	1.63 ± 0.05	0.78 ± 0.02	20.51 ± 1.8	traces[a]
MLA2	n.d.	0.22 ± 0.03	0.021 ± 0.002	2.226 ± 0.125	traces[a]
MLA3	0.039 ± 0.006	0.038 ± 0.008	0.633 ± 0.046	1.485 ± 0.396	0.233 ± 0.003
MLW1	1.223 ± 0.128	7.145 ± 0.820	0.581 ± 0.066	1.847 ± 0.001	traces[a]
MLW2	n.d.[b]	0.251 ± 0.038	0.023 ± 0.006	0.230 ± 0.037	traces[a]
MLW3	0.152 ± 0.023	0.052 ± 0.007	0.586 ± 0.013	0.201 ± 0.007	0.258 ± 0.010
LA1	0.877 ± 0.007	2.980 ± 0.086	0.999 ± 0.009	23.56 ± 0.33	traces[a]
LA2	0.017 ± 0.001	0.104 ± 0.010	0.126 ± 0.002	4.765 ± 0.089	traces[a]
LA3	0.104 ± 0.024	0.022 ± 0.009	0.432 ± 0.036	1.271 ± 0.407	0.198 ± 0.048
LW1	2.216 ± 0.085	8.417 ± 0.179	0.534 ± 0.008	1.086 ± 0.033	traces[a]
LW2	0.350 ± 0.010	1.051 ± 0.070	0.145 ± 0.011	0.539 ± 0.043	traces[a]
LW3	0.096 ± 0.008	0.024 ± 0.003	0.666 ± 0.053	0.205 ± 0.072	0.280 ± 0.066
MA1	1.091 ± 0.141	2.909 ± 0.343	1.088 ± 0.169	27.44 ± 3.67	traces[a]
MA2	n.d.[b]	0.191 ± 0.021	0.041 ± 0.005	2.812 ± 0.285	traces[a]
MA3	0.136 ± 0.003	0.036 ± 0.009	0.986 ± 0.026	2.275 ± 0.144	0.131 ± 0.006
MW1	3.073 ± 0.052	12.923 ± 0.001	0.613 ± 0.001	1.386 ± 0.110	traces[a]
MW2	n.d.[b]	0.191 ± 0.040	0.057 ± 0.013	0.346 ± 0.087	traces[a]
MW3	0.092 ± 0.001	0.032 ± 0.001	0.429 ± 0.013	0.247 ± 0.055	0.219 ± 0.003

Sample abbreviations: ML: Molise Landrace, L: Luxe, M: Multitalia; A: late autumn, W: late winter; 1, 2, 3: number of sampling; a: < LOQ; b: no ion current.

analyses (Sirtori *et al.* 2004). The HPLC analyses of isoflavones were performed on an Agilent HP-1100 HPLC equipped with a HP-1050 Diode Array Detector (DAD). Data were processed with a HP Chemstation (for LC 3D). The analyses were carried out on an Alltima C18 LC-MS column (3 µm, 150 x 2.1 mm). The injection volume was 5 µL, 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. HPLC gradient was reported in Bednarek *et al.* 2001. HPLC-ESI-MS analyses were performed on a HP-1100-MSD Ion Trap system SL version. HPLC column and conditions were the same described for LC-UV analyses. The MS parameters were set according to a previous paper (Sirtori *et al.* 2004). Peaks were attributed taking into account the MS data and by comparing the retention times with those of authentic standards. The quantification of isoflavones was done on the basis of UV signal of the eluted peaks. The concentrations of calibration isoflavone solutions ranged from 0.25 to 0.005 mg/mL for genistein and genistein 7-O-glucoside, and 0.125 to 0.0025 mg/mL for all the other isoflavones. All experimental data were submitted to ANOVA test.



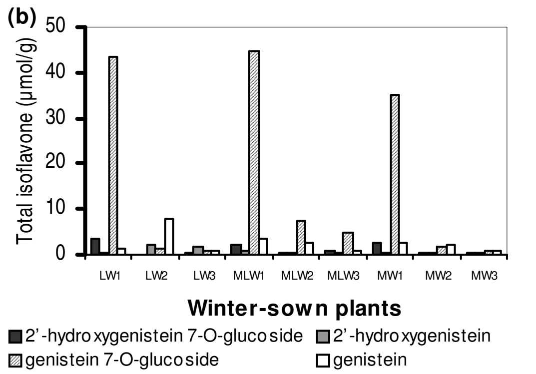


Fig. 1. Main isoflavones quantified in leaves of three varieties of L. albus: L: Luxe, ML: Molise Landrace, M: Multitalia, expressed as µmol/g; (a) autumn-sown plants A1, A2, A3: sampling dates (mm/dd/yy) 1 (04/14/2005), 2 (05/05/2005) and 3 (06/23/2005), respectively; (b) winter sown plants W1, W2, W3: sampling dates 1, 2 and 3, respectively.

RESULTS AND DISCUSSION

In leaf and stem samples, seven isoflavones and four flavonoids were identified, while in roots only the main isoflavones were detected whereas the flavonoids were absent (Table 2). Both green and mature seeds were analysed (data not shown) and no isoflavones were detected, according to literature data (Sirtori *et al.* 2004).

AUTUMN-SOWN PLANTS

In leaves, four isoflavones were quantified in the following order of abundance: genistein > genistein 7-*O*-glucoside > 2'-hydroxygenistein > 2'-hydroxygenistein 7-*O*-glucoside (Fig. 1a); genistein represented 61% to 81% of the total isoflavone content.

The total amount of isoflavones ranged from 25.20 mg/g in Molise Landrace (ML) to 34.48 mg/g in Multitalia (M) in the first sampling date and from 1.85 mg/g (ML) to 8.89 mg/g (M) in the last sampling date, with a decrease of 74% for M, 89% for Luxe (L) and 93% for the ML. The isoflavone content of stems (Table 3) showed similar patterns and evolutions to leaves: genistein was the most abundant isoflavone and a sharp decrease from the first to the third sampling date was observed, 90% for ML, 91% for M and 93% for L.

WINTER-SOWN PLANTS

The pattern of isoflavones was different from A experiment plants. Indeed five isoflavones were quantified in lupin fresh tissues: 2'-hydroxygenistein 7-*O*-glucoside, genistein 7-*O*-glucoside, 2'-hydroxygenistein, genistein and luteone. On average in L and M 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 ML the order was genistein 7-*O*-glucoside > genistein > 2'-hydroxygenistein 7-*O*-glucoside > 2'-hydroxygenistein. Genistein 7-*O*-glucoside was the main isoflavone in the first sampling date (about 90% of total isoflavones in leaves and from 66% to 72% in stems of ML and M, respectively). Luteone could be quantified in leaves of the sweet cultivars at the second development stage and in the stems of all cultivars at the third development stage. In general, the total isoflavone content in W experiment was lower than in A experiment (Fig. 1b and Table 3), either in leaves (-29% in the first development stage, -65% in the second, and -64% in the third) or in stems (-51% in the first development stage, -73% in the second, and -52% in the third). On the whole, the fresh organs from winter sown plants exhibited a decrease in total isoflavone content between the first and the last sampling date, both in leaves (-87% in ML, and -94% in L and M) and in stems (-88% in ML, -90% in L and -94% in M). In the roots of ripening plants of late winter sowing, the total isoflavone content was comparable to that observed in leaves and stems of plants in the same sowing time and sampling date (data not shown). The

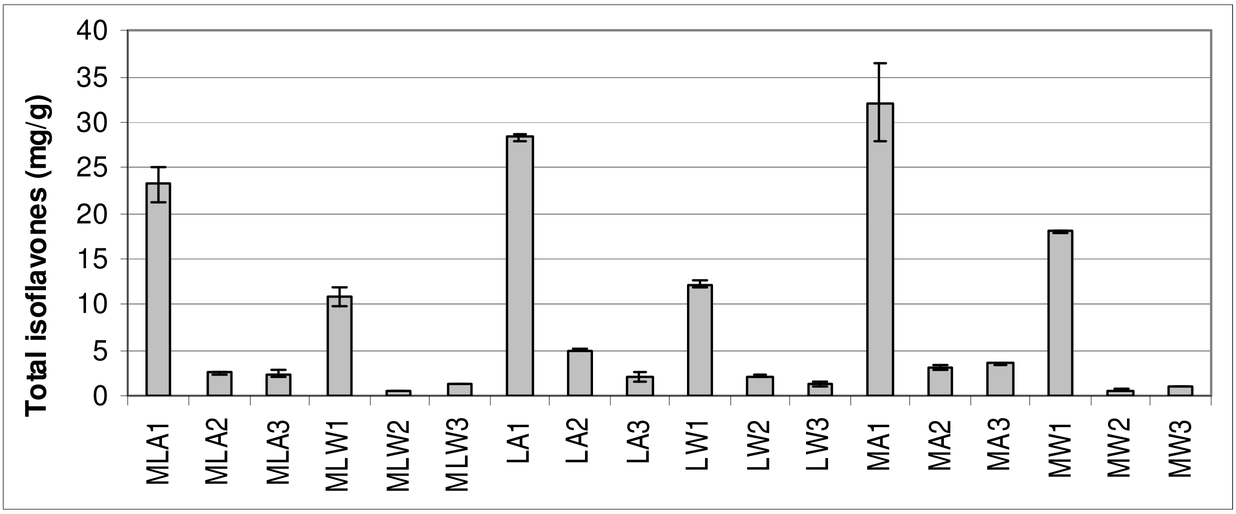


Fig. 2. Total isoflavone content in stems of three cultivars of *L. albus*; ML: Molise Landrace, L: Luxe, M: Multitalia; W: late winter; A: late autumn, W: late winter; 1, 2, 3: number of sampling.

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main isoflavone was genistein 7-*O*-glucoside in ML and the hydroxylated aglycone in M and L (D'Agostina *et al.* 2008).

On the whole, our qualitative data were in good agreement with available literature, in particular regarding the data of stems and leaves (Katagiri *et al.* 2000; Bednarek *et al.* 2001). Other legume species, for example red clover (Sivesind *et al.* 2005), have been reported to contain more isoflavones in leaves than in other plant tissues, a fact explained by the greater susceptibility of leaves to pathogen attack (Bednarek *et al.* 2001). In the same way, the higher isoflavone content of young lupins might derive from the crucial impact that biotic and environmental stresses may have on plant survival and the consequent increasing in plant defense (Posmyk *et al.* 2005). Furthermore the greater isoflavone content in A experiment plants compared to winter-sown ones were probably due to low temperature stresses that these plants have to face during their vegetative development. On the other hand, roots from juvenile plants produce a great amount of isoflavones (Katagiri *et al.* 2000; Bednarek *et al.* 2001), which may derive from the essential role of these metabolites as signalling molecules in nodulation during the very early stage of growth (Subramanian *et al.* 2007). The evolution of isoflavones along the crop growth stage showed a similar behavior in the three cultivars. Multitalia, however, exhibited the greatest amount of isoflavones in the first development stage under autumn sowing. This could be a physiological response to the greater stress implied by low temperature, since Multitalia is less winter-hardy than Luxe or Molise ecotype (Annicchiarico *et al.* 2007).

This work was supported by Fondazione Cariplo, project "Novel methodologies for the quality control of the production of lupin and lupin based food products" (2005-1803).

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