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Alkaloid variation during germination in different lupin species

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Abstract

Quinolizidine alkaloids, occurring in lupins, are the largest single group of legume alkaloids with clear ecological functions in the defence of the plant. During germination, some degree of transformation of alkaloids to other more bioactive compounds, such as esters, occurs. The aim of this work was to investigate the transformations of alkaloids during germination, in *Lupinus albus*, *L. angustifolius* and *L. campestris*. Total quinolizidine alkaloid contents in raw seeds were 1.51 g/100 g (*L. angustifolius*), 2.36 g/100 g (*L. albus*) and 2.45 g/100 g (*L. campestris*). During germination in *L. albus*, lupanine increased and albine and 13-hydroxylupanine decreased substantially. In *L. angustifolius*, 13-hydroxylupanine also decreased. In *L. campestris*, hydroxyaphylline and hydroxyaphyllidine increased while epihydroxyaphylline and dehydroepihydroxyaphylline decreased. The ester 13-tigloyloxylupanine increased progressively during the germination of *L. angustifolius* (from 0 to 0.044 g/100 g) and *L. albus* (from 0.001 to 0.022 g/100 g). Germination of lupin seeds for 3 days, maximum, could be desirable in order to minimize the presence of alkaloids, as well as to avoid the formation of the quinolizidine alkaloid esters.

Keywords: Germination; *Lupinus*; Alkaloids

1. Introduction

Lupin seeds are employed as a protein source for animal and human nutrition in various parts of the world, not only for their nutritional value (high in protein, lipids and dietary fibre), but also their adaptability to marginal soils and climates. Human consumption of lupins has increased in recent years. Lupin flour is added for its nutritive value (high protein efficiency ratio) and also to provide functional properties in bakery and pastry products, protein concentrates and other industrial products, as well as for the elaboration of lactose-free milk and yoghurt analogues (Jiménez-Martínez, Hernández Sánchez, & Dávila Ortiz, 2003; Lquari, Vioque, Pedroche, & Millán, 2002).

Quinolizidine alkaloids are used as a nitrogen source for seedlings (Wink & Witte, 1985), and they also play a defensive role against predators in the plant. The presence of these compounds is a limiting factor for lupin consumption. Elevated concentrations of alkaloids produce a bitter taste, and some pharmacological effects of lupin alkaloids have been documented (Kinghorn & Balandrin, 1984; Wink, 1994). Lupanine and other lupin alkaloids show moderate toxicity in vertebrates, while α-pyridone alkaloids, such as cytisine and anagyrine are toxic, with agonistic activity on both nicotinic and muscarinic acetylcholine receptors, as quinolizidine alkaloids display similar agonistic activities as the alkaloid nicotine (Wink, 1994); they also affect Na^+ and K^+ channels, inducing gastrointestinal, nervous and respiratory symptoms in humans and animals. *L. angustifolius* alkaloids have been found to be neither carcinogenic

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nor teratogenic and their LD_{50} levels are above the levels that would be consumed in a normal diet (Culvernor & Petterson, 1986; Petterson, Ellis, Harris, & Spadex, 1987). Wink (1994) reported the LD_{50} for oral intake in rats, as 350–510 mg/kg for sparteine and 410 mg/kg for lupanine; cytisine (ED_{50} 2.5 mM) produced a reduction of nematode and helminth motility. Doses of anagyrine in the range of 2–30 mg/kg were required to induce moderate-to-severe crooked calf defects (Keeler, 1976; Keeler, James, Shupe, & Kampen van, 1977).

Lupin alkaloids can be eliminated by technological treatments of bitter seeds, but some sweet varieties have also been obtained through breeding programs. These varieties have the advantage of having low alkaloid content but they are also less resistant to diseases and herbivore attack.

Alkaloid synthesis takes place in the stroma of leaf chloroplasts, following a light-regulated biosynthesis, with a stimulated period during the day. Then they are transported via the phloem and stored in vacuoles in all the organs of the plant, preferentially in epidermal and subepidermal tissues of stems and leaves. Alkaloids tend to disappear during leaf senescence. The seeds are especially rich in alkaloids, containing up to 5% (dry weight) which represents about 8–10% of the total nitrogen stored in lupin seeds (Wink, 1998; Wink & Hartmann, 1980; Wink & Witte, 1984).

Germination involves a great number of physiological changes, including synthesis, degradation and transformation of different compounds. This process has been shown to reduce the presence of antinutrients such as α-galactosides or phytic acid in legume seeds, but also it may reduce the total alkaloid content. (De la Cuadra et al., 1994). Trugo (1993) and Cunha-Queda and Beirao da Costa (1994) have also observed that controlled germination reduces the alkaloid content in lupin seeds. This degradation has been attributed to mobilization of alkaloidal nitrogen.

However, previous studies suggest that alkaloids can form esters during germination, resulting in the production of potentially toxic compounds for animals (Wink & Twardowski, 1992). Wink and Hartmann (1981) observed the formation of ester-synthesizing enzymes in *L. polyphyllus* in about 12-day-old seedlings and an increase in the amount of 13α-tigloyloxylupanine. A high proportion of alkaloid esters and a low content of 13-hydroxylupanine was found in leaves by the same authors. The esters 13-tigloyloxylupanine, 13-benzoyloxylupanine, 13-*trans*-cinnamoyloxylupanine and 4-acetoxylupanine were reported in *L. albus* and *L. angustifolius* by Mülbauer, Witte, and Wink (1987).

This work attempts to establish the effect of germination on the alkaloid content of three species of lupin: two European species (*L. albus*, *L. angustifolius*), and one American species (*L. campestris*).

2. Materials and methods

2.1. Samples and germination process

Bitter seeds of *L. albus* L. (LO-3923) and *L. angustifolius* L. (1413), from Servicio de Investigación Agraria of Badajoz (Spain); and wild *L. campestris* Schltdl. et Cham., from Herbario de la Escuela Nacional de Ciencias Biológicas del Instituto Politécnico Nacional of Oaxtepec-Xochimilco (Mexico) were used in this study.

Several assays were performed in order to select the best conditions for the germination of the seeds: (1) on wet filter paper; (2) between wet filter papers and (3) in wet sand. Different temperature conditions were also assayed: (a) 20 °C, and; (b) a pre-treatment at 7 °C for 7 days followed by the temperature alternance (20 °C for 16 h night/30 °C for 8 h light). *L. angustifolius* and *L. campestris* were washed with 20 ml of sodium hypochlorite solution (0.2 g Cl/l), then with distilled water (25 ml, three times) and scarified prior to germination, in order to avoid fungus proliferation and improve the germination of the seeds.

Germination on wet filter paper gave more fungus proliferation, while in wet sand and between filter paper the results obtained were similar. Pretreatment at 7 °C and variation of temperatures did not improve the germination at 20 °C. For this reason, the optimal germination process was performed according to the Rules of the International Seed Testing Association (Anon., 1999), as follows: 800 seeds from each species were used for the germination assay, distributed in 10 trays, with 80 seeds each one. The seeds were spread on a moist sheet of filter paper (Albet 1516, 42×52 cm) and covered with another sheet of moist filter paper. They were put into a germination chamber under environmentally controlled conditions: 20 °C, 8 h of light per day exposure, and watering of the seeds during germination to keep the paper always wet. Samples (80 seeds/tray) were taken at 0 (control), 1, 2, 3, 4, 5, 6, 7, 8 and 9 germination days. The germination process was repeated twice for each species, and the germination capacity was evaluated by percentage of germination and seed weights. Samples for analysis were constituted by germinated and moist seeds, discarding those that did not show any water absorption during the process.

2.2. Analytical method

Samples were freeze-dried and ground to pass through a 150 μm sieve (Tecator, Cyclotec 1093). Extraction of the milled seed was done as described by Muzquiz, Burbano, Cuadrado, and de la Cuadra (1993): 0.5 g of ground seeds were homogenized with 5 ml of 5% trichloroacetic acid for 1 min. The mixture was then centrifuged for 15 min (10,000*g*) and the supernatant separated. The extraction was repeated twice. The su-

pernatants were collected in a decantation funnel and 0.8 ml of 10 M NaOH were added. Three extractions with 15 ml of dichloromethane were performed, and the organic phase was evaporated to dryness at room temperature. The residue was dissolved in 1 ml of methanol and a codeine solution as an internal standard (final concentration of codeine, 1 mg/ml) was added.

A Perkin–Elmer gas chromatograph equipped with a nitrogen–phosphorus detector (NPD) and operated by a Turbochrom program was used. The column used was a SPB-1 (30 m × 0.25 mm id) and helium was the carrier gas. The temperatures of the injector and detector were 240 and 300 °C, respectively. The oven temperature was 150 °C, increased by 5 °C/min to 235 °C and final hold time of 23 min at 235 °C. Calibration curve was performed for lupanine with linear response over the range 0–1.250 mg/ml and correlation coefficients of above 0.99.

For the identification of the alkaloids, capillary CG-MS was applied. A Perkin–Elmer Autosystem XL gas chromatograph (working with the same column and conditions as above) was coupled with a mass selective detector (Perkin–Elmer Turbomass Gold) that was combined with the Turbomass software for the identification of alkaloids in the samples.

2.3. Statistical analysis

A one way ANOVA analysis was applied to the obtained analytical data, as well as Duncan's multiple range test in order to stablish the statistical significance of alkaloid variations. The computer package Statgraphics Plus 4.1 was used for this purpose.

3. Results and discussion

Maximum germination rates were 100% for *L. albus* and *L. campestris*; *L. angustifolius* seeds showed lower viability, with 72.5% germinated seeds after 9 days. (Fig. 1). These results are in agreement with data reported by De la Cuadra et al. (1994), who reported the higher rate of germination of *L. albus* in comparison to other lupin species, and the more effective germination of lupin seeds in the presence of light compared to conditions without light.

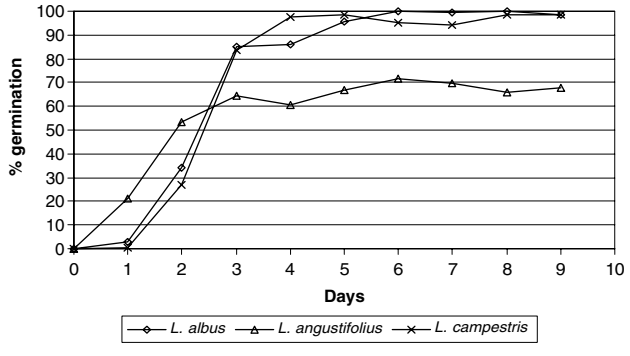

Fig. 1. Germination of lupin seeds (expressed as %).

Fig. 2 shows the chromatographic profiles of the three analysed species, at 0 and 9 days of germination. *L. albus* and *L. campestris* were the species with the highest total alkaloid content (2.36 and 2.46 g/100 g, respectively), followed by *L. angustifolius* (1.51 g/100 g) (Fig. 3).

The individual alkaloids in *L. albus* (Table 1) were lupanine as the major one (1.36 g/100 g), which represented more than 50% of the total alkaloid content, followed by albine (0.46 g/100 g), multiflorine (0.18 g/100 g) and other compounds in levels below 0.05 g/100 g (ammodendrine, angustifoline, α-isolupanine, 5,6-dehydrolupanine, 11,12-*seco*-12,13-didehydromultiflorine and 13-hydroxylupanine). Although the presence of angustifoline in *L. albus* seeds is not very usual, it has been previously reported in low levels by Wink and Witte (1985), De la Vega et al. (1996) and Wink (1994). The presence of the ester 13-tigloyloxylupanine was detected by GC-MS (Fig. 4), and quantified as 0.002 g/100 g in the non-germinated seeds.

During germination of *L. albus* statistically significative variations were detected on all the alkaloids ($p < 0.05$). The total alkaloid content varied significatively through germination from 2.36 to 3.03 g/100 g. The statistical analysis of the data showed that the most clear tendency was that ammodendrine, albine, 5,6-dehydrolupanine and 13-hydroxylupanine decreased during germination, while lupanine and 13-tigloyloxylupanine, increased during the process. Lupanine content increased to 2.36 g/100 g in 9 days, and total alkaloid amount reached 3.04 g/100 g; 13-tigloyloxylupanine (Fig. 4) showed a progresive increase (Fig. 5), from 0.001 to 0.022 g/100 g, in agreement with the results observed in *L. polyphyllus* by Wink and Hartmann (1980). Statistically significant correlations ($p < 0.05$) were found between albine and the total alkaloids content for *L. albus*, due to the high presence of this alkaloid in this species of *Lupinus*. The alkaloid angustifoline also correlates positively to lupanine, multiflorine and 13-hydroxylupanine content.

L. angustifolius quinolizidine alkaloids were mainly lupanine (0.55 g/100 g), and its derivatives angustifoline (0.45 g/100 g) and 13-hydroxylupanine (0.38 g/100 g), contributing 36%, 30% and 25%, respectively, to the total amount (Table 2). Other alkaloids such as multiflorine were found in levels below 0.06 g/100 g, in agreement with Wink, Meibner, and Witte (1995). No esters were detected in the non-germinated seeds, although Hirai, Suzuki, Yamazaki, and Saito (2000) had reported the presence of ester derivatives of 13-hydroxylupanine in non germinated bitter *L. angustifolius* seeds.

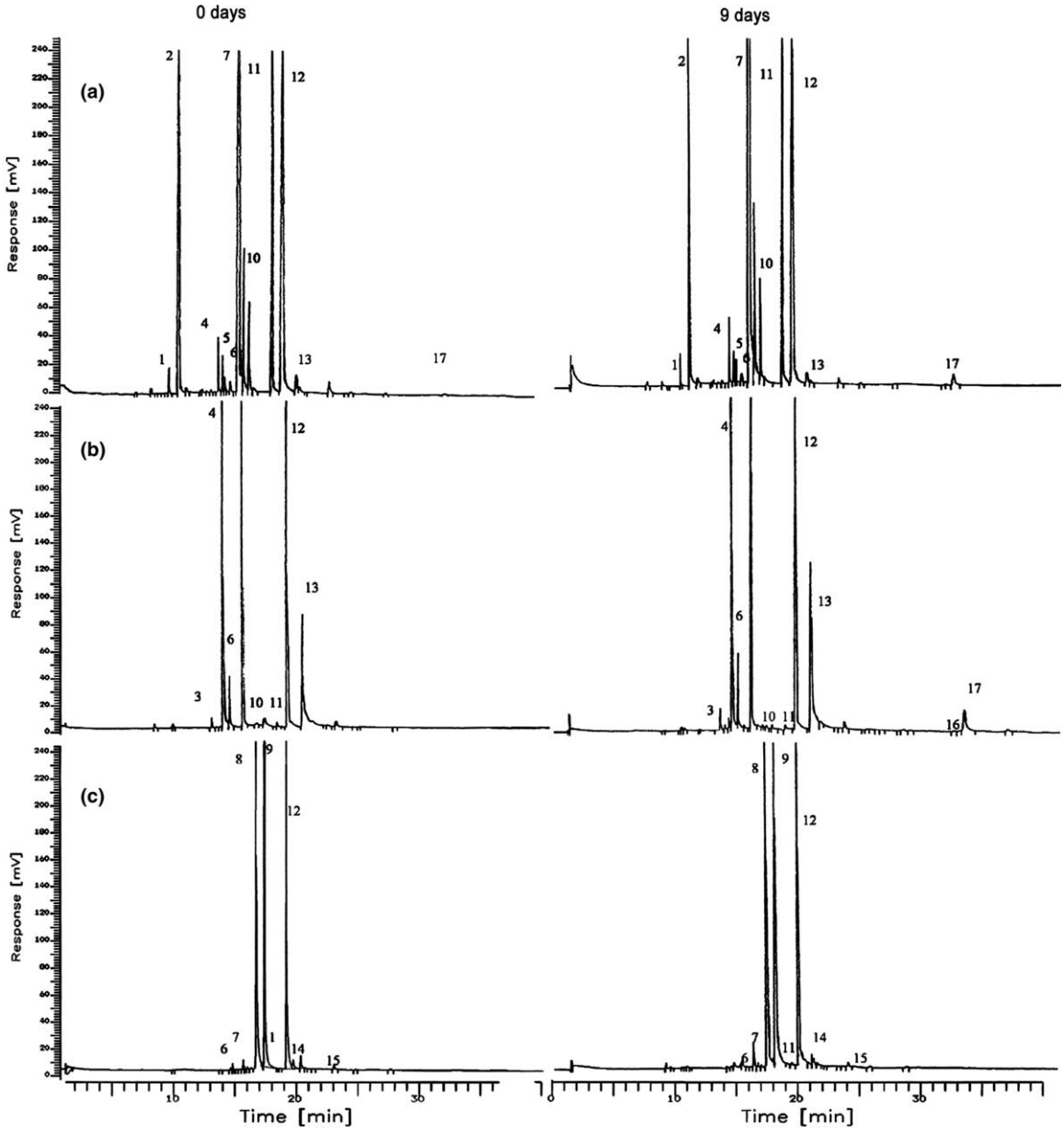

Fig. 2. Chromatographic profile of alkaloids in *Lupinus* seeds: (a) *L. albus*, (b) *L. angustifolius*, (c) *L. campestris*, 1 = Ammodendrine; 2 = Albine; 3 = Isoangustifoline; 4 = Angustifoline; 5 = α-Isolupanine; 6 = 5,6-dehydrolupanine; 7 = Lupanine; 8 = Hydroxyaphilline; 9 = Hydroxyaphillidine; 10 = 11,12-*seco*-Didehydromultiflorine; 11 = Multiflorine; 12 = Internal standard (codeine); 13 = 13-hydroxylupanine; 14 = Epihydroxyaphilline; 15 = Dehydro-epihydroxyaphilline 16 = ester; 17 = 13-tigloyloxylupanine.

During germination of *L. angustifolius*, most of the alkaloid content fluctuated showing statistically significant variations ($p < 0.05$). The total alkaloid content as well as 5,6-dehydrolupanine, showed a minimum at 3 days of germination and increased progressively to recover the initial levels at the end of the germination period (Fig. 3). Statistically significant correlation was found for *L. angustifolius*, where lupanine and its derivatives (13-hydroxylupanine, 5,6-dehydrolupanine and angustifoline) were positively correlated (>74%). A positive correlation was also found for the two esters detected in this species. 13-Tigloyloxylupanine was detected after 3 days of germination, with progressively increasing levels up to 0.05 g/100 g after 8 days of germination. The other ester detected could not be clearly identified, although its mass spectrum suggest that it could be 13-angeloyloxylupanine or 13-*cis*/*trans*-cinnamoyloxylupanine. According to Mülbauer et al. (1987) it may be identified as the *trans*-isomer of 13-angeloyloxylupanine, which has always been found accompanying 13-tigloyloxylupanine. This compound appeared after 6 days of germination, at very low levels, and stabilised at 0.0005–0.0016 g/100 g until the end of germination process.

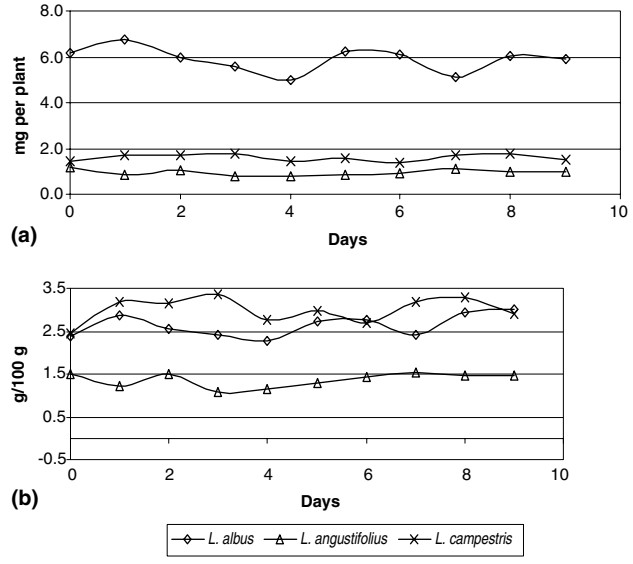

Fig. 3. Total alkaloid content in lupin seeds during germination: (a) mg/plant; (b) g/100 g.

In lupin seeds, the alkaloid lupanine is the precursor of other alkaloids found in the analysed samples, such as 13-hydroxylupanine, 5,6-dehidrolupanine, angustifoline and the esters of lupanine (Kinghorn & Balandrin, 1984). The amount of 13-hydroxylupanine in *L. angustifolius* is close to that of its precursor lupanine (0.25–0.38 g/100 g and 0.37–0.57 g/100 g respectively), while in *L. albus*, 13-hydroxylupanine is at much lower levels than lupanine (0.01–0.02 g/100 g and 1.36–2.19 g/100 g respectively). This could suggest a more active transformation of lupanine into 13-hydroxylupanine in *L. angustifolius*, than in *L. albus*, where the synthesis of lupanine is more intense than its transformation since its levels increase during germination. The decrease of 13-hydroxylupanine content in both species indicates a subsequent progressive degradation of this compound during the germination process. Angustifoline showed the same behaviour, being the alkaloid characteristic of *L. angustifolius*, with very similar levels to lupanine (0.37–0.57 g/100 g) in all raw and germinated seeds of this species. On the contrary, in *L. albus* it remains in very low levels (0.02–0.04 g/100 g). 5,6-Dehydrolupanine was also found in higher levels in *L. angustifolius*. Although the esters of lupanine appeared in raw seeds of *L. albus*, their synthesis from lupanine was also faster in *L. angustifolius* than in *L. albus* seeds, reaching higher levels after 6 germination days.

All these facts suggest that in *L. angustifolius*, lupanine is highly transformed into angustifoline, isoangustifoline and its direct derivatives, 13-hydroxylupanine and 5,6-dehydrolupanine. All these compounds were more than 90% of total alkaloid content of *L. angustifolius*, with close levels of lupanine, angustifoline and 13-hydroxylupanine, and much lower levels for 5,6-de-

Table 1
Alkaloids levels during the germination of *L. albus* (g/100 g)

Days	Amodendrine	Albine	Angustifoline	α-Isolupanine	5,6-Dehydro-lupanine	Lupanine	11,12-*seco*-12,13-Didehydromultiflorine	Multiflorine	13-Hydroxy-lupanine	13-Tigloyloxy-lupanine
0	0.027 ± 0.005	0.459 ± 0.045	0.025 ± 0.004	0.007 ± 0.002	0.016 ± 0.001	1.360 ± 0.101	0.048 ± 0.008	0.183 ± 0.023	0.016 ± 0.011	0.002 ± 0.001
1	0.025 ± 0.014	0.264 ± 0.052	0.034 ± 0.006	0.010 ± 0.002	0.014 ± 0.008	1.798 ± 0.289	0.066 ± 0.015	0.234 ± 0.033	0.019 ± 0.008	0.002 ± 0.001
2	0.020 ± 0.009	0.218 ± 0.028	0.027 ± 0.004	0.015 ± 0.008	0.013 ± 0.008	1.692 ± 0.250	0.051 ± 0.011	0.184 ± 0.016	0.016 ± 0.003	0.002 ± 0.000
3	0.011 ± 0.001	0.192 ± 0.044	0.031 ± 0.001	0.009 ± 0.002	0.005 ± 0.003	1.769 ± 0.143	0.058 ± 0.014	0.200 ± 0.008	0.019 ± 0.008	0.003 ± 0.001
4	0.012 ± 0.004	0.168 ± 0.052	0.028 ± 0.010	0.007 ± 0.003	0.005 ± 0.002	1.727 ± 0.367	0.044 ± 0.004	0.180 ± 0.029	0.018 ± 0.001	0.005 ± 0.000
5	0.012 ± 0.003	0.239 ± 0.073	0.032 ± 0.007	0.010 ± 0.004	0.005 ± 0.002	1.990 ± 0.508	0.064 ± 0.021	0.223 ± 0.035	0.018 ± 0.001	0.005 ± 0.003
6	0.013 ± 0.003	0.250 ± 0.053	0.035 ± 0.004	0.010 ± 0.003	0.004 ± 0.002	1.994 ± 0.233	0.074 ± 0.020	0.218 ± 0.021	0.020 ± 0.005	0.008 ± 0.001
7	0.011 ± 0.001	0.200 ± 0.017	0.027 ± 0.004	0.008 ± 0.001	0.005 ± 0.002	1.775 ± 0.168	0.060 ± 0.007	0.186 ± 0.013	0.010 ± 0.001	0.012 ± 0.003
8	0.013 ± 0.002	0.227 ± 0.024	0.029 ± 0.003	0.010 ± 0.003	0.005 ± 0.002	2.192 ± 0.361	0.077 ± 0.011	0.197 ± 0.022	0.011 ± 0.000	0.015 ± 0.002
9	0.015 ± 0.002	0.267 ± 0.045	0.037 ± 0.004	0.011 ± 0.002	0.005 ± 0.003	2.158 ± 0.376	0.076 ± 0.008	0.241 ± 0.024	0.013 ± 0.002	0.022 ± 0.003

Mean value of two separated germinations ± SD ($n-1$), $n = 8$.

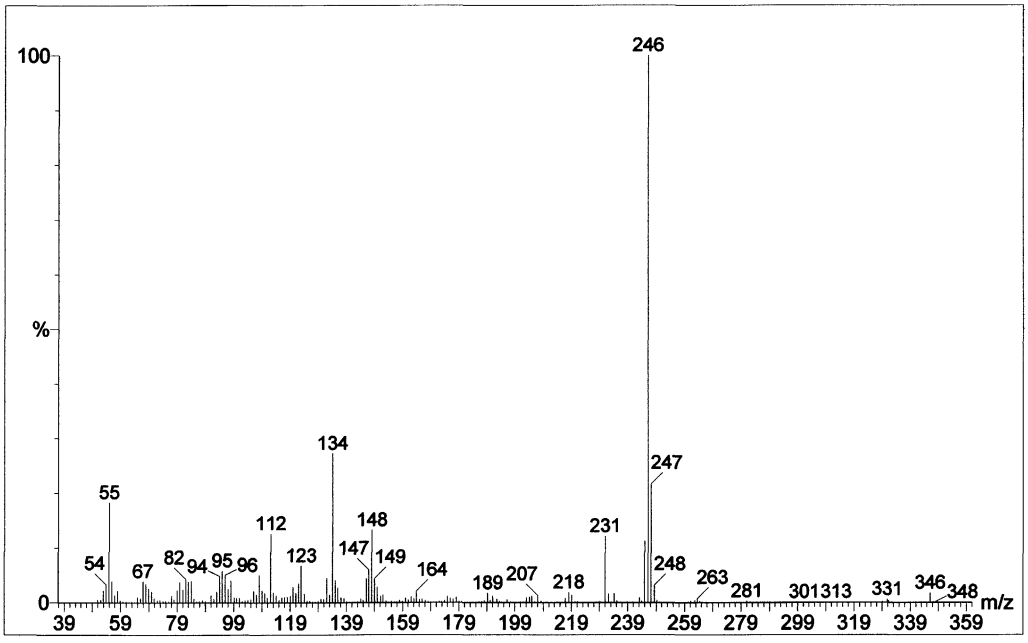

Fig. 4. Spectrum of 13-tigloyloxylupanine from *L. angustifolius* and *L. albus* obtained by capillary GC-MS.

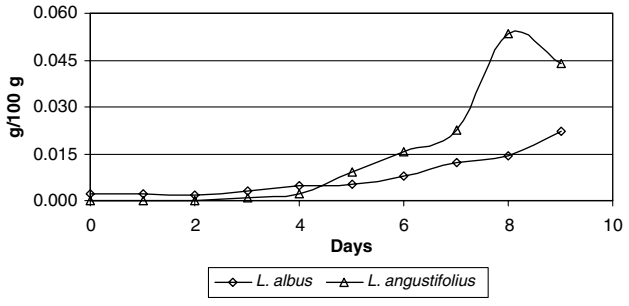

Fig. 5. 13-tigloyloxylupanine content of lupin seeds during germination.

hydrolupanine. On the contrary, in *L. albus*, lupanine remains at higher levels, with less transformation into its derivatives, being accompanied by other alkaloids such as multiflorine and albine (at levels of 0.18–0.24 g/100 g and 0.16–0.46 g/100 g, respectively), as well as its derivatives at lower concentrations.

In *L. campestris*, lupanine and its derivative 5,6-dehydrolupanine, multiflorine and its derivative 11,12-*seco*-12,13-didehydromultiflorine, were found to be minor alkaloids and aphylline type alkaloids were the more important ones. The total alkaloid content in non-germinated seeds of *L. campestris* was 2.45 g/100 g (Table 3). Hydroxyaphylline, hydroxyaphyllidine, dehydro-epihydroxyaphylline and epi-hydroxyaphylline were identified by GC-MS (Meibner & Wink, 1992) and represent 85%, 12%, 2% and 1%, respectively of the total amount. Multiflorine, lupanine, 5,6-dehydrolupanine and 11,12-*seco*-12,13-didehydromultiflorine were also detected in levels below 0.005 g/100 g. Alkaloids in *L. campestris* was significantly ($p < 0.05$) during germination between 2.45 and 3.45 g/100 g, with no clear behaviour in some cases. The most clear trends were an increase in hydroxyaphylline and hydroxyaphyllidine content and a decrease in epihydroxyaphylline, and dehydro-epihydroxyaphylline. Aphylline-related alkaloids (hydroxyaphylline, hydroxyaphyllidine, epihydroxyaphylline, dehydro-epihydroxyaphylline) were correlated significantly to one another, where the strongest correlation (71%) was for hydroxyaphylline and hydroxyaphyllidine. No esters were detected, although Bermúdez Torres, Robledo Quintos, Barrera Necha, and Wink, 2001 indicated the presence of tigloyloxylupanine in the seeds of some American lupins.

No α-pyridone alkaloids, such as the highly toxic anagyrine and cytisine were detected in any of the analyzed lupins. This is significant, since these α-pyridones (e.g. anagyrine) cause malformations, the so-called "crooked calf disease", in young sheep and calfs, when their mothers feed on lupins or broom containing anagyrine (ingested at 7–11 mg/kg per day during day 40 and 75 of gestation) (Keeler, 1976; Keeler et al., 1977).

Our results are in agreement with previous studies (Muzquiz, 2000; Muzquiz et al., 1994; Wink et al., 1995), which reported the presence of multiflorine, albine and α-isolupanine in some European species including *L. albus*, and the appearance of aphylline and its derivatives in American lupins. Hirai et al. (2000) reported the presence of lupanine, 13-hydroxylupanine and angustifoline in *L. angustifolius* seeds. The proportion of alkaloids found by these authors are similar to the samples analysed in this work.

Table 2
Alkaloids levels during the germination of *L. angustifolius* (g/100 g)

Days	Isoangustifoline	Angustifoline	5,6-Dehydro-lupanine	Lupanine	11,12-*seco*-12,13-Dide-hydromultiflorine	Multiflorine	13-Hydroxy-lupanine	Ester	13-Tigloyloxy-lupanine
0	0.015 ± 0.001	0.455 ± 0.019	0.061 ± 0.001	0.550 ± 0.020	0.000 ± 0.000	0.014 ± 0.001	0.379 ± 0.017	ND	ND
1	0.011 ± 0.002	0.395 ± 0.076	0.034 ± 0.007	0.467 ± 0.098	0.001 ± 0.000	0.011 ± 0.002	0.283 ± 0.057	ND	ND
2	0.014 ± 0.002	0.500 ± 0.088	0.052 ± 0.011	0.537 ± 0.099	0.001 ± 0.000	0.011 ± 0.003	0.376 ± 0.056	ND	ND
3	0.009 ± 0.002	0.377 ± 0.027	0.035 ± 0.003	0.394 ± 0.011	0.001 ± 0.000	0.006 ± 0.001	0.255 ± 0.010	ND	0.001 ± 0.001
4	0.010 ± 0.003	0.396 ± 0.015	0.037 ± 0.003	0.378 ± 0.012	0.001 ± 0.001	0.009 ± 0.004	0.321 ± 0.064	ND	0.002 ± 0.002
5	0.011 ± 0.003	0.441 ± 0.045	0.046 ± 0.007	0.501 ± 0.056	0.001 ± 0.000	0.008 ± 0.003	0.245 ± 0.038	ND	0.009 ± 0.002
6	0.012 ± 0.002	0.501 ± 0.062	0.053 ± 0.005	0.547 ± 0.054	0.003 ± 0.001	0.009 ± 0.003	0.292 ± 0.027	0.001 ± 0.000	0.016 ± 0.008
7	0.013 ± 0.004	0.567 ± 0.085	0.057 ± 0.009	0.562 ± 0.085	0.003 ± 0.002	0.009 ± 0.004	0.300 ± 0.068	0.001 ± 0.000	0.023 ± 0.008
8	0.013 ± 0.001	0.499 ± 0.028	0.048 ± 0.004	0.566 ± 0.038	0.002 ± 0.000	0.006 ± 0.001	0.251 ± 0.002	0.002 ± 0.001	0.054 ± 0.013
9	0.011 ± 0.002	0.497 ± 0.104	0.052 ± 0.014	0.557 ± 0.088	0.002 ± 0.000	0.005 ± 0.001	0.258 ± 0.043	0.001 ± 0.001	0.044 ± 0.007

Mean value of two separated germinations ± SD $(n-1)$, $n = 8$.

Table 3
Alkaloids in the germination of *L. campestris* (g/100 g)

Days	5.6-Dehydrolupa-nine	Lupanine	11,12-*seco*-12,13-Dide-hydromultiflorine	Hydroxyaphylline	Hydroxyaphylli-dine	Multiflorine	Epihydroxyaphyl-line	Dehydro-epihydr-oxyaphylline
0	0.002 ± 0.000	0.002 ± 0.000	0.002 ± 0.000	0.285 ± 0.013	2.088 ± 0.052	0.005 ± 0.000	0.026 ± 0.002	0.047 ± 0.002
1	0.011 ± 0.004	0.002 ± 0.001	0.001 ± 0.000	0.408 ± 0.029	2.681 ± 0.323	0.003 ± 0.001	0.023 ± 0.004	0.048 ± 0.011
2	0.012 ± 0.001	0.003 ± 0.000	0.001 ± 0.000	0.448 ± 0.028	2.597 ± 0.161	0.004 ± 0.002	0.027 ± 0.006	0.057 ± 0.005
3	0.012 ± 0.002	0.004 ± 0.001	0.001 ± 0.000	0.494 ± 0.068	2.758 ± 0.203	0.002 ± 0.001	0.025 ± 0.006	0.056 ± 0.005
4	0.010 ± 0.001	0.003 ± 0.001	0.001 ± 0.000	0.360 ± 0.069	2.340 ± 0.360	0.006 ± 0.001	0.016 ± 0.005	0.037 ± 0.006
5	0.011 ± 0.001	0.003 ± 0.001	0.001 ± 0.000	0.409 ± 0.046	2.473 ± 0.252	0.003 ± 0.001	0.021 ± 0.004	0.057 ± 0.002
6	0.010 ± 0.005	0.009 ± 0.000	0.002 ± 0.000	0.348 ± 0.072	2.270 ± 0.535	0.002 ± 0.001	0.018 ± 0.005	0.043 ± 0.013
7	0.012 ± 0.005	0.003 ± 0.001	0.001 ± 0.000	0.460 ± 0.071	2.648 ± 0.078	0.006 ± 0.002	0.024 ± 0.003	0.036 ± 0.002
8	0.010 ± 0.006	0.002 ± 0.000	0.001 ± 0.000	0.460 ± 0.035	2.736 ± 0.110	0.006 ± 0.001	0.020 ± 0.003	0.026 ± 0.003
9	0.014 ± 0.005	0.004 ± 0.001	0.001 ± 0.000	0.378 ± 0.081	2.405 ± 0.449	0.006 ± 0.002	0.019 ± 0.002	0.021 ± 0.003

Mean value of two separated germinations ± SD $(n-1)$, $n = 8$.

De la Cuadra et al. (1994) also found a slight increase in alkaloids during germination of *L. albus*, which is in agreement with this study. According to Strack, Becher, Brall, and Witte (1991), some esters appeared, in the cotyledons and primary leaves of *L. angustifolius*, reaching 50–80% of the total alkaloid content. These authors reported that esters are exclusively present in the aerial plant organs, with the exception of tigloyl esters, which can be found in other plant organs. This is in agreement with Mülbauer et al. (1987), since this compound was found in *L. angustifolius* and *L. albus* germinated seeds.

The results of this study suggest that lupin seeds should not be germinated longer than 3 days in order to keep the alkaloid level low and to avoid transformation into alkaloid esters. Three days of germination has been previously shown to have positive effects on the other antinutritive factors in lupin seeds. De la Cuadra et al. (1994) found a 100% reduction of α-galactosides (raffinose, stachyose and verbascose) in the seeds after two days of germination, while sucrose increased. Furthermore a significant decrease in phytic acid occurs in *L. albus* during the first 48 h of germination (36.5% reduction), as has been shown by Muzquiz et al. (1998). Honke, Kozlowska, Vidal-Valverde, Frias, and Górecki (1998) also reported an enzymatic hydrolysis of higher inositol phosphates to give tetra- and triinositol phosphates, during germination of lupin seeds. Therefore, for human and animal nutrition, germination of lupin seeds for maximum 3 days could be desirable in order to minimize the presence of antinutrutive factors in the products.

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References

Anon. (1999). International Rules for Seeds Testing. Published by The International Seed Testing Association (ISTA), Switzerland.

Bermúdez Torres, K., Robledo Quintos, N., Barrera Necha, L. L., & Wink, M. (2001). Alkaloid profile of leaves and sedes of *Lupinus hintonii* C.P. Smith. *Zeitschrift fur Naturforschung c, 57*, 243–247.

Culvernor, C. C. J., & Petterson, D. S. (1986). Lupin toxins – alkaloids and phomopsins. In *Proceedings of the fourth international lupin conference* (pp. 188–198). South Perth, Australia: Department of Agriculture.

Cunha-Queda, A. C., & Beirao da Costa, M. L. (1994). A RSM study to the effect of controlled germination in *Lupinus luteus* alkaloid content. In J. M. Neves-Martins & M. L. Beirão da Costa (Eds.), *Advances in lupin research* (pp. 521–523). Lisboa: Instituto Superior de Agronomía (ISA press).

De la Cuadra, C., Muzquiz, M., Burbano, C., Ayet, G., Calvo, R., Osagie, A., & Cuadrado, C. (1994). Alkaloid, α-galactosides and phytic acid changes in germinating lupin seeds. *Journal of the Science of Food and Agriculture, 66*, 357–364.

De la Vega, R., Gutierrez, M. P., Sanz, C., Calvo, R., Robredo, L. M., de la Cuadra, C., & Muzquiz, M. (1996). Bactericide-like effect of lupin alkaloids. *Industrial Crops and Products, 5*, 141–148.

Hirai, M. Y., Suzuki, H., Yamazaki, M., & Saito, K. (2000). Biochemical and partial molecular characteristics of bitter and sweet forms of *Lupinus angustifolius*, an experimental model for study of molecular regulation of quinolizidine alkaloid biosynthesis. *Chemical Pharmaceutical Bulletin, 48*(10), 1458–1461.

Honke, J., Kozlowska, H., Vidal-Valverde, C., Frias, J., & Górecki, R. (1998). Changes in quantities of inositol phosphates during maturation and germination of legume seeds. *Food Research and Technology, 206*(4), 279–283.

Jiménez-Martínez, C., Hernández Sánchez, H., & Dávila Ortiz, G. (2003). Production of a yogurt-like product from *Lupinus campestris* seeds. *Journal of the Science of Food and Agriculture 83*(6), 515–522.

Keeler, R. F. (1976). Lupin alkaloids from teratogenic and nonteratogenic lupins: III. Identification of anagyrine as the probable teratogen by feeding trials. *Journal of Toxicology and Environmental Health, 1*, 887–898.

Keeler, R. F., James, L. F., Shupe, J. L., & Kampen van, K. R. (1977). Lupine-induced crooked calf disease and a management method to reduce incidence. *Journal of Range Management, 30*(2), 97–102.

Kinghorn, A. D., & Balandrin, M. F. (1984). Quinolizidine alkaloids of the *Leguminosae*: Structural types, analysis chemotaxonomy and biological activities. In S. W. Pelletier (Ed.), *Alkaloids: Chemical and biological perspectives* (Vol. 2, pp. 105–148). New York: Wiley.

Lquari, H., Vioque, J., Pedroche, J., & Millán, F. (2002). *Lupinus angustifolius* protein isolates: Chemical composition, functional properties and protein characterization. *Food Chemistry, 76*, 349–356.

Meibner, C., & Wink, M. (1992). Analysis of quinolizidine alkaloids from North-American lupins by GC-MS. In M. Wink (Ed.), *Lupinen 1991-Forschung, Anbau und Verwertung* (pp. 91–129). Universität Heidelberg.

Mülbauer, P., Witte, L., & Wink, M. (1987). New ester alkaloids from *Lupinus* (genus *Lupinus*). *Planta Medica, 3*, 237–239.

Muzquiz, M., Burbano, C., Cuadrado, C., & de la Cuadra, C. (1993). Determinación de factores antinutritivos termorresistentes en leguminosas I: Alcaloides. *Investigación Agraria. Producción y Protección Vegetales., 8*, 351–361.

Muzquiz, M., Cuadrado, C., Ayet, G., De la Cuadra, C., Burbano, C., & Osagie, A. (1994). Variation of alkaloid components of lupin seeds in 49 genotypes of *Lupinus albus* L. from different countries and locations. *Journal of Agricultural and Food Chemistry, 42*(7), 1447–1450.

Muzquiz, M., Pedrosa, M. M., Cuadrado, C., Ayet, G., Burbano, C., & Brenes, A. (1998). Variation of alkaloids, alkaloid esters, phytic acid and phytase activity in germinated seed of *Lupinus albus* and *L. luteus*. In A. J. Jansman, G. D. Hil, J. Huisman, & A. F. B. van der Poel (Eds.), *Proceedings of the third international workshop on antinutritional factors in legume seeds and rapeseed* (pp. 387–390). Wageningen: EAA Publications.

Muzquiz, M. (2000). Separation of alkaloids by gas chromatography. In I. D. Wilson, E. R. Adlard, C. F. Poole, & M. Cooke (Eds.),

Encyclopedia of separation science (pp. 1938–1949). San Diego, USA: Academic Press.

Petterson, D. S., Ellis, Z. L., Harris, D. J., & Spadex, Z. E. (1987). Acute toxicity of the major alkaloids of cultivated *Lupinus angustifolius* seed to rats. *Journal of Applied Toxicology, 7*(1), 51–53.

Strack, D., Becher, A., Brall, S., & Witte, L. (1991). Quinolizidine alkaloids and the enzymatic synthesis of their cinnamic and hydroxycinnamic acid esters in *Lupinus angustifolius* and *L. luteus*. *Phytochemistry, 30*(5), 1493–1498.

Trugo, L.C. (1993). Effect of germination on nutritive value of lupin seeds. In Abstracts of the VII international lupin conference, Portugal: Evora.

Wink, M. (1994). Biological activities and potential application of lupin alkaloids. In J. M. Neves-Martins & M. L. Beirão da Costa (Eds.), *Advances in lupin research* (pp. 161–178). Lisboa: Instituto Superior de Agronomía (ISA press).

Wink, M. (1998). A short history of alkaloids. In M. F. Roberts & M. Wink (Eds.), *Alkaloids, biochemistry, ecology and medicinal applications* (pp. 11–44). New York: Plenum Press.

Wink, M., & Hartmann, T. (1980). Enzymatic synthesis of quinolizidine alkaloids in lupin chloroplasts. *Zeitschrift fur Naturforschung, 35*, 93–97.

Wink, M., & Hartmann, T. (1981). Sites of enzymatic synthesis of quinolizidine alkaloids and their accumulation in *Lupinus polyphyllus*. *Zeitschrift fur Pflanzenphysiologie, 102*, 337–344.

Wink, M., Meibner, C., & Witte, L. (1995). Patterns of quinolizidine alkaloids in 56 species of the genus *Lupinus*. *Phytochemistry, 38*(1), 139–153.

Wink, M., & Twardowski, T. (1992). Allelochemical properties of alkaloids. Effects on plants, bacteria and protein biosynthesis. In S. J. H. Rizvi & V. Rizvi (Eds.), *Allelopathy. Basic and Applied Aspects* (pp. 129–150). London: Chapman and Hall.

Wink, M., & Witte, L. (1984). Turnover and transport of quinolizidine alkaloids. Diurnal fluctuations of lupanine in the phloem sap, leaves and fruits of *Lupinus albus* L. *Planta, 161*, 519–524.

Wink, M., & Witte, L. (1985). Quinolizidine alkaloids as nitrogen source for lupin seedlings and cell cultures. *Zeitschrift fur Naturforschung c, 40*, 767–775.