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Quinolizidine Alkaloids in Seeds of Lupin Genotypes of Different Origins

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The intake of lupin-based foods could imply the exposure of consumers to quinolizidine alkaloids. The objectives of this study were to assess the genetic variation among and within 11 geographic regions of *Lupinus albus* ecotypes, verify the quinolizidine alkaloids amount of alkaloid-poor *L. albus* and *Lupinus angustifolius* varieties, and assess the effect of two climatically contrasting Italian environments on the alkaloid content. The quantitation was performed by GC-MS, and in all samples lupanine was the most abundant quinolizidine alkaloid, followed by albine and 13α-hydroxylupanine for *L. albus* and by 13α-hydroxylupanine and angustifoline for *L. angustifolius*. Some regions tended to have a high (Azores) or low (Egypt, Near East, Maghreb) total alkaloids content, but the variation among ecotypes within regions was larger than that among regions following the estimation of variance components. Alkaloid-poor varieties tended to have higher total alkaloid contents when grown in the subcontinental climate site, exceeding in some cases the limit of 0.200 mg/g.

KEYWORDS: GC-MS analyses; genetic variation; genetic resources; lupanine; *Lupinus albus*; *Lupinus angustifolius*; quinolizidine alkaloids

INTRODUCTION

Quinolizidine alkaloids are an important class of secondary metabolites of plants belonging to *Lupinus* and other genera of the Fabaceae family. They act as a nitrogen reserve and confer resistance toward pathogens and herbivores (*1*−*3*). They are biosynthesized in the green tissues of the plant and then transported via the phloem and stored in all organs, seeds included (*4*). The main quinolizidine alkaloids reported for *Lupinus albus* and *Lupinus angustifolius* are lupanine, albine, 13α-hydroxylupanine, α-isolupanine, angustifoline, 13α-angeloyloxylupanine, and 13α-tigloyloxylupanine (*2*). Their chemical structures are reported in **Figure 1**.

Toxicity studies performed on lupanine and sparteine (the latter being a major quinolizidine alkaloids in *Lupinus luteus*) have shown a moderate acute oral toxicity due to neurological effects leading to loss of motor co-ordination and muscular control (*5*). In mammals, quinolizidine alkaloid intoxication is characterized by trembling, shaking, excitation, and convulsion (*6*). At high concentrations, anagyrine is also teratogenic (*7*), an activity shown also by ammodendrine, a piperidine alkaloid, which is usually extracted and analyzed together with quinolizidines (*2*). Human acute toxicity is restricted to a few poisoning cases (*8*), and the general symptoms include nausea, respiratory arrest, progressive weakness, and coma (*5*).

Lupin has gained attention not only as feed supplement (*9*) but also, in the past decade, as ingredient for human food; this is due to its high protein percentage (34−43% of dry matter) and acceptable contents of essential amino acids (*10*). The outcome might be a growing exposure of consumers to quinolizidine alkaloids: in lupin ecotypes, old varieties, the alkaloid levels are so high that their seeds have to be soaked in water for several days to become edible. Many sweet varieties containing reduced amounts of alkaloids have been selected in the past two decades (*9*). The amount of quinolizidine alkaloids in cultivated lupin species is genetically controlled by one or a few major recessive genes that have been exploited to select sweet varieties, as well as by minor genes that may concur in selecting varieties able to meet the alkaloid levels required for marketing of grains (*9*). A maximum limit of 200 mg/kg for the total quinolizidine alkaloid content in lupin flours and foods has been fixed by the Health Authorities of Great Britain (*11*), France (*12*), and Australia and New Zealand (*5*).

Information on the effect of the growing environment on the alkaloid level of sweet varieties is limited and concerns essentially *L. angustifolius*. Cowling and Tarr (*13*) have reported large environmental effects, substantially unrelated to rainfall

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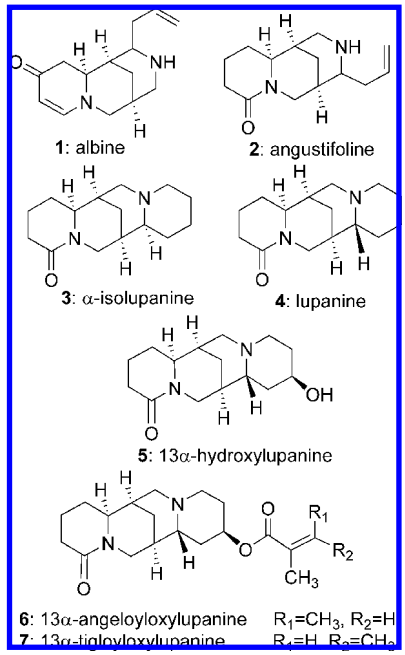

Figure 1. Chemical structures of some quinolizidine alkaloids of lupin seeds.

amount. Early drought stress tended to increase the alkaloid content, but terminal stress had the opposite effect in two of three cultivars in a study by Christiansen et al. (*14*). Adequate soil P level tended to raise the alkaloid contents relative to suboptimal soil P (*15*). Indications on environmental effects associated with major climatic areas may help end-users to locate areas where lupin productions are more likely to meet the required security standard for quinolizidine alkaloids.

In general, gas chromatography−mass spectrometry (GC-MS) is the favorite technique for the quantification of quinolizidine alkaloids in lupin seeds and lupin-based foods or feeds (*2, 16*−*18*). A main issue of this method is that lupanine and the other alkaloids are not commercially available and must be extracted from alkaloid-rich seeds with complex and time-consuming procedures (*16, 19*).

This study investigated the variation in pattern and total amount of quinolizidine alkaloids of seeds from *L. albus* and *L. angustifolius*. The objectives were to assess the genetic variation among and within 11 major geographic regions of *L. albus* ecotypes, locating germplasm with fairly low alkaloids content, which may be useful for crossing with sweet varieties, if also possessing other useful agronomic characteristics; to assess material with particularly high alkaloid amount as a convenient source for lupanine extraction; and to verify the alkaloid amounts and patterns of *L. albus* and *L. angustifolius* sweet varieties and the occurrence of environmental effects on quinolizidine alkaloid content exerted by climatically contrasting (Mediterranean or subcontinental) cropping locations.

MATERIALS AND METHODS

Materials. Hexane and dichloromethane were purchased from Baker (Deventer, The Netherlands); 36% hydrochloric acid was purchased from Merck (Darmstadt, Germany) and 25% ammonia from Carlo Erba (Rodano, Italy). *n*-Alkanes for the determination of the Kovats indices (KIs) were bought from Sigma-Aldrich (St. Louis, MO): pentadecane (purity = 99%), octadecane (99%), docosane (99%), tetracosane (99%), triacontane (99%), hexatriacontane (98%). Sparteine (purity > 99.5%) was purchased from Fluka (Sigma-Aldrich, St. Louis, MO); a sample of lupanine was provided by Prof. F. Sparatore (University of Genova, Italy) and a sample of 13-hydroxylupanine by Dr. H. Reinhard (Swiss Federal Office of Public Health, Bern, Switzerland).

Sampling. A world collection of 48 *L. albus* ecotypes from 11 geographical regions was evaluated. It included four Italian ecotypes collected by CRA-ISCF (named Carpino, Decollatura, S. Croce di Magliano, and Soleto after their collecting sites) and the following accessions kindly provided by INRA of Lusignan (France): ITA57, ITA1, LA246, and ITA49, from Italy; GR33, GR05, GR56, and GR57, from Greece; E3, E2, E16, and E36, from Spain; E91, E126, E107, and E132, from Portugal; AC050, AC139, AC085, and AC155, from Azores; LA646, LA642, LA648, and LA653, from Madeira and Canary Islands (named Mad-Can samples); LA686, LA150, MAROC74, and MAROC78, from Maghreb (Algeria and Morocco); EGYPTE64, EGYPTE76, EGYPTE38, and LA364, from Egypt; LA654, LA656, LA020, and ETHIOP98, from East Africa; LA406, LA415, LA427, and LA673, from the Near East; LA120, LA431, LA110, and LA259, from Turkey. They were evaluated under autumn sowing in Lodi (northern Italy) during the cropping season 2004−2005 adopting an α-lattice design with three replications, each established from 36 seeds. Lodi, which has a subcontinental climate, exhibited the following major climatic characteristics: absolute minimum temperature, −9 °C; number of frost days, 78; winter−spring rainfall (from December 1 to June 15), 212 mm.

The set of alkaloid-poor tested genotypes included six French varieties of *L. albus*, namely, Adam, Arés, Energy, Lucky, Ludic, and Lustar, and three varieties of *L. angustifolius*, namely, Arabella (from Germany) and Jindalee and Quilinoc (from Australia). The *L. albus* varieties were evaluated in Lodi in 2004−2005 and in Sanluri (Sardinia) in 2004−2005 under autumn sowing, whereas the *L. angustifolius* varieties were evaluated in Lodi in 2004−2005 under late-winter (mid-February) sowing and in Sanluri (Sardinia) in 2003−2004 under autumn sowing, adopting in all cases a randomized complete block experiment with two replications. Sanluri, which has a Mediterranean climate with mild winters, was characterized by an absolute minimum temperature of −2.2 and 10 frost days in 2003−2004, no frost during 2004−2005, and winter−spring rainfall (from December 1 to June 15) of 356 mm in 2003−2004 and 240 mm in 2004−2005. Both sites were characterized by moderate soil P content (21 ppm in Lodi; 19 ppm in Sanluri). Each *L. albus* plot was established from 36 seeds, whereas each *L. angustifolius* plot (targeted also to agronomic evaluation) comprised 360 sown seeds. In all experiments, lupin seeds were treated with iprodione (0.95 g/kg of seed) and carbendazim (0.45 g/kg of seed) as active ingredients and were inoculated with NPPL HiStick (Becker Underwood, Toulouse, France) prior to sowing. Chemical analyses were carried out on each plot on a random sample of grains collected at crop maturity.

Quinolizidine Alkaloid Extraction. Lupin seeds were dehulled by hand, ground in a house-hold mill (Braun, Germany), sieved through a 60 mesh screen, and then defatted with hexane for 6 h in a Soxhlet apparatus using cellulose extraction thimbles (123 mm × 43 mm i.d.; Whatman International, Brentford, U.K.). Each dried sample (1 g) was suspended in 8 mL of 0.1 N HCl and stirred at room temperature for 17 h; the mixture was centrifuged at 10000 rpm for 50 min at 4 °C, the supernatant was collected, and the solid was washed again twice with 5 mL of 0.1 N HCl. The gathered extracts were alkalinized with 5% NH_4OH to pH 10−11 and then applied to an Extrelut NT 20 column (Merck, Darmstadt, Germany). After 20 min, the alkaloids were eluted with CH_2Cl_2 (4 × 20 mL) and the solvent was evaporated to dryness under vacuum. The residue was then diluted in an appropriate volume of dichloromethane and analyzed by GC-MS. Each sample was independently extracted at least three times.

GC-MS Analyses. The analyses were performed on a Shimadzu QP-5000 GC-MS instrument equipped with an AOC20i autosampler (Shimadzu) and a 30 m × 0.25 mm i.d., 0.25 μm, AT-1 ms capillary column (Supelco, Milan, Italy). The temperature program was as follows: 150 °C for 5 min, from 150 to 300 °C at 5 °C/min, then 300 °C for 15 min. Analyses were performed in split mode (split ratio 1:25), the injection volume was 1 μL, the injection temperature was 250 °C, the interface temperature was 300 °C, and the acquisition was from *m/z* 50 to 450. The source operated in EI mode at 70 eV. Each analysis

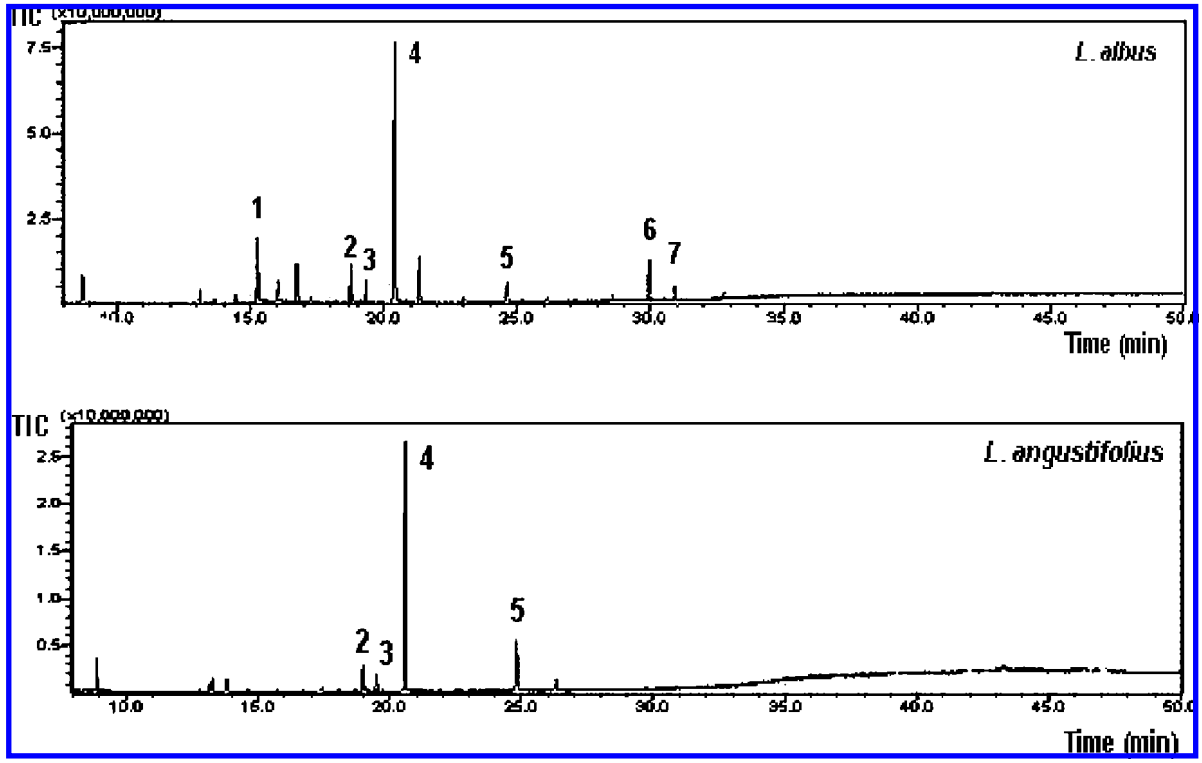

Figure 2. Comparison of GC-MS chromatograms of *Lupinus albus* (cv. Adam) and *Lupinus angustifolius* (cv. Quilinoc). Peaks: 1, albine; 2, angustifoline; 3, α-isolupanine; 4, lupanine; 5, 13α-hydroxylupanine; 6, 13α-angeloyloxylupanine; 7, 13α-tigloyloxylupanine.

was repeated at least four times. KIs were determined by injecting a mixture of linear alkanes: pentadecane (C15), octadecane (C18), docosane (C22), tetracosane (C24), triacontane (C30), and hexatriacontane (C36) according to the method of Kovats (*20*). Exemplary GC-MS chromatograms of the alkaloid extract from seeds of *L. albus* cv. Adam and *L. angustifolius* cv. Quilinoc are shown in **Figure 2**.

Quinolizidine Alkaloid Quantification. The alkaloid quantification was performed in full-scan mode by the external standard method, using lupanine as standard. The calibration curve was prepared by injecting five solutions of lupanine at different concentrations in the range of 0.1−0.9 mg/g, adding a known amount of sparteine to each solution to check the response of the instrument; the relationship between peak area and concentration was linear, and the regression coefficient (R^2) was always >0.99. Because lupanine was the only standard in our hands in sufficient amount and purity, the concentration of the other alkaloids was estimated by using the lupanine calibration curve adjusted for the molecular weight of each alkaloid. For this reason, the quantitative results reported have to be regarded as estimated concentrations (*17*).

In standard solutions, the limit of detection (LOD) and limit of quantification (LOQ) values (S/N > 3) of lupanine were 2 and 3 mg/L, respectively, and those of sparteine 1 and 3 mg/L, respectively, whereas in the lupin flour, the LOD and LOQ values of lupanine were 1 and 2 mg/kg, respectively.

The precision of the method was estimated by analyzing sparteine solutions within the same day (intraday with $n = 10$) and on different days (interday with $n = 5$), obtaining relative standard deviations (RSD%) of 2−3 and 5−6%, respectively. The recovery was evaluated by spiking the flour of *L. albus* cv. Arés with known amounts of sparteine and was always >92%.

Statistical Analysis. The estimated total alkaloid content of ecotypes was subjected to an analysis of variance (ANOVA) aimed to test the variation among regions of origin of the ecotypes and the average variation among ecotypes within region. The latter source of variation acted as the error term for the former, assuming ecotypes within region as a random sample. The extent of among-region and within-region variability was estimated by their components of variance according to a restricted maximum-likelihood method (assuming also regions as a random or, at least, representative sample of the regions of origin of white lupin ecotypes). Additional ANOVAs tested the variation among ecotypes within each region.

The difference in estimated total quinolizidine alkaloid content between subcontinental and Mediterranean climate locations was assessed separately for each alkaloid-poor variety by *t* test. For each lupin species, an ANOVA including the factors variety and location tested the variation between sites and among sweet varieties and the occurrence of genotype × location interaction. All statistical analyses were carried out by Statistical Analysis System (SAS, 1999) software.

RESULTS AND DISCUSSION

The alkaloids were identified by comparing the experimental KIs and mass spectra with literature data (*2, 21*). Lupanine and 13-hydroxylupanine were confirmed also by spiking the samples with authentic standards. In total, seven alkaloids were detected and quantified: albine, angustifoline, α-isolupanine, lupanine, 13α-hydroxylupanine, 13α-angeloyloxylupanine, and 13α-tigloyloxylupanine. The concentrations of lupanine are correct, whereas the concentrations of the other alkaloids were "estimated" by comparing their areas with the calibration curve of lupanine.

Alkaloid-Rich Accessions of *L. albus*. In these samples lupanine was the most abundant alkaloid, representing 88−92% of the total; on average, the content of the other alkaloids followed this order: albine >13α-hydroxylupanine > angustifoline >13α-angeloyloxylupanine. Besides 13α-angeloyloxylupanine, no other ester of 13α-hydroxylupanine was detected.

The total estimated quinolizidine alkaloid content of the alkaloid-rich accessions are reported in **Figure 3**. The alkaloid-richest ecotypes were LA259 (Turkey), ITA49 and Decollatura (Italy), and AC155 (Azores), all above 15.00 mg/g. As expected, no ecotype was sweet; however, the East African accession LA656 (0.80 mg/g) and the Egyptian accessions LA364 (1.84 mg/g) and EGYPTE38 (1.95 mg/g) could be classified as "semibitter" (i.e., with alkaloids in the range of 1.00−2.00 mg/

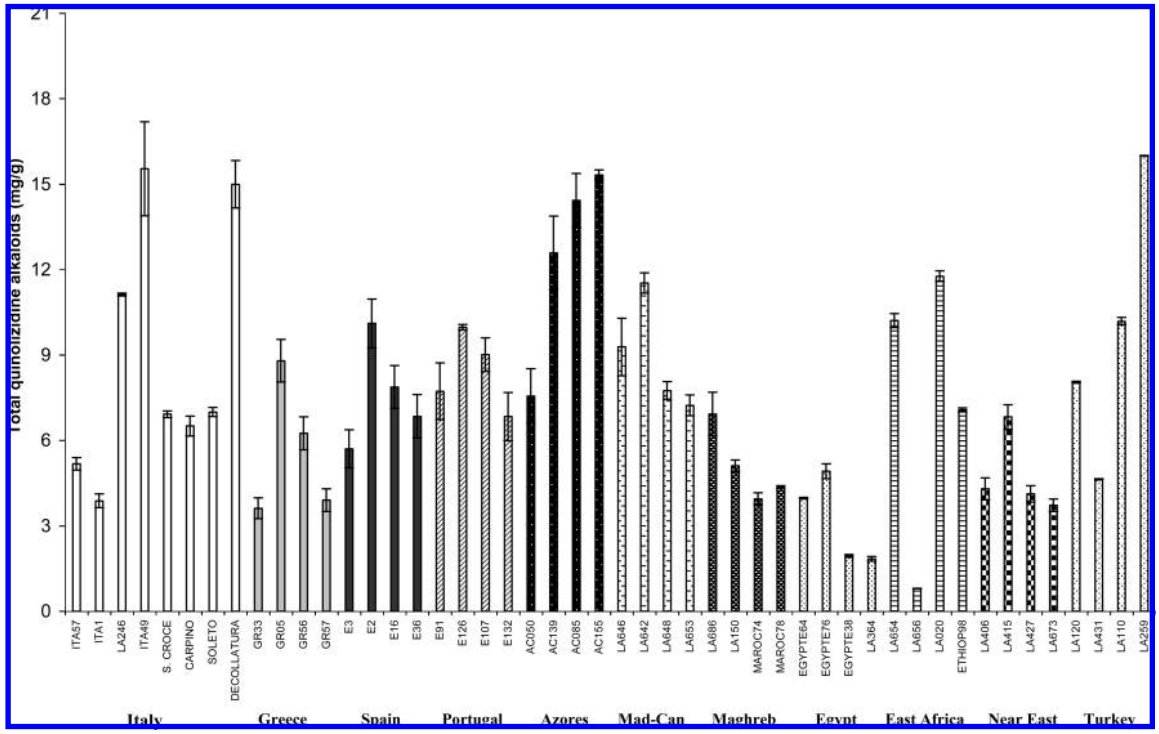

Figure 3. Total estimated quinolizidine alkaloid content of 48 ecotypes of *L. albus* from 11 regions of origin expressed as milligrams per gram of flour from dehulled seed (mean ± standard error; $n \geq 3$).

g) according to the definition proposed by von Baer and Perez (*22*). None of these samples reached values of >20 mg/g as observed by Muzquiz et al. (*23*) on different ecotypes.

The analysis of variance indicated that both among-region and average within-region variations for total quinolizidine content were significant ($P < 0.001$), but the estimated components of variance indicated the larger size of the variation among ecotypes within regions relative to that among regions, 10.61 versus 3.76 $(mg/g)^2$. The within-region variation was significant for all regions (at $P < 0.05$ for Portugal and Spain; at $P < 0.01$ for the other regions). Wide within-region variation was detected for Italy (range = 3.88−15.54 mg/g of flour), East Africa (range = 0.80−11.77 mg/g), and Turkey (range = 4.64−16.00 mg/g). The ecotypes from Azores displayed, on average, the highest alkaloid content (12.48 mg/g). This region differed significantly ($P < 0.05$) from those characterized by lowest alkaloids levels, namely, Egypt (3.17 mg/g on average), the Near East (4.74 mg/g on average), Maghreb (5.09 mg/g on average), and Greece (5.64 mg/g on average).

These results suggest that the selective pressure leading to greater or lower alkaloid content [as determined mainly by the extent of biotic stress from pathogens and herbivores (*2, 3*)] varied markedly within each region. Although some regions tended to high (Azores) or low (Egypt, the Near East, Maghreb) content of alkaloids, it is mainly the within-region variation that should be explored to locate material with extremely high or low alkaloid values. The alkaloid-poorest ecotypes may be preferred as exotic genetic resources in crossing programs aimed at increasing the genetic variation available for plant selection. Information on the agronomic value in climatically contrasting regions of these accessions is being generated (Annicchiarico et al., unpublished data). The alkaloid-richest ecotypes may be exploited, instead, for extracting pure lupanine or other alkaloids, considering that authentic samples of these compounds for use as analytical standards are not commercially available. Lupanine and other quinolizidine alkaloids may also be employed as intermediates for producing semisynthetic derivatives with improved characteristics and useful pharmacological properties (*2, 24*).

Alkaloid-Poor Genotypes of *L. albus* and *L. angustifolius*. The chromatograms of the alkaloid-poor cultivars (**Figure 2**) were more diversified than those of the ecotypes, owing to the greater contribution of minor components to total quinolizidine alkaloid content: in fact, the biosynthetic block that occurs in sweet varieties mainly concerns lupanine (*25*).

The content of single alkaloids for each cultivar is reported in **Table 1**. The differences in the pattern between *L. albus* and *L. angustifolius* are shown in **Table 1** and in the chromatograms of **Figure 2**. In *L. albus*, the most abundant alkaloid was always lupanine, followed by smaller amounts of albine and 13α-angeloyloxylupanine, which were present in all samples, and of 13α-hydroxylupanine, which was detected in most samples. Small amounts of angustifoline and 13α-tigloyloxylupanine were detected only in about half of the samples, whereas α-isolupanine was found only in three samples. The presence of angustifoline is rather uncommon in *L. albus*, but it was already reported (*2*).

Surprisingly, some minor alkaloids were quantified in only one cropping site for the same variety (**Table 1**); the occasional detection of a minor alkaloid in only one environment does not indicate necessarily an environmental effect, as some compounds could be present in amounts below the instrumental LOQ in the other environment.

The pattern of *L. angustifolius* seeds is simpler (**Table 1** and **Figure 2**). Besides lupanine, only three minor alkaloids were present in all samples: 13α-hydroxylupanine, angustifoline, and α-isolupanine, in order of abundance.

The total alkaloid contents of the alkaloid-poor cultivars, both *L. albus* and *L. angustifolius*, are compared in **Figure 4**. The variation was larger than expected, because it ranged from 0.050 mg/g (Lucky in Lodi) to 0.565 mg/g (Adam in Sanluri) in *L. albus* and from 0.141 mg/g (Quilinoc in Sanluri) to 0.701 mg/g

Table 1. Composition of Quinolizidine Alkaloids in Alkaloid-Poor Seeds of *L. albus* and *L. angustifolius* Grown in Two Climatically-Contrasting Italian Sites (LO, Lodi, Subcontinental; SA, Sanluri, Mediterranean) Expressed in Milligrams per Gram of Flour from Dehulled Seed

		mg/g of flour (mean ± standard error; $n \geq 3$)						
no.	sample	albine	angustifoline	α-isolupanine	lupanine	13α-hydroxylupanine	13α-angeloyloxylupanine	13α-tigloyloxylupanine
1	Adam LO	0.068 ± 0.003	0.022 ± 0.001	0.022 ± 0.001	0.258 ± 0.016	0.079 ± 0.003	0.088 ± 0.004	0.028 ± 0.001
2	Adam SA	0.046 ± 0.002	0.018 ± 0.001	0.011 ± 0.001	0.209 ± 0.018	0.056 ± 0.005	0.071 ± 0.004	
3	Ares LO	0.019 ± 0.001	0.012 ± 0.001		0.238 ± 0.005	0.021 ± 0.001	0.026 ± 0.002	0.013 ± 0.001
4	Ares SA	0.010 ± 0.001	0.006 ± 0.001		0.064 ± 0.002	0.010 ± 0.001	0.019 ± 0.001	0.009 ± 0.001
5	Energy LO	0.018 ± 0.001			0.257 ± 0.010	0.017 ± 0.001	0.019 ± 0.001	0.017 ± 0.001
6	Energy SA	0.010 ± 0.001			0.148 ± 0.013	0.009 ± 0.001	0.010 ± 0.001	0.010 ± 0.001
7	Lucky LO	0.013 ± 0.001			0.026 ± 0.002		0.011 ± 0.001	
8	Lucky SA	0.018 ± 0.001			0.029 ± 0.001	0.017 ± 0.001	0.012 ± 0.001	
9	Ludic LO	0.025 ± 0.002	0.013 ± 0.001	0.007 ± 0.001	0.161 ± 0.008	0.021 ± 0.001	0.063 ± 0.003	
10	Ludic SA	0.014 ± 0.001	0.008 ± 0.001		0.090 ± 0.006		0.034 ± 0.002	
11	Lustar LO	0.033 ± 0.002	0.009 ± 0.001		0.061 ± 0.002	0.013 ± 0.001	0.015 ± 0.001	
12	Lustar SA	0.013 ± 0.001			0.026 ± 0.001	0.007 ± 0.001	0.011 ± 0.001	0.008 ± 0.001
13	Arabella LO[a]		0.051 ± 0.006	0.034 ± 0.004	0.426 ± 0.016	0.082 ± 0.004		
14	Arabella SA[a]		0.041 ± 0.001	0.023 ± 0.001	0.329 ± 0.018	0.059 ± 0.002		
15	Jindalee LO[a]		0.062 ± 0.006	0.008 ± 0.001	0.452 ± 0.036	0.179 ± 0.012		
16	Jindalee SA[a]		0.063 ± 0.002	0.011 ± 0.001	0.394 ± 0.020	0.183 ± 0.007		
17	Quilinoc LO[a]		0.012 ± 0.006	0.008 ± 0.001	0.112 ± 0.006	0.031 ± 0.001		
18	Quilinoc SA[a]		0.012 ± 0.001	0.011 ± 0.001	0.092 ± 0.012	0.026 ± 0.001		

[a] *L. angustifolius*.

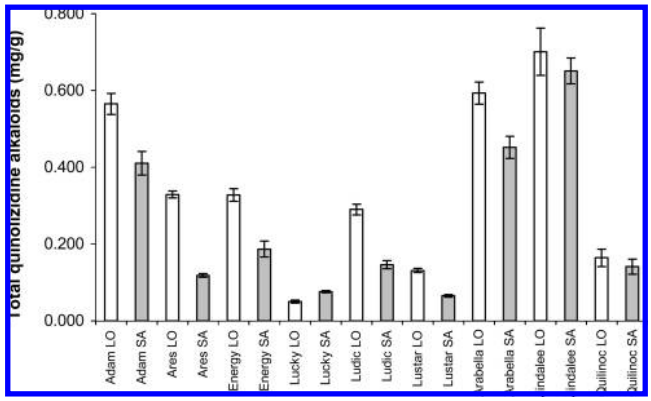

Figure 4. Total estimated quinolizidine alkaloid content in nine alkaloid-poor varieties of *L. albus* or *L. angustifolius* grown in two climatically contrasting Italian sites (LO, Lodi, subcontinental; SA, Sanluri, Mediterranean) expressed as milligrams per gram of flour from dehulled seed (mean ± standard error; $n \geq 3$).

(Jindalee in Lodi) in *L. angustifolius*. Of the 22 variety−location combinations, 6 of *L. albus* (including all samples of Lucky and Lustar) and 2 of *L. angustifolius* (Quilinoc in both sites) contained <0.2 mg/g alkaloids, whereas 1 of *L. albus* (Adam in Lodi) and 3 of *L. angustifolius* (Jindalee in both sites and Arabella in Lodi) exceeded the value of 0.5 mg/g proposed as the maximum limit for "sweet" seeds by Von Baer and Pérez (*22*).

The subcontinental climate location led to significantly ($P < 0.05$) greater quinolizidine alkaloid contents than the Mediterranean site for the *L. angustifolius* variety Arabella and for all *L. albus* varieties, with the exception of Lucky, according to the *t* test. Although nonsignificant, this trend occurred also for the other two varieties of *L. angustifolius* (**Figure 4**). The ANOVA confirmed the overall greater alkaloid contents of the materials grown in Lodi relative to Sanluri [0.280 vs 0.165 mg/g in *L. albus* and 0.486 vs 0.415 mg/g in *L. angustifolius* ($P < 0.01$)]. It also confirmed the occurrence of variation among varieties within each species ($P < 0.01$) and, in this context, the lower total quinolizidine alkaloid contents of Lustar and Lucky, within *L. albus*, and Quilinoc, within *L. angustifolius*. Genotype × location interaction for total alkaloid content was sizable ($P < 0.01$) in *L. albus*, implying a few changes of variety rank between the two locations, and limited ($P < 0.05$) in *L. angustifolius*, where variety ranks were consistent across locations (**Figure 4**).

A major environmental effect that was consistent in the two species was the greater total alkaloid content of grains grown in the subcontinental climate site relative to the Mediterranean location. The main difference between these cropping environments concerned the extent of low temperatures during winter (given the absence of large differences in rainfall amount), but their impact in the cold-prone site was limited on *L. angustifolius* material because of its late-winter sowing. This study confirms also for *L. albus* material the occurrence of environmental effects on total quinolizidine alkaloid content, which had emerged earlier for alkaloid-poor *L. angustifolius* genotypes (*13*−*15*).

As already cited, in addition to the long-standing interest in lupins as a protein-rich feed supplement (*9*), the past decade has seen a fast increase in the exploitation of lupin for human nutrition, in particular, to replace animal or soybean proteins in imitation meat products, pasta, bread, snacks, and beverages (*26*−*28*). Furthermore, lupin protein concentrates and isolates have shown some emulsifying and foaming properties, important in food production (*29*), and recent literature has indicated some possible health benefits related to the consumption of lupin proteins (*30, 31*). A clear proof of the development of the lupin market comes from the decision of the European Commission to include lupin in the list of food allergens requiring mandatory declaration on the food label (*32*).

An outcome of the increasing application of lupin ingredients in food formulation may be a growing exposure of consumers to quinolizidine alkaloids. In the agricultural practice, the total quinolizidine alkaloid content of 0.500 mg/g is considered the limit between alkaloid-rich and alkaloid-poor white lupin seeds (*21*). Von Baer and Pérez (*22*) attributed the term "sweet" to grains having quinolizidine alkaloid content below 0.500 mg/g, "semisweet" in the range of 0.500−1.00 mg/g, "semibitter" in the range of 1.00−2.00 mg/g, and "bitter" above this limit.

Although some recent papers (*16, 17, 33*) have confirmed the safety of commercially available lupin-based foods, it would be useful to revise the lupin seed classification by taking into account the limit of 0.200 mg/g for human consumption introduced by the Health Authorities of some countries (*5, 11, 12*), defining as "sweet" the grains having quinolizidine alkaloid

content below 0.200 mg/g. Only flours from seeds of this class could be used in any ratio in the formulation of human foods, whereas seeds from semisweet material could be added only in controlled ratios or after some treatments aimed to reduce the quinolizidine alkaloid content.

The relatively high alkaloid values exhibited by some "low-alkaloid" varieties in this study refer to the specific lot of commercial seed that was evaluated and may not reflect the intrinsic genetic characteristics of the cultivars, as displayed, for instance, in prebasic seed prior to commercial multiplication. Indeed, seed multiplication is exposed to the risk of genetic shift toward higher alkaloid content due to pollen flow from bitter material, which increases over generations of multiplication as a consequence of higher advantage of greater bitterness under natural selection (*9*). In this context, our results reinforce the need to carefully monitor the alkaloid content of seeds not only in production lots but also across the various stages of seed multiplication of the varieties, as well as consider the different environmental conditions of their cultivation.

ABBREVIATIONS USED

EI, electron impact; KI, Kovats index; LOD, limit of detection; LOQ, limit of quantification.

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