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Field Crops Research 87 (2004) 23–34
Field Crops Research
www.elsevier.com/locate/fcr

Cultivar discrimination of Portuguese *Lupinus albus* by seed protein electrophoresis: the importance of considering ''glutelins'' and glycoproteins

Ana C. Vaz[a], Carla Pinheiro[a], João M.N. Martins[b], Cândido P.P. Ricardo[a,b,*]

[a]*Instituto de Tecnologia Química e Biológica, Ap 127, 2781-901 Oeiras, Portugal*
[b]*Departamento de Botânica e Engenharia Biológica, ISA, 1349-017 Lisboa, Portugal*

Received 25 July 2002; received in revised form 20 August 2003; accepted 1 September 2003

Abstract

The Osborne protein fractions of seeds of 17 *Lupinus albus* cultivars from different regions in Portugal (obtained from plants grown at the same location) were analysed. The objective of this work was to examine the variability in the seed protein fractions of such a *L. albus* germplasm sample in order to disclose existing genetic relationships and to obtain information useful for a breeding program. Globulins and, interestingly, the fraction extracted with NaOH (''glutelins'') were the major seed protein constituents. The several protein fractions were separated by electrophoresis (reducing SDS polyacrylamide gel electrophoresis) and the patterns examined by multivariate analysis. Bands of ''glutelins'' together with those of glycosylated polypeptides were found to have the highest discriminating capacity. Lupin populations were grouped according to their clinal geographical distribution from north (small-seeded, higher plant architecture and later flowering type) to south (large-seeded, shorter and early flowering type). It is also possible that polypeptide patterns could reflect microclimatic specificities related with altitude and temperature. In fact, one cultivar from the south but from high altitude was grouped with the north plant types adapted to colder and wetter weather, whereas two cultivars from a warmer north region were grouped with the southern types. The results show, in addition, that a general correlation exists between cultivar early flowering characteristics and higher amounts of both total seed protein and glutelins, and lower glutelin glycosylation. Since high seed protein content and early flowering are important aims of lupin breeding programs, these findings on the seed protein fractions could stimulate and direct future research in lupin improvement.

Keywords: Accession clustering; Glutelins; Glycoproteins; *Lupinus albus*; Molecular marker; Environment correlation; Seed storage proteins

1. Introduction

Grain legumes are important crops because they fix their own nitrogen and their seed storage proteins are the main nitrogen source in human and livestock nutrition (Duranti and Gius, 1997). Relative to other legumes, lupin seeds have a high protein content (Hill,

* Corresponding author. Tel.: +351-21-4469654; fax: +351-21-4433644.
E-mail address: ricardo@itqb.unl.pt (C.P.P. Ricardo).

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doi:10.1016/j.fcr.2003.09.006

1986; Petterson, 1998) and low levels of toxic antinutritional compounds (Gross, 1988). In many aspects sweet lupin seeds compare favourably with soya bean as a protein supplement for non-ruminants (Hill, 1986). However, lupins are more agronomically versatile than soya beans, since they are better adapted to growth in mild winter or cold summer rainfall areas and on poor soils (Gladstones, 1998). They are also able to mobilise phosphates unavailable to other plants (Huyghe, 1997; Perry et al., 1998; Vance, 2001). In spite of this, the major use of lupins in Europe has been as fodder and green manure. However, there is now an increased interest in lupins as grain legumes due to their potential to improve farming systems and soil sustainability (Duranti and Gius, 1997; Perry et al., 1998; Howieson et al., 2000; Vance, 2001).

Lupinus albus L. is a traditional and widespread crop in Portugal, where it exists in many distinct forms as a result of adaptation to a variety of soil and climatic conditions throughout the country. Martins (1994) showed that the architecture and behaviour of the plants changes from north (tall and late flowering plants) to south (short and early flowering types), reflecting differences in the phylogenetic origins of the studied accessions. Since seed storage proteins are considered to be useful and reliable genetic markers (Radić et al., 1997), the renewed interest in *L. albus* led us to evaluate a sample of 17 representative Portuguese cultivars (experimentally grown in the same soil) through the analysis of their seed protein constituents separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE). This technique has proven its reliability for the identification of characteristic differences in band patterns of seed storage proteins (Bonetti et al., 1995).

The objective of our work was to examine the variability in the seed protein fractions in order to disclose existing genetic relationships and to obtain information useful for a breeding program. Analysis of the results by a program of the Numerical Taxonomy System (NTSYS) with multivariate analysis (Rholf, 1998), led to the identification of several marker polypeptides important in the definition of groups of cultivars, which were strongly related with the climatic conditions of cultivar origin. Furthermore, a general correlation was observed between cultivar early flowering characteristics and a higher content of total seed protein and the frequency of certain polypeptides, a finding that appears to be of agronomic relevance.

2. Material and methods

2.1. Seed material

All seeds were obtained from the germplasm collection of the Departamento de Botânica e Engenharia Biológica (Instituto Superior de Agronomia, ISA, Lisboa). Sixteen cultivars of *L. albus* L. that are traditionally grown in distinct regions of the country (Fig. 1) were studied plus the epigonal line (EPI) which is a mutant of low size, small seed and high precocity. All the seeds used in the experiments were produced on the same soil at ISA.

2.2. Extraction of mature seed proteins

The seed tegument was removed, the embryonic axis discarded and the cotyledons powdered and used for protein fractionation following the method of Derbyshire et al. (1976). The seed meal was extracted successively with water, 5% (w/v) NaCl, 75% (v/v) ethanol and 0.25% (w/v) NaOH. The detailed scheme of extraction is represented in Fig. 2. Due to the presence of salts in the seeds themselves, extraction with water removed some salt-soluble proteins that precipitated on dialysis (termed A globulins).

The pellet fractions were dried overnight in the air at 4 °C and the supernatant fractions were lyophilised; each fraction was resuspended in sample buffer [50 mM Tris–HCl, pH 6.8, 100 mM 2-mercaptoethanol, 2% (w/v) sodium dodecyl sulphate (SDS), 15% (v/v) glycerol and 0.06% (w/v) *m*-cresol purple].

2.3. Electrophoretic separations

The protein content of the fractions was quantified by the Lowry method (Bensadoun and Weinstein, 1976) and 150 μg protein of each fraction was analysed by SDS-PAGE according to Laemmli (1970) (4% T, 2.6% C in the stacking gel; 12% T, 2.6% C in the running gel). Sigma molecular markers (MW-SDS-70L) were included. Two slab gels (16 cm × 18 cm × 1.0 mm) were run together at 50 mA for 3 h and

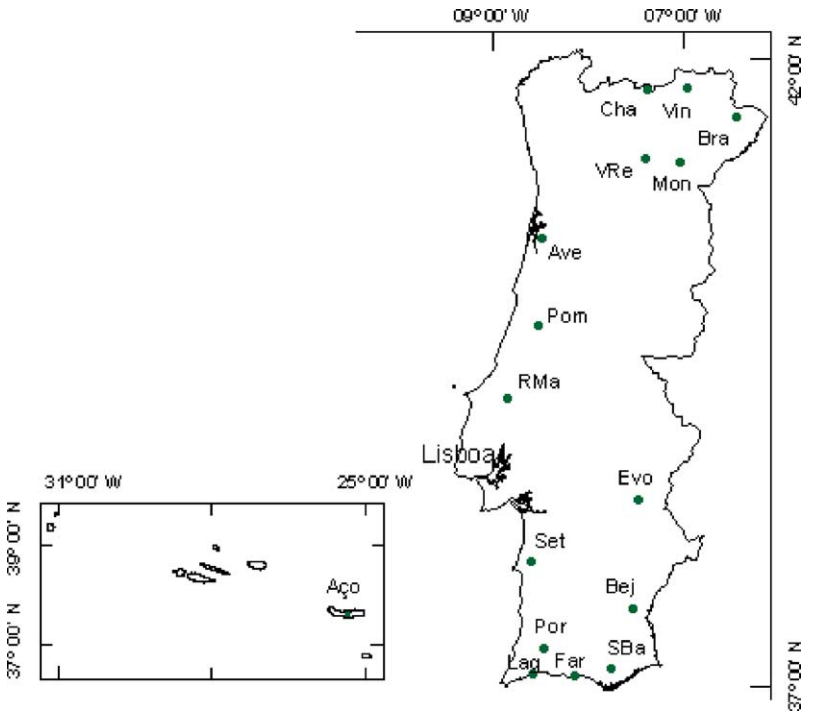

Fig. 1. Geographical distribution of the accessions studied. The accessions are from continental Portugal either north populations (Cha, Vin, Bra, Mon, VRe), the south populations (Lag, Far, Por, SBa, Bej, Set, Evo), or the *megalosperma* type (Pom, Ave, RMa) and from the Azores islands (Aço). The EPI is not shown.

the gels were stained with Coomassie Brilliant Blue G-250.

For the detection of glycoproteins, the electrophoretically separated proteins were electroblotted onto an immobilising polyvinylidene difluoride (PVDF) membrane for 1 h at 80 mA at room temperature with 25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS and 10% methanol as transfer buffer. After electrophoretic transfer, the PVDF membrane was rinsed with water and then with TBS (20 mM Tris–HCl pH 7.4 and 0.5 M NaCl). To saturate non-occupied binding sites in the PVDF membrane it was incubated in TTBS [0.1% (w/v) Tween 20 in TBS] for 1 h at room temperature. Glycoproteins were detected according to Faye and Chrispeels (1985). The membrane was incubated with concanavalin-A (25 μg/ml, Sigma C-2010) in TTBSS (1 mM $CaCl_2$ and 1 mM $MgCl_2$ in TTBS) for 1 h at room temperature with shaking, washed four times (5 min each) with TTBSS and once in TBS and further incubated with peroxidase I (50 μg/ml, Sigma P-8125), in TTBSS for 1 h at room temperature with shaking. Afterwards, the membrane was washed four times (5 min each) with TTBSS and once in TBS. The glycoproteins were detected by staining with 0.06% (w/v) 4-chloro-1-naphthol, 0.03% (v/v) H_2O_2 and 20% methanol in TTBSS. Incubation with the peroxidase substrate solutions was carried out until the glycoproteins bands were clearly visible, and the reaction stopped by washing the membrane in distilled water.

2.4. Multivariate analysis of the data

All the bands (a total of 171) detected in the several gels were numbered. Their presence (1) or absence (0) was recorded in a matrix that relates the polypeptide bands and the cultivars. The calculations and analysis of the polypeptides patterns were computed by the Windows Version 2.01b of Numerical Taxonomic System (NTSYSpc) program modules (Rholf, 1998). The principal co-ordinate analysis on the binary data was performed using the original matrix with

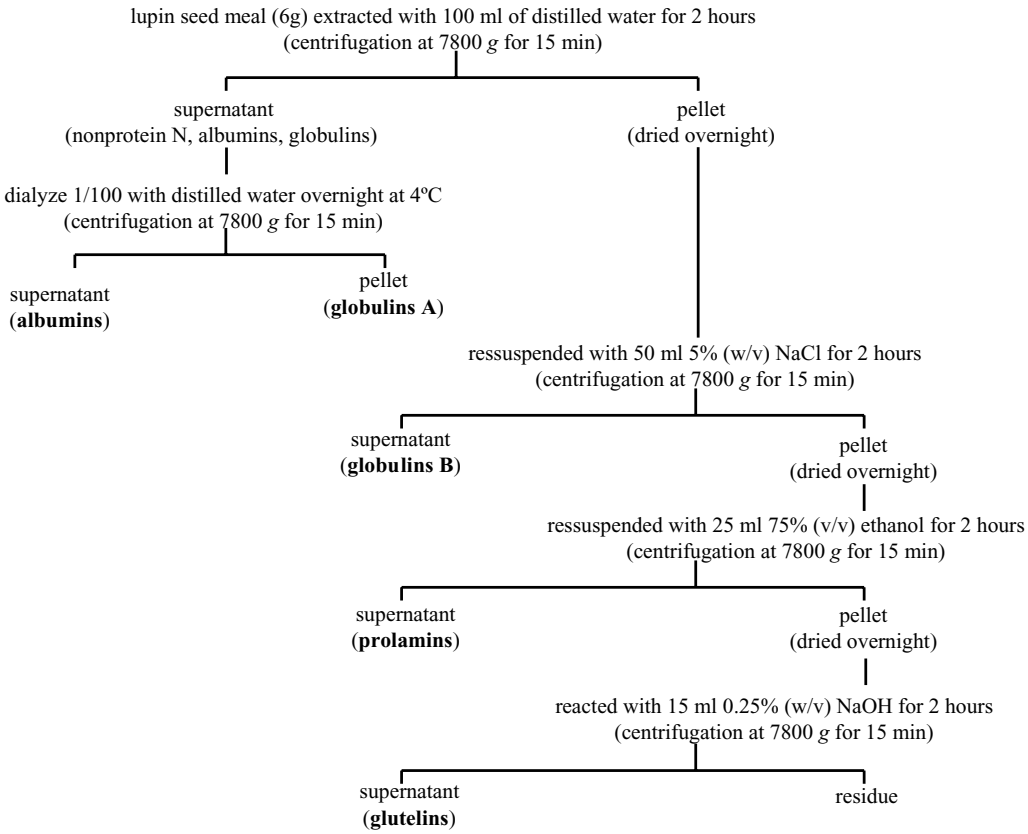

Fig. 2. Extraction procedure followed to obtain the classical Osborne seed protein fractions.

17 columns, quantifying the lupin accessions and the 171 bands. As only some of the bands were found to be discriminating, the original matrix was reduced to a 60 by 17 matrix. The dissimilarity matrix between accessions was calculated using the *simple matching coefficient* and performed by the SimQual module that computes a variety of similarity and dissimilarity coefficients (association coefficients) for qualitative (nominal) data. For a two-way frequency table for all pairs of two objects i and j, the simple matching coefficient is calculated dividing m by n, where $m = a + b$ (number of matches), $u = b + c$ (number of unmatches) and $n = u + m$ (total sample size) (Sneath and Sokal, 1973; Legendre and Legendre, 1983). This dissimilarity matrix was *double centred* (Dcentre module) in which row and column means were subtracted from each element and the grand mean was added on. When this new matrix was factored (EIGEN module), the elements of the eigenvectors corresponding to the positive eigenvalues could be interpreted as the co-ordinates of each point in the Cartesian space (resulting in a principal co-ordinates analysis). The EIGEN module computed the eigenvectors of the transformed matrix and the resulting vectors are the principal co-ordinate axes for the populations analysed.

3. Results and discussion

3.1. Seed protein fractionation

The sequential extraction of the lupin seed proteins based on differences in solubility allowed the easy separation of the Osborne fractions: albumins, globulins A, globulins B, prolamins and glutelins, from the 17 cultivars studied (Table 1). It is evident that lupin glutelins (30–71% of the total protein) and globulins

Table 1
Total cotyledon protein content (g protein/g dry weight) and percentage of albumins, globulins, glutelins and prolamins of cotyledons in the total protein for 17 *L. albus* accessions

L. albus line	Accession number	Total protein (g/g DW)	Albumins (%)	Globulins A (%)	Globulins B (%)	Glutelins (%)	Prolamins (%)
Aço	6	0.197	16.2	3.7	36.0	43.7	0.25
Ave	29	0.203	16.7	4.5	25.6	52.6	0.55
Bej	43	0.209	12.5	2.5	32.1	52.7	0.22
Bra	12	0.234	17.1	2.0	50.1	30.4	0.43
Cha	7	0.196	20.9	3.9	27.5	47.4	0.36
EPI	–	0.224	12.0	3.9	13.8	70.1	0.21
Evo	40	0.254	11.8	2.4	26.0	59.3	0.39
Far	49	0.184	12.7	7.8	20.4	59.0	0.18
Lag	–	0.213	10.8	4.6	13.1	71.2	0.30
Mon	18	0.173	18.6	3.8	29.0	48.1	0.54
Pom	30	0.174	17.2	4.9	23.5	53.9	0.48
Por	47	0.282	9.9	2.9	28.3	58.6	0.27
RMa	32	0.183	15.3	9.2	27.3	48.0	0.34
SBa	48	0.201	15.4	4.6	21.4	58.3	0.19
Set	42	0.231	12.1	1.5	23.4	62.8	0.25
Vin	9	0.248	14.9	2.3	33.9	48.5	0.30
VRe	20	0.267	11.2	3.3	30.3	55.0	0.20

(18–52%) are the main protein fractions of the seed. Such high glutelin contents are not usually cited for legume seeds. It should be kept in mind that although the Osborne solubility classes remain useful as working definitions they do not take into consideration the modern knowledge on proteins. Recent molecular and biochemical analyses of seed storage proteins and their genes indicate that these proteins fall into three major groups: albumins, globulins and prolamins (Shewry and Casey, 1999), the Osborne glutelins being considered either of the globulin type (Shewry and Casey, 1999) or of the prolamin type (Shewry and Tatham, 1999). In spite of differences in solubility, the salt-soluble globulins from legumes, rice and oat and the salt-insoluble glutelins from rice, have similarities in primary sequences that make all of them members of the globulin gene family (Katsube et al., 1999).

Total protein content was seen to vary greatly (17–28% of the dry weight) between the lupin cultivars (Table 1). Despite differences that might be due to genotype and location these values appear to be somewhat lower than those described by Huyghe (1997) and Lilley (1999) for the seeds of white lupins. However, it should be noticed that protein concentration of the seeds is usually obtained by multiplying the total nitrogen content by a conversion factor of 6.25, which causes an overestimation of protein content due to the contribution of non-protein nitrogen (Mossé, 1990). Such interference did not apply to our figures that were obtained by adding together the values of the protein fractions.

3.2. SDS-PAGE of seed proteins

Polypeptide separation for the several seed protein fractions was performed. Since the use of protease inhibitors during extraction has been discussed (Derbyshire et al., 1976; Casey et al., 1986), we tested the effect of an inhibitor cocktail (1 mM leupeptin, 1 mM pepstatin A, 1 mM aprotinin and 1 mM EDTA) on the polypeptide patterns. We found no significant differences for the patterns obtained in the presence or absence of the inhibitors (Fig. 3).

The SDS-PAGE polypeptide patterns of albumins, globulins B and glutelins, stained with Coomassie Blue are shown in Fig. 4A–C for the 17 lupin cultivars. The corresponding glycoprotein patterns are shown in Fig. 5.

3.3. Clustering of lupin accessions

In order to analyse the variability in the overall polypeptide bands, the patterns of the Coomassie Blue stained polypeptides (120 bands) and of the

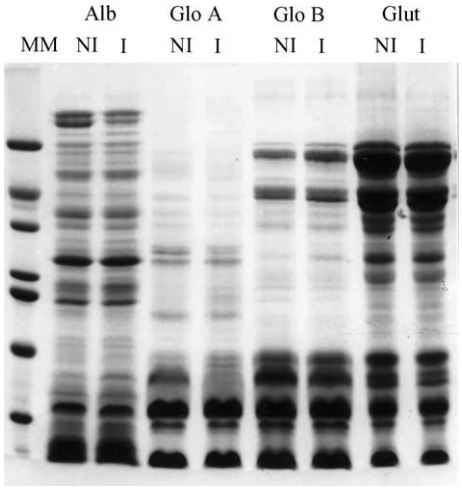

Fig. 3. Electrophoretic patterns of the *L. albus* (Aço accession) seed albumins, globulins A and B and glutelins extracted in the presence (I) or absence (NI) of an inhibitor cocktail. Molecular weight markers are 66, 45, 36, 29, 24, 21.5 and 14.3 kDa.

glycosylated polypeptides (51 bands) were converted into a numerical matrix (data not shown) according to Rholf (1998) (see Section 2). Only 60 of these bands were found to be discriminating: 3 of the 34 albumins; 2 of the 25 globulins A; 8 of the 30 globulins B; 21 of the 30 glutelins and 25 of the 51 glycoproteins (5 of the 12 albumins, 5 of the 12 globulins A, 8 of the 15 globulins B and 8 of the 13 glutelins). Table 2 shows for these 60 bands the discriminant character loading on the principal co-ordinate axes.

It is noteworthy that previous attempts to distinguish cultivars by electrophoretic patterns of only globulins and albumins in lupins (Przybylska and Zymniac-Przybylska, 1994; Santos et al., 1997) and in alfalfa (Krochko and Bewley, 2000) were not successful. Interestingly, although glutelins are not considered to be a usual class of seed proteins in legumes, these NaOH soluble proteins appear to be very important for discriminating between the lupin accessions. Glycosylated proteins were also seen to be useful for the distinction of lupin cultivars, an observation also made by Weiss et al. (1991) in barley.

The projection of the 17 populations into two spaces (1,2 and 1,3 principal co-ordinate axes) when analysed throughout the 60 discriminating polypeptide bands by 2D plot module is presented in Fig. 6A and B. Using the projection module we performed the projection of the variables (60 discriminating bands) that describe the spread of the populations in a multi-dimensional space. The projection plane is a two-dimension space of first versus second principal co-ordinate axes, and the orthogonal plane first versus third principal co-ordinate axes (Fig. 6C and D).

Two main groups of clusters were observed (Fig. 6A and B). Along the first co-ordinate axis (which explains the maximum variability, 23%), one cluster is mostly composed of accessions from the north of Portugal and the Atlantic islands. The other cluster has populations mainly from central and south Portugal. It is clear that variation in glutelins and glycosylated proteins greatly contributed to the definition of these two groups. Indeed, the first group is explained either by the glycosylated glutelins s_6, s_7, s_{11}, s_{10} or by the glycosylated globulins r_{13}, r_7, r_3, while the second group is explained by the glutelins t_{26}, t_{27}, t_8, t_9, the glycosylated glutelins s_5, s_8 and by the glycosylated albumin g_8.

3.4. Relationship between cultivar characteristics and climatic conditions

Protein plots are, then, able to discriminate populations according to their clinal geographical distribution, i.e., between the small-seeded, taller plant architecture and later flowering group from the north (rainy and cooler region) and the large-seeded, shorter and early flowering type group from the south (dry and warmer region). Interestingly, the placement of some cultivars, which appear as exceptions to this general rule, could be explained by microclimatic variation within the macroclimatic environment. This can be illustrated by the cooler (2–3 °C) and wetter (2–3 times) southern Monchique mountain area, which approaches the northern climatic conditions and the warmer (3–6 °C) and dryer (less than 50% precipitation) north region known as ‘‘Terra Quente Durience’’ that resembles the Mediterranean regions of the south (INMG, 1991; Daveau, 1995). Thus, the Por cultivar from the Monchique mountain area, electrophoretically resembles the vigorous, small-seeded and late flowering populations from the north. Conversely, Mon and VRe, from ‘‘Terra Quente Durience’’ are larger-seeded and shorter architecture cultivars that resemble the southern type. Aço, which originates in the Azores islands (a rainy Atlantic archipelago), is the

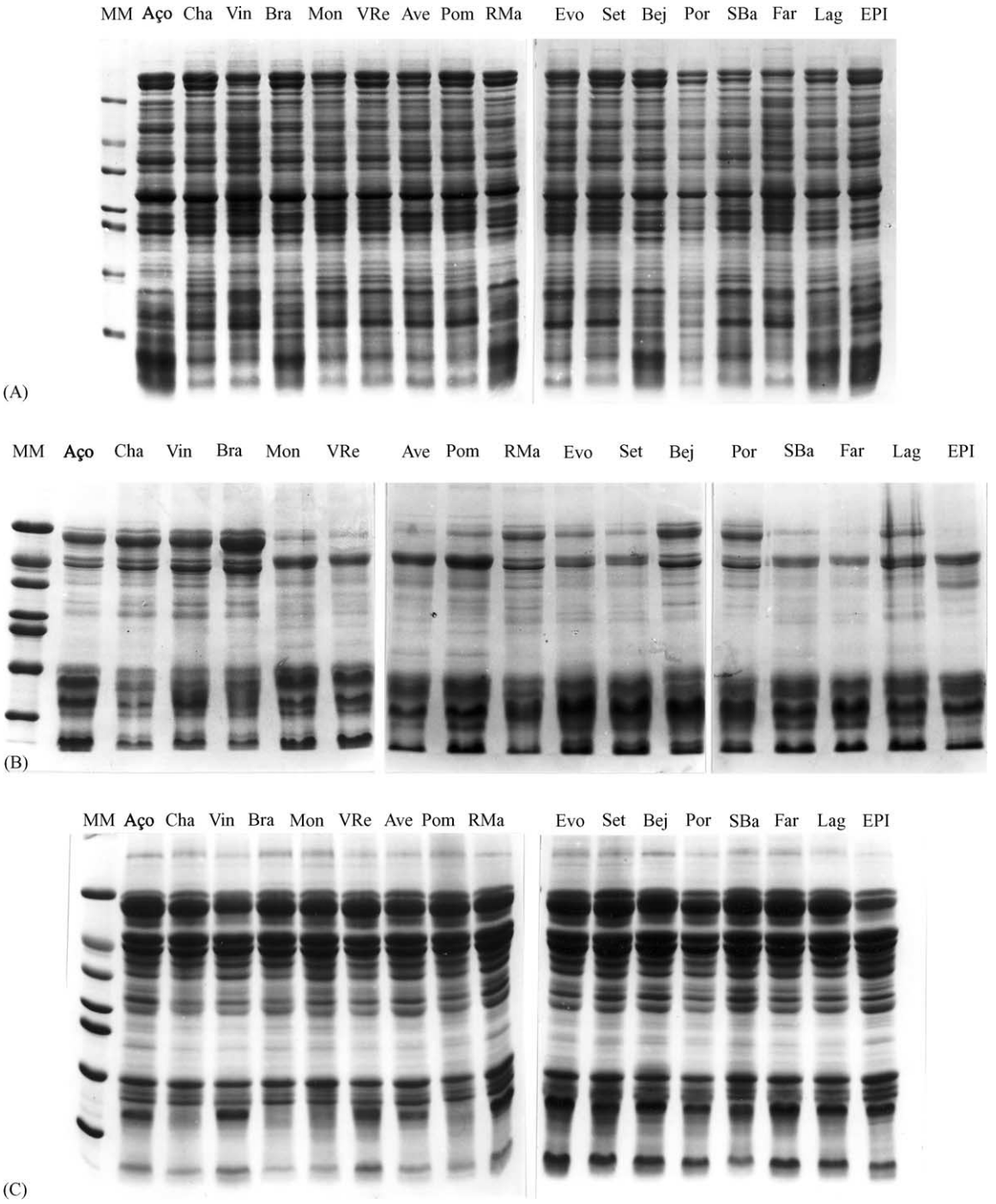

Fig. 4. SDS-PAGE of *L. albus* seed albumins (A) (see Fig. 1), seed globulins B (B) and seed glutelins (C) of the 17 accessions. Molecular weight markers are 66, 45, 36, 29, 24, 21.5 and 14.3 kDa.

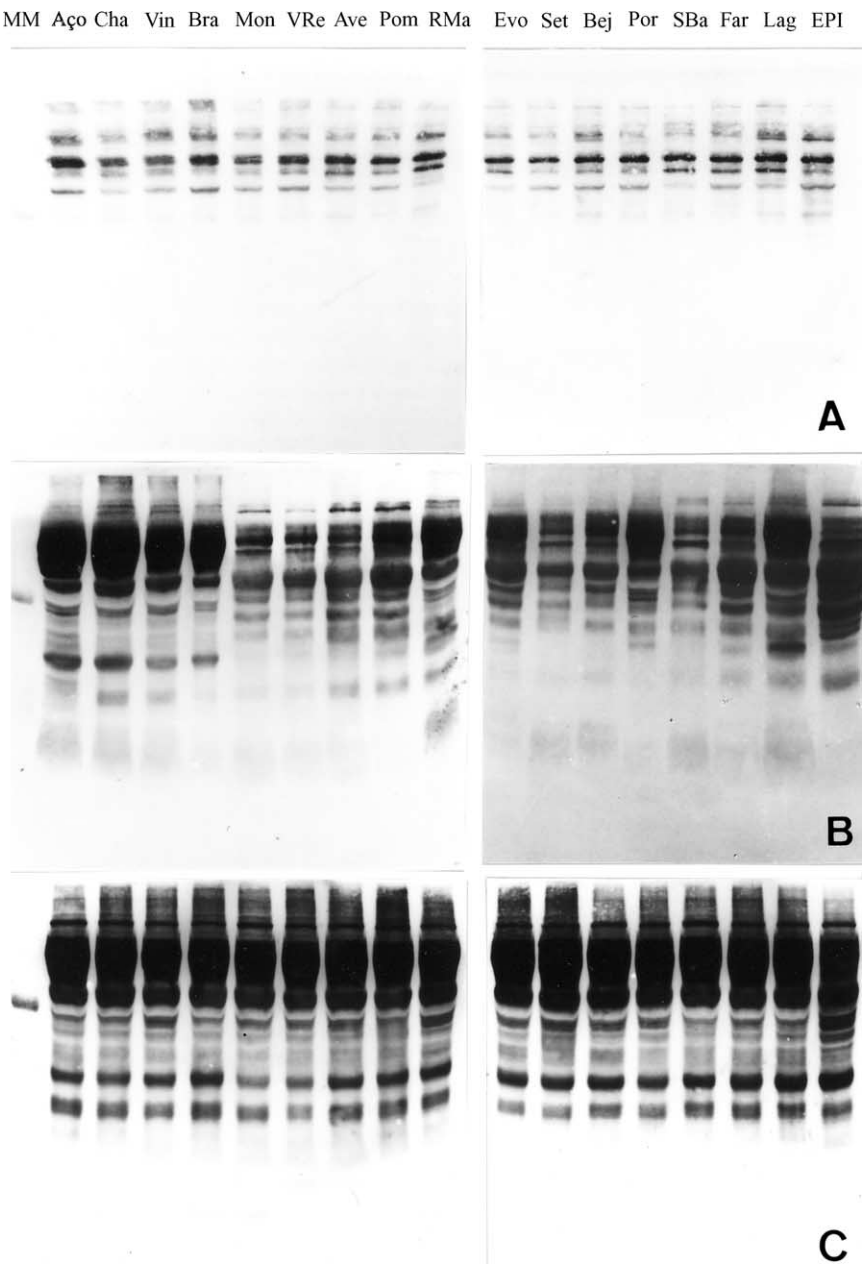

Fig. 5. Glycoprotein patterns of *L. albus* seed albumins (A), globulins B (B) and glutelins (C) of the 17 accessions. Of the several molecular weight markers used (66, 45, 36, 29, 24, 21.5 and 14.3 kDa), only ovalbumin (45 kDa) is glycosylated.

latest flowering cultivar resembling the northern populations despite significant differences in plant architecture. Ave and Pom, which are from the coastal region, are cultivars of a *megalosperma* type, isolated as a result of a high level of selection pressure.

A similar division of the lupin accessions into two main groups is obtained in the UPGMA phenogram based on the sample matching (Fig. 7). It is notable that the EPI accession is rather distinctive from these two groups. The heterogeneity of this population in

Table 2
Discriminant character loading on the principal co-ordinate axes showing variability in the different protein fraction bands for 17 *L. albus* accessions[a]

Band	First PCooA	Second PCooA	Third PCooA	Band	First PCooA	Second PCooA	Third PCooA
n_3	0.0405	**−0.0609**	0.0018	t_{27}	−0.1041	−0.0362	0.0193
n_{12}	−0.0162	**0.0901**	−0.0109	t_{28}	−0.0414	−0.0036	**0.0569**
n_{16}	−0.0581	0.0453	−0.0150	t_{29}	0.0135	−0.0061	0.0137
a_1	0.0165	0.0065	0.0026	t_{30}	0.0219	0.0408	**0.0525**
a_{17}	0.0487	0.0120	**−0.0705**	g_7	−0.0426	−0.0470	−0.0096
b_6	−0.0307	0.0182	0.0038	g_8	−0.0830	−0.0378	0.0103
b_7	0.0032	−0.0416	−0.0123	g_9	−0.0227	−0.0053	−0.0141
b_{16}	−0.0039	−0.0395	−0.0169	g_{10}	−0.0592	0.0106	0.0142
b_{17}	−0.0400	−0.0129	−0.0247	g_{11}	−0.0148	0.0176	0.0101
b_{19}	−0.0137	**−0.0670**	−0.0339	h_2	0.0349	0.0027	−0.0072
b_{20}	−0.0307	0.0019	0.0112	h_4	−0.0162	**0.0901**	−0.0109
b_{21}	−0.0013	0.0271	0.0118	h_8	0.0349	0.0027	−0.0072
b_{22}	0.0410	**0.0772**	−0.0235	h_9	0.0189	0.0117	−0.0026
t_5	−0.0208	−0.0053	−0.0114	h_{12}	−0.0740	0.0505	0.0207
t_7	−0.0548	**0.0812**	0.0230	r_3	0.0734	0.0015	0.0218
t_8	−0.0855	0.0054	−0.0005	r_6	**0.1300**	0.0111	−0.0049
t_9	−0.0855	0.0054	−0.0005	r_7	0.0365	0.0188	0.0000
t_{10}	−0.0227	−0.0053	−0.0141	r_{10}	0.0176	0.0279	−0.0181
t_{11}	−0.0227	−0.0053	−0.0141	r_{11}	0.0041	−0.0152	0.0066
t_{13}	−0.0483	−0.0056	0.0014	r_{12}	0.0041	−0.0225	0.0027
t_{15}	0.0348	−0.0326	0.0170	r_{13}	**0.1817**	−0.0193	0.0125
t_{16}	0.0135	−0.0061	0.0137	r_{15}	−0.0176	0.0009	−0.0019
t_{17}	0.0154	−0.0309	−0.0013	s_5	**−0.1537**	0.0094	−0.0092
t_{19}	−0.0238	−0.0312	0.0287	s_6	**0.2081**	−0.0127	0.0124
t_{20}	0.0135	−0.0061	0.0137	s_7	**0.2081**	−0.0127	0.0124
t_{21}	−0.0079	−0.0015	0.0171	s_8	**−0.1537**	0.0094	−0.0092
t_{22}	−0.0182	0.0594	**−0.0488**	s_9	−0.0211	0.0047	0.0028
t_{24}	−0.0437	0.0126	0.0134	s_{10}	0.0801	**0.0810**	**0.0491**
t_{25}	0.0651	**−0.0633**	−0.0107	s_{11}	**0.1181**	0.0439	−0.0144
t_{26}	−0.0825	−0.0084	0.0214	s_{13}	0.0041	−0.0225	0.0027

[a] n: albumins; a: globulins A; b: globulins B; t: glutelins; g: glycosylated albumins; h: glycosylated globulins A; r: glycosylated globulins B; s: glycosylated glutelins. Numbers in bold are the character loads for each of the principal components that explain the range of variation.

relation to the other accessions can also be seen using co-ordinate analysis (Fig. 6).

3.5. Agronomic implications

Since lupin plants are autogamous, their genotypes are predominantly homozygous. In traditional isolated agricultural systems farmers imposed a strong selection for populations adapted to microclimatic conditions present in mountainous regions, leading to a strong gene fixation process. Thus, it appears that the correlation between microclimate (related to latitude and altitude) and protein banding patterns is due to a multilocus association as a result of gene fixation. Such a gene fixation process is evident at a phenotypic level, as has already been shown by Martins (1994) who, identically, was able to distinguish the two main lupin groups when the principal component analysis was based on morphological characters. Furthermore, our present results show that the early flowering characteristics in lupin cultivars are generally correlated with higher mean levels of total seed protein and glutelin, and lower glutelin glycosylation (Tables 1 and 2, Fig. 6). Since high seed protein content and early flowering are important lupin breeding strategies our findings on the protein fractions could have value in future research on lupin agronomy and improvement.

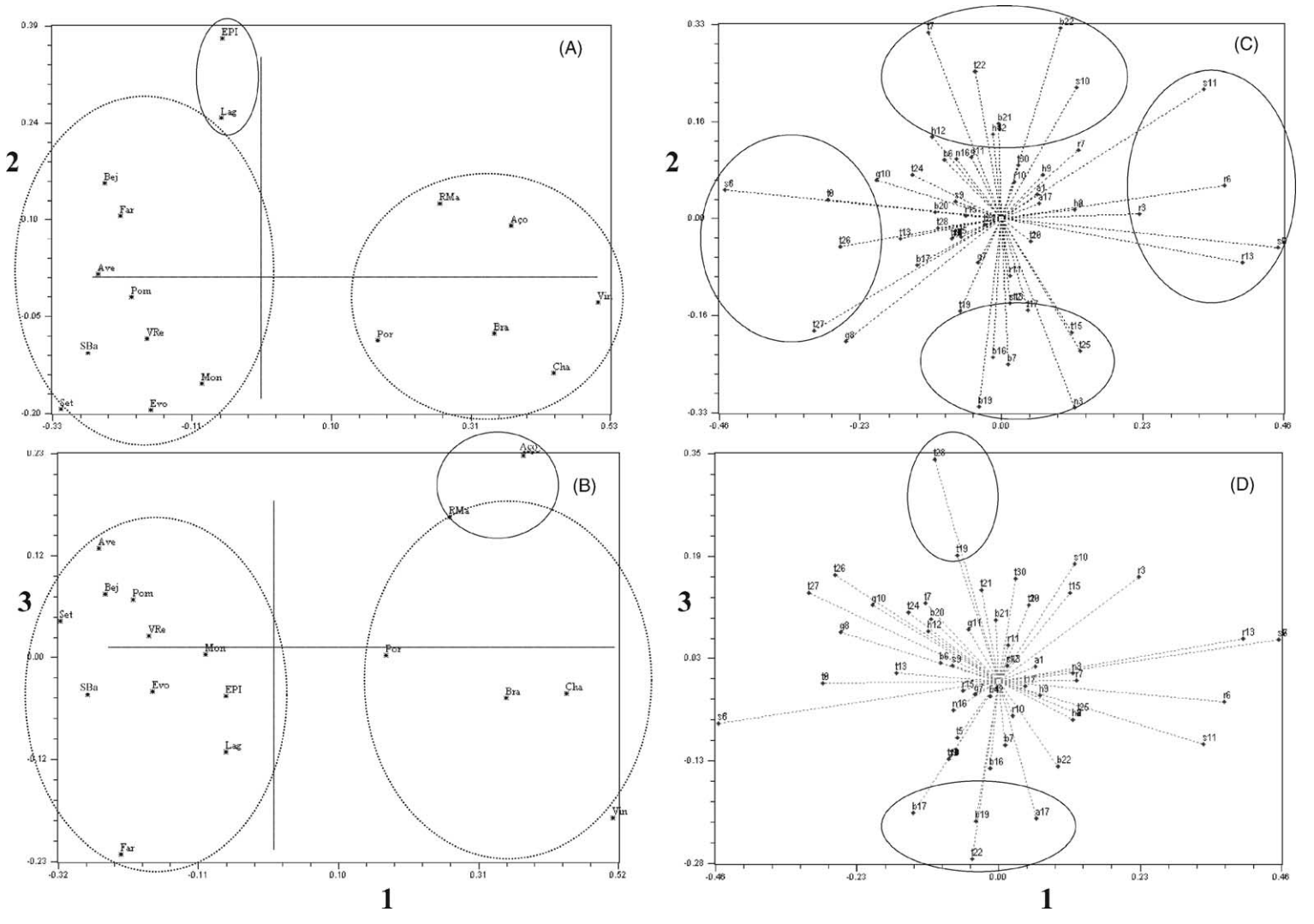

Fig. 6. Principal co-ordinate analysis of protein fraction variability for the 17 *L. albus* accessions. On the scatter plots, the axes are projected in the plane defined by first and second principal co-ordinate axes (A) and first versus third axes (B). These axes explain 46% of total variation (the first accounts for 23%, the second for 13% and the third for 10%). The corresponding vectors of the projected variables (protein fraction bands) are shown in C (first versus second principal co-ordinate axes) and in D (first versus third axes).

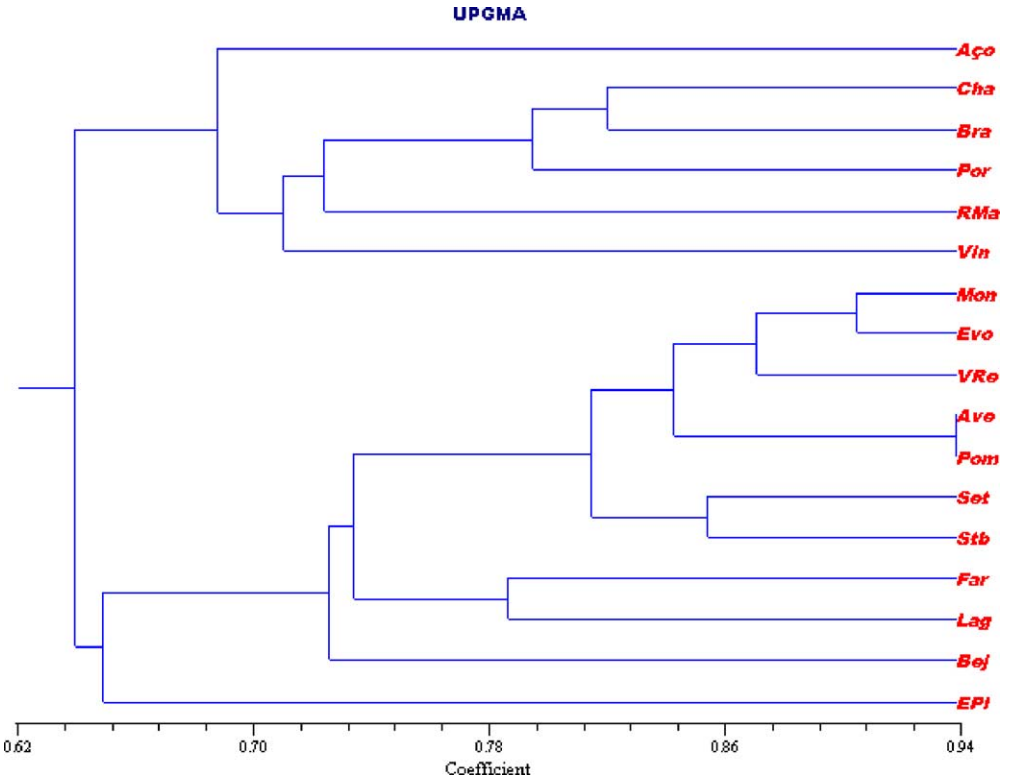

Fig. 7. UPGMA phenogram displaying the dissimilarity matrix based on the simple matching coefficient performed between the 17 accessions.

4. Conclusions

Legume storage proteins tend to be naturally heterogeneous for the properties of their constituent polypeptides. Since globulins of legume seeds are considered to be the most important storage proteins, they have been used in attempts to discriminate among cultivars. Such attempts for lupins (Przybylska and Zymniac-Przybylska, 1994; Santos et al., 1997) have had little success. Similarly, in alfalfa, the electrophoretic analysis of globulins and albumins revealed only minor differences in polypeptide components (Krochko and Bewley, 2000). Our results indicate that an analysis of white lupin based on only globulins and albumins would have led to a similar conclusion. However, by the additional consideration of ''glutelins'' and glycosylated polypeptides it was possible to establish coherent cultivar groups which were shown to be related to the cultivars geographical origin. Furthermore, a general correlation was found between glutelins and cultivar early flowering, which is an important agronomic characteristic. Although glutelins are not considered a usual class of seed proteins in legumes they do appear fairly abundant in lupins, very polymorphic and of agronomic relevance. So, further research is justified regarding the origin of such proteins and their physico-chemical, biochemical, and genetic characteristics.

Acknowledgements

We thank Mr. Pedro Duarte for help with map drawing and Dr. Phil Jackson for scientific comments and for correcting the English.

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