BIOLOGIA PLANTARUM 46 (3): 435-443, 2003

Fractionation and electrophoretic patterns of storage proteins of *Ebenus cretica*. A preliminary survey as a tool in taxonomy

T. SYROS*, T. YUPSANIS**,[1] and A. ECONOMOU*

School of Agriculture, Department of Horticulture, School of Chemistry, Department of Biochemistry***, *Aristotle University, 54 124 Thessaloniki, Greece*

Abstract

Seed storage proteins of *Ebenus cretica* were fractionated to albumins, globulins, prolamins and glutelins according to their solubility in water, 0.5 M NaCl solution, 55 % propanol-2 and 0.125 M sodium borate (pH 9.0) containing 0.5 % SDS (sodium dodecyl sulfate) solution, respectively. Glutelins consist of the major (about 81 %) fraction of the total extracted proteins. Analysis by SDS-PAGE revealed that the total extracted protein patterns from different racemes of the same plant were similar, while those from seeds of different plants were different. In addition, distinct differences were observed within protein patterns of alkaline extractable glutelin fractions and salt soluble globulin fractions. In *E. cretica* four ecotypes (A - D) were distinguished by SDS-PAGE of total extracted seed proteins. The last method was more simple and rapid than others and was suggested for screening analysis.

Additional key words: albumins, globulins, glutelins, *Leguminosae*, ornamental plant, prolamins, SDS-PAGE.

Introduction

The introduction of new plants in ornamental horticulture is important and for this reason native plants are selected for their desirable characteristics. One of them is the *Ebenus cretica*, an evergreen subshrub (20 - 80 cm in height). It has impressive pink or purple flowers that form later thick racemes (Syros *et al.* unpublished).

The information concerning seed storage proteins in ornamental plants is limited to date. Since different storage proteins electrophoretic patterns are direct products of genetic differentiation they may serve as molecular markers for distinguishing phylogenetic similarities and dissimilarities in genera (Moustakas *et al.* 1986), species (Ladizinsky and Hymowitz 1979) and subspecies (Haider and El-Shanshoury 2000) as well as for assess genotypic variations among inbred lines (Haider *et al.* 2000) and for varietal identification (Wrigley *et al.* 1982, Yupsanis and Moustakas 1988, Yupsanis *et al.* 1992, Cooke 1995, Vladova *et al.* 2000). In this, respect bands from different species or varieties but with the same migrating capacity are likely to be produced by genes common to both species. On the other hand bands that migrate differently are likely to be controlled by different genes or different alleles of a locus (Boulter *et al.* 1966).

Storage proteins of legume seeds as well as cereals are of the main nitrogen source in human and livestock nutrition, and consequently they have extensively studied (Derbyshire *et al.* 1976, Nielsen 1984, Casey *et al.* 1986, Krochko and Bewley 1990). Legumes preferentially accumulate globulins, but no information was available on preferentially accumulated seed proteins and the degree of similarities of storage protein electrophoretic patterns within species of *E. cretica*.

The objective of the present study was to fractionate the storage proteins *of Ebenus cretica* seeds and to develop a seed protein electrophoresis technique in order to assess the degree of genotypic variation within the species of *Ebenus cretica*.

Received 21 September 2001, *accepted* 4 April 2002.
Abbreviations: PAGE - polyacrylamide gel electrophoresis; SDS - sodium dodecyl sulphate.
[1]Corresponding author; fax: (+31) 09 97689, e-mail: yupsanis@chem.auth.gr

Materials and methods

Plant material: Racemes of *Ebenus cretica* were collected according to the different phenotypes of plants from two different locations, the first in west Crete (45 T-plants), 35 km southwest of Chania at an altitude of about 600 m (White mountain) and the second, from central Crete (40 K-plants), 14 km south-eastern ward of Heraklion at an altitude of about 500 m (Gioucta mountain). The distance between west and central Crete is ~200 km. *Ebenus cretica* grows in rocky areas of hills, at an altitude of < 800 m, in alkaline soil, rich in calcium carbonate. Seeds were manually removed and diluted. The frozen seeds (in liquid nitrogen) were ground in a pestle and mortar and the resulting flour either was extracted with 10 cm^3 g^{-1} of ice-cold diethyl ether to remove most of the lipids or was extracted directly as follows below. All traces of ether were removed in vacuum. In any case, the flour was stored at -20 °C.

Total extractions of proteins: Non-defatted/defatted flour (100 mg) was extracted (1 h; 4 °C) with ice cold buffer (at a tissue to medium ratio of 1:10) consisting of 65 mM Tris HCl, pH 6.8, containing 1 % sodium dodecyl sulphate (SDS) and 1.0 mM phenyl-methyl-sulphonyl-fluoride in the presence/absence of 2 % 2-mercapto-ethanol (Yupsanis and Shewry 1993). After centrifugation at 13 000 *g* for 15 min, the supernatant was used for protein (180 μg) electrophoresis.

Extraction of albumins, globulins, prolamins and glutelins: The salt soluble proteins (albumins and globulins) of non-defatted/defatted (1 h; 4 °C) flour (200 mg) were extracted with NaCl (extraction ratio 1:10; 1 h; 4 °C) in the presence/absence of 2-mercaptoethanol. After centrifugation (13 000 *g*; 15 min) the precipitate (A) was extracted as below and the supernatant was dialyzed against water (48 h; 4 °C). Then, it was centrifuged (13 000 *g*; 30 min). The freeze-dried supernatant contained the albumins and the precipitate (B) the globulins. To extract prolamins, the precipitate (A) was extracted with 55 % propanol-2 (1 h; 25 °C). After centrifugation (13 000 *g*; 30 min) the supernatant contained prolamins and the precipitate (C) was extracted with 125 mM sodium borate, pH 9.0, containing 0.5 % SDS in the presence/absence of 2 % 2-mercaptoethanol (1 h; 40 °C). The remainder contained insoluble glutelins. The protein extraction experiments were repeated three times.

In another procedure, prolamins were extracted straight from the flour with 25 % 2-chloroethanol, containing 30 % sucrose and 0.5 mg cm^{-3} pyronin G (extraction ratio 1:6) (Autran 1982) and the supernatant was electrophorised under native conditions at pH 3.1 according to Yupsanis (1983).

Protein determination: The concentration of extractable protein was determined using a filter paper dye binding assay (Minamide and Bamburg 1990). The protein content of dry seed was determined according to Kjeldahl method as modified by Matveef (1957).

Electrophoresis: Protein patterns were analyzed by 12 % SDS-polyacrylamide gel electrophoresis (SDS-PAGE; 12 × 16 × 0.15 cm; vertical slab gel) according to the Laemmli (1970). The gels were stained with Coomassie brilliant blue R-250 (Yupsanis and Shewry 1993).

Results

The amounts of different protein fractions (as % of total protein extracted) from the sequential extraction procedure were as follows: albumins (9.75 %), globulins (6.48 %), prolamins (3.02 %) and glutelins (80.75 %). On the average, the percentage of protein in *Ebenus cretica* was found to be 40 - 45 % of the dry matter.

SDS-electrophoresis patterns of extracted of seed total protein extraction revealed three major groups of protein bands: a low molecular mass group (Mr 14 200 - 20 100; LMG), a medium Mr group (24 000 - 45 000; MMG) and a high Mr group (45 000 - 66 000; HMG) (Fig. 1*A*,*B*). The presence of 2-mercaptoethanol in the extraction medium increased the amount of protein extracted (Fig. 1*A*; *lines 1* and *4* in comparison with lines *2* and *3*, respectively). Unchanging electrophoretic patterns of total extracted proteins were shown with/without treatment of the flour with diethyl ether (Fig. 1*B*; *lines 5* and *8* in comparison with *lines 6* and *7*, respectively).

According to the electrophoretic patterns of total extracted proteins, *E. cretica* species showed four ecotypes (named A, B, C and D). Within A-ecotype seeds (38) belonged to one or more racemes of the same *Ebenus cretica* plant there was very little variability (of about 5 %) as was judged by the SDS-electrophoresis of totally extracted proteins (Fig. 2*A*). Under similar conditions within B (Fig. 2*B*) as well as in C and D ecotype seeds (results not shown), there was detected no variability in protein pattern. A great variability was found in the MMG of the total protein electrophoresis patterns among plants with different phenotype (A, B, C and D ecotypes) of the same or different locations (Fig. 3). *E. cretica* species of west Crete showed the A ecotype (Fig. 3, *lines 5*, *6*, *7*, *13*, *14*, *15*, *16*, *3'*, *4'*, *14'* and *15'*), the B ecotype (Fig. 3, *lines 8*, *1'*, *2'* and *13'*)

and the C-ecotype (Fig. 3, *line 16'*) in a ratio of about 70, 25 and 5 %, respectively, of the total population. On the other hand, in the central Crete the same ecotypes, A ecotype (Fig. 3, *lines 3, 4, 11, 12, 5', 6', 7', 9'* and *11'*), B ecotype (Fig. 3, *line 10'*) and C ecotype (Fig. 3, *lines 1, 2, 10, 8'* and *12'*) with one more named D ecotype (Fig. 3, *line* 9) were shown in a ratio of about 56, 6, 32 and 6 %, respectively. The above patterns remained unchanged under reducing conditions of electrophoresis (results not shown).

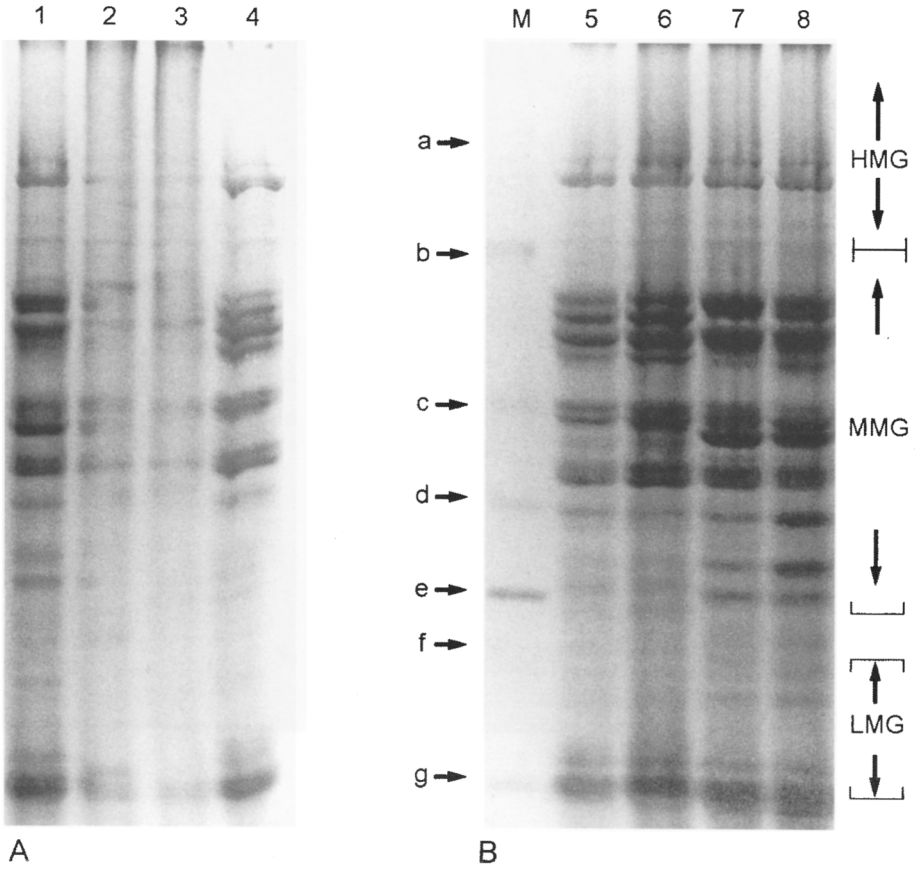

Fig. 1. Total extracted seed protein patterns of K-plant and T-plant of *Ebenus cretica* following SDS-PAGE. *A*: *lines 1* and *2* are proteins extracted from several seeds of K-plant without pretreatment with diethyl ether but in the presence/absence of 2-mercaptoethanol, respectively, *lines 3* and *4* are protein extracted from several seeds of T-plant as in *2* and *1*, respectively. K-plant (from central Crete) and T-plant (from western Crete) belong to B and A-ecotypes, respectively. *B*: *lines 5* and *6* are as in *4* of *A-gel* but with/without pretreatment with diethylether, respectively, *7* and *8* are as in *1* of *A-gel* but with/without pretreatment with diethyl ether, respectively. The *arrows* indicate the molecular mass markers (M): *a* - bovine albumin (Mr ~ 66 000), *b* - egg albumin (Mr ~ 45 000), *c* - glyceraldehyde-3-phoshate dehydrogenase (Mr ~ 36 000), *d* - carbonic anhydrase (Mr ~ 29 000), *e* - trypsinogen (Mr ~ 24 000), *f* - trypsin inhibitor (Mr ~ 20 100), *g* - α-lacto albumin (Mr ~ 14 200). Protein extraction of similar volume was run in SDS-electrophoresis under reducing conditions.

Furthermore, the densitograms of protein electrophoregrams (Fig. 3) were statistically treated (programme: *Gel Pro Analyzer for Windows*) in order to show the standard deviation of all protein bands (Fig. 4). According to the protein electrophoretic pattern (Fig. 3) and densitograms (Fig. 4) the ecotypes A, B, C and D showed 15, 16, 14 and 14 bands, respectively. In Table 1 the common bands among ecotypes A - D are shown. In this investigation bands with a difference bellow of 700 Da were thought to be of similar Mr. The method used to estimate the degree of affinities between

Table 1. Common bands (SDS electrophoresis) between *Ebenus cretica* ecotypes.

Ecotype	A	B	C	D
A	0			
B	15	0		
C	12	11	0	
D	11	11	10	0

ecotypes based on total seed storage proteins profile (Fig. 3*A*,*B*) was the hyper geometric distribution as employed by Smith *et al.* (1970) and the unbiased per group method analysis (UPGMA). According to the

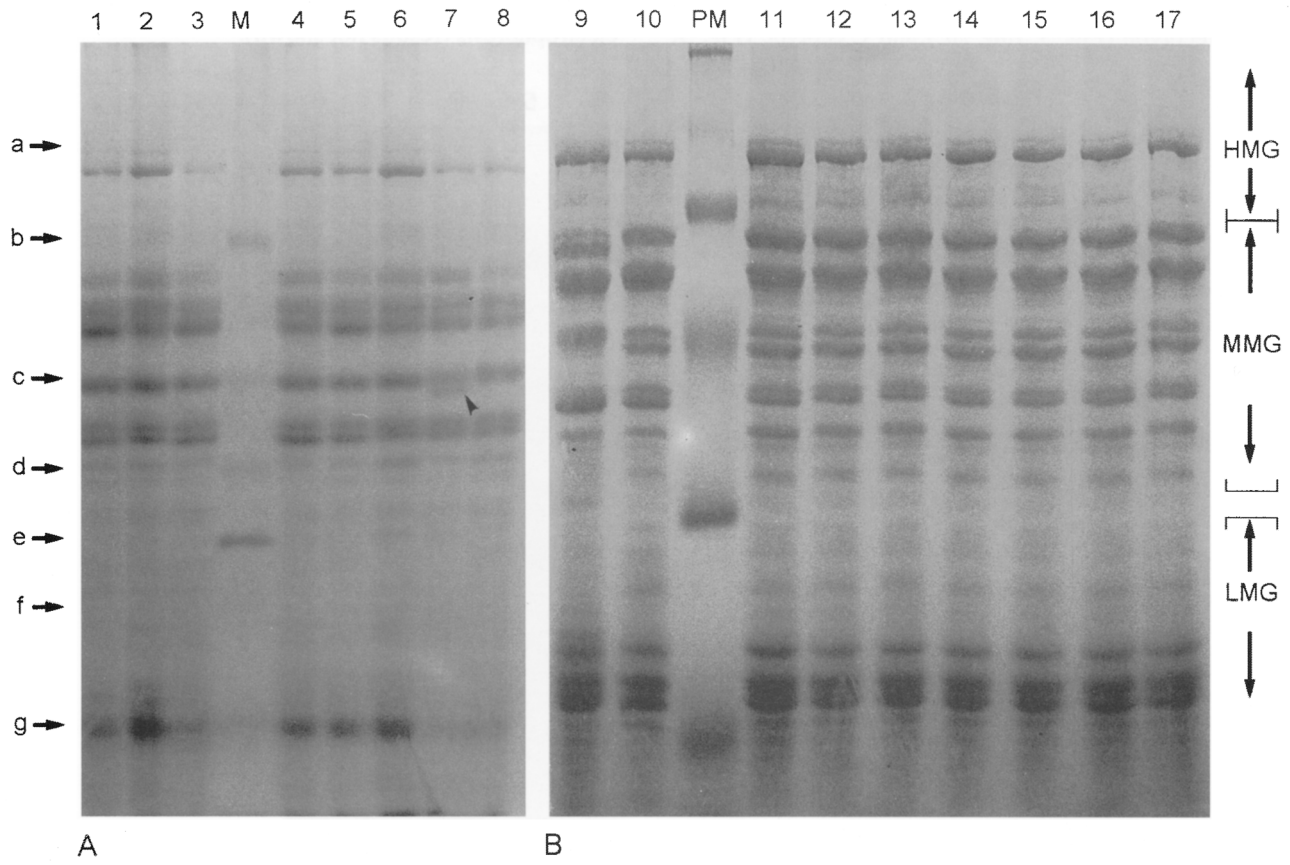

Fig. 2. Seed protein patterns of *Ebenus cretica* (same plant) following SDS-PAGE. *A*: *lines* (*1 - 3*) and (*4 - 8*) are proteins extracted from single seeds from racemes of the same plant (belong to A ecotype) included 3 and 5 seeds, respectively. The *arrowhead* indicates an additional band in the pattern of *line 7*. The *arrows* indicate the molecular mass markers (M) as in Fig. 1. *B*: *line 9* is protein extracted from single seed from raceme included 1 seed (A ecotype) and *lines 10 - 11*, *12 - 14*, and *15 - 17* are proteins extracted from single seeds from racemes of the same plant (belong to B ecotype) included 2, 3 and 3 seeds respectively. PM: Prestained molecular markers.

Table 2. Probability of random matching of SDS electrophoresis bands between *Ebenus cretica* ecotypes.

Ecotype	A	B	C	D
A	0			
B	0	0		
C	0.373	0.376	0	
D	0.446	0.376	0.327	0

above statements the relationship between the ecotypes as shown in Tables 1 and 2, and dendrogram (Fig. 5).

The distribution of albumin fractions within two *Ebenus cretica* plants from different locations seemed to be almost similar under reducing/non-reducing SDS-PAGE conditions (Fig. 6*A*; lines 2 and 4 in comparison with 1 and 3, respectively), while those of globulins were different and unchanged like albumins under reducing/non-reducing conditions (Fig. 6*B*; lines 1 and 3 in comparison with 2 and 4, respectively). Fig. 7 revealed that the SDS-PAGE patterns of alkaline soluble glutelin fractions (of non defatted flour) included almost similar protein bands as those of totally extracted proteins (lines 1 and 4 of Fig. 7 in comparison with the respective lines 8 and 6 of Fig. 1*B*). However, in a number of samples, pretreatment of the flour with diethyl ether resulted to a strong diminution of the intensity of the MMG bands in the alkaline soluble glutelin patterns (Fig. 7; track 1 in comparison with track 3). SDS-electrophoresis of propanol extracted prolamins as well as native electrophoresis of 2-chloro-ethanol extracted prolamins showed no defined bands (results not shown).

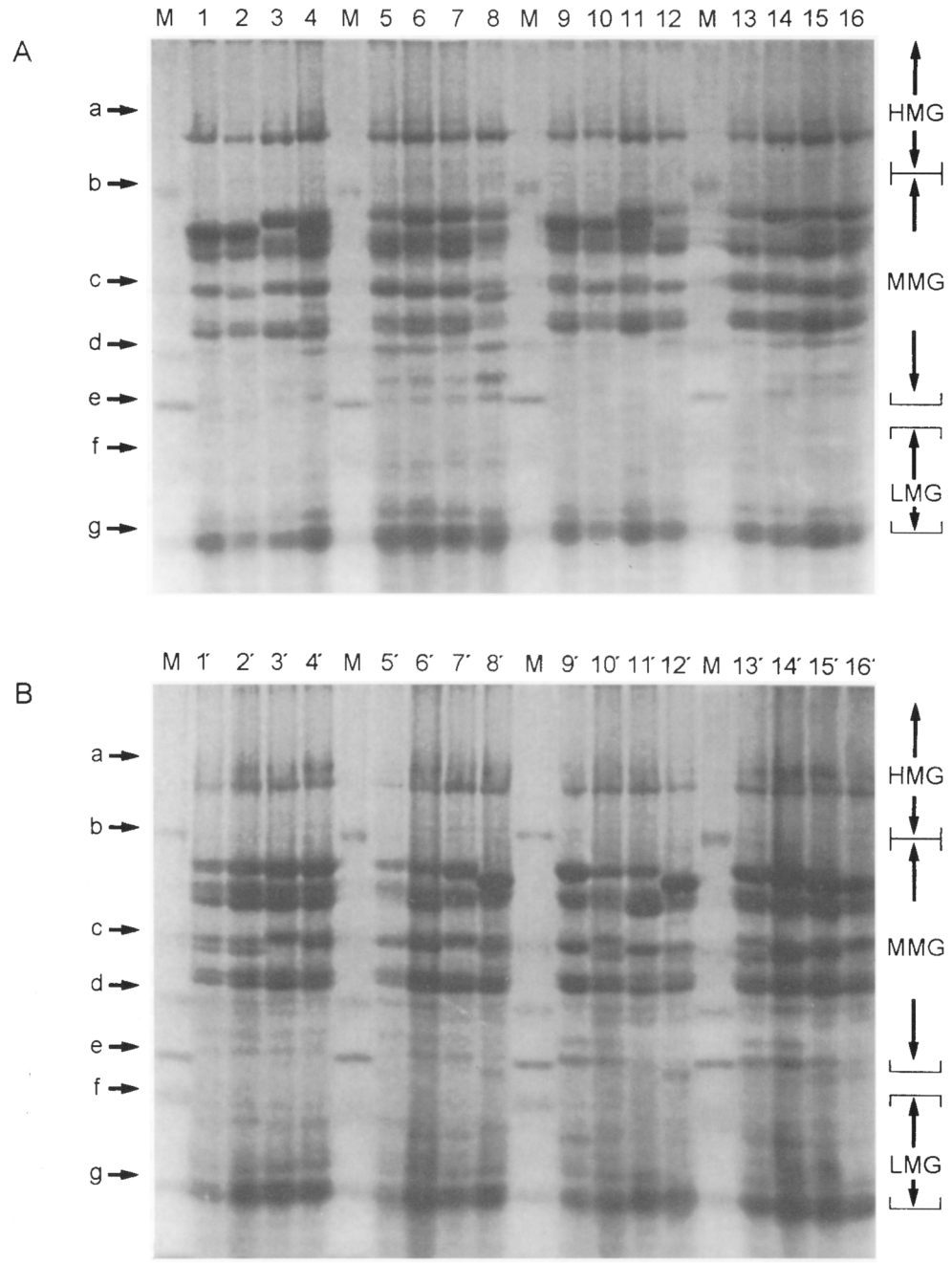

Fig. 3. Seed protein patterns of *Ebenus cretica* (different plants from different locations) following SDS-PAGE. *A*: *lines 1*, *2*, *3*, *4*, *9*, *10*, *11* and *12*, and *lines 5*, *6*, *7*, *8*, *13*, *14*, *15* and *16* are proteins extracted from single seeds from racemes of different plants collected from central and west Crete, respectively. *B*: lines *1'*, *2'*, *3'*, *4'*, *13'*, *14'*, *15'* and *16'*, and lines *5'*, *6'*, *7'*, *8'*, *9'*, *10'*, *11'* and *12'* are proteins extracted from single seeds from racemes of different plants collected from west and central Crete respectively. The *arrows* indicate the molecular mass markers (M) as in Fig. 1.

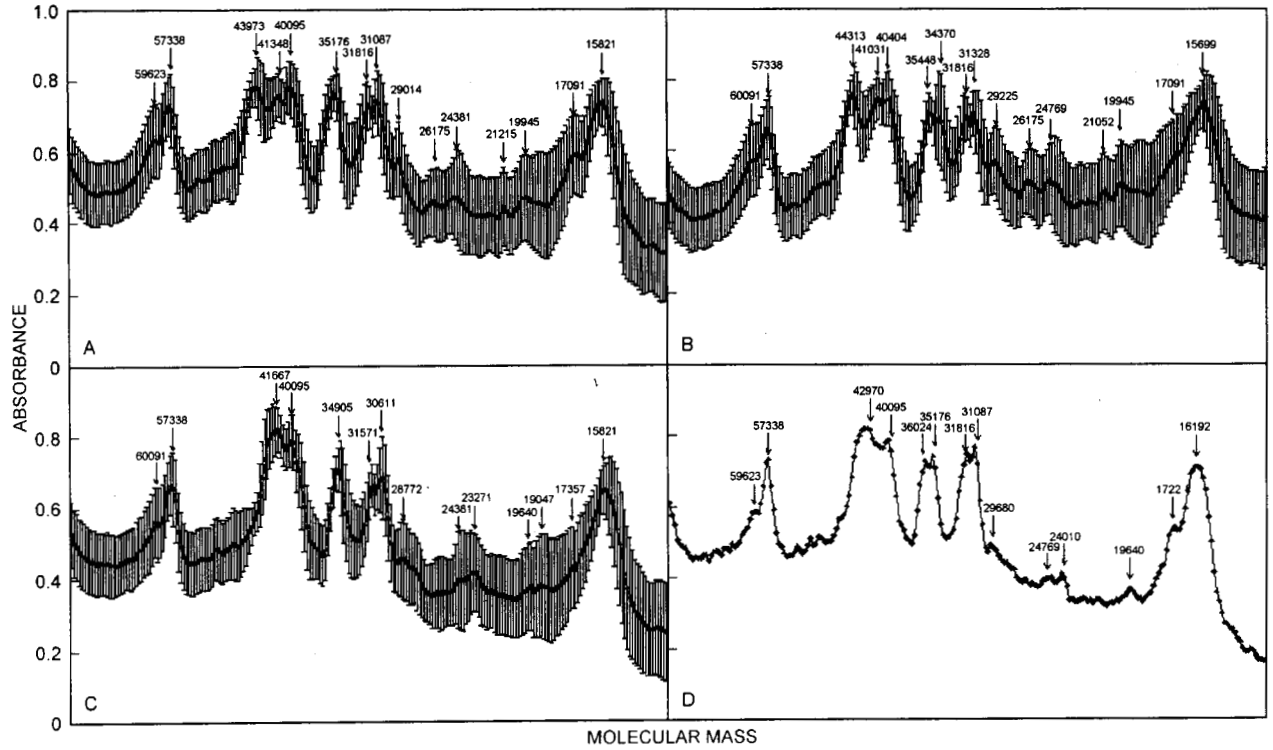

Fig. 4. Densitograms of protein electrophoretic patterns of Fig. 3. The electrophoregrams were statistically treated (Gel Pro Analyzer for Windows). A, B, C and D are densitograms of A, B, C and D ecotypes respectively.

Discussion

The average protein content (40 - 45 %) of *Ebenus cretica* is similar to that in soybean, also a member of the *Leguminosae* family (most of them contain 20 - 25 %) (Derbyshire *et al.* 1976). Fractionation of *E. cretica* storage proteins revealed that the major extractable storage protein was the alkaline soluble glutelins while the minor was the alcohol soluble prolamins. Thus, it seems that *Ebenus cretica* is an unusual member of *Leguminosae* due to its large proportion of glutelins. The major storage proteins in other members of the *Leguminosae* family are globulins, where they may participate up to 70 % of the whole seed protein (Nielsen 1984), and albumins. For example, in alfalfa (*Medicago sativa* L.) medicagin (a legumin-like globulin), alfin (a vicilin-like globulin) and a family of low Mr albumins comprise 30, 10 and 20 %, respectively, of the total extractable protein from cotyledons of mature seeds (Krochko and Bewley 1990). Medicagin shows obvious similarities to the major 11/12S seed globulins of other species *i.e.* pea, bread bean, soybean (Casey *et al.* 1986). Glutelins are the main protein fraction (~99 % of the total seed protein extracted) in the species of *Quercus ilex* and *Q. robur*, which are ornamental plants of the *Fagaceae* family (Collada *et al.* 1988). Regarding prolamins, they consisted of half of the total nitrogen in the grain of barley, wheat and rye (member of the tribe *Triticeae*) (Burgess and Shewry 1986).

Regarding the optimum conditions of total protein extraction, the presence of 2-mercaptoethanol in the extraction medium increased the amount of protein extracted (Fig. 1*A*). Similar results were reported to soybean protein extractions (Nash *et al.* 1974). Although, air dried seed is usually milled and defatted prior to extraction of storage proteins our results in most of the samples, supported the suggestion of Derbyshire *et al.* (1976) that this step is not necessary. In other words, unchanging electrophoretic patterns were shown with/ without treatment with diethylether.

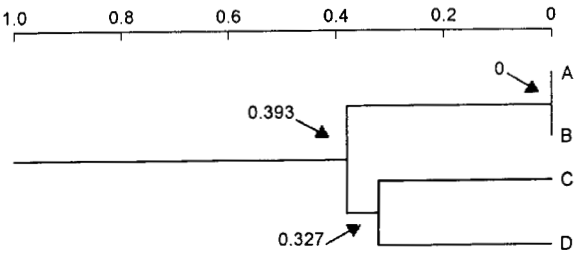

Fig. 5. Dendrogram of *Ebenus cretica* ecotypes (A-D) based in unbiased per group method analysis (UPGMA).

Based on our data, SDS-electrophoresis analysis of total extracted proteins (Fig. 3), alkaline soluble glutelin fractions (Fig. 5) and globulin fractions (Fig. 4*B*) could be utilized for screening analysis within species of *Ebenus cretica*. In *Solanum* species, glutelin fraction (soluble in basic solutions) was useful in genetic and

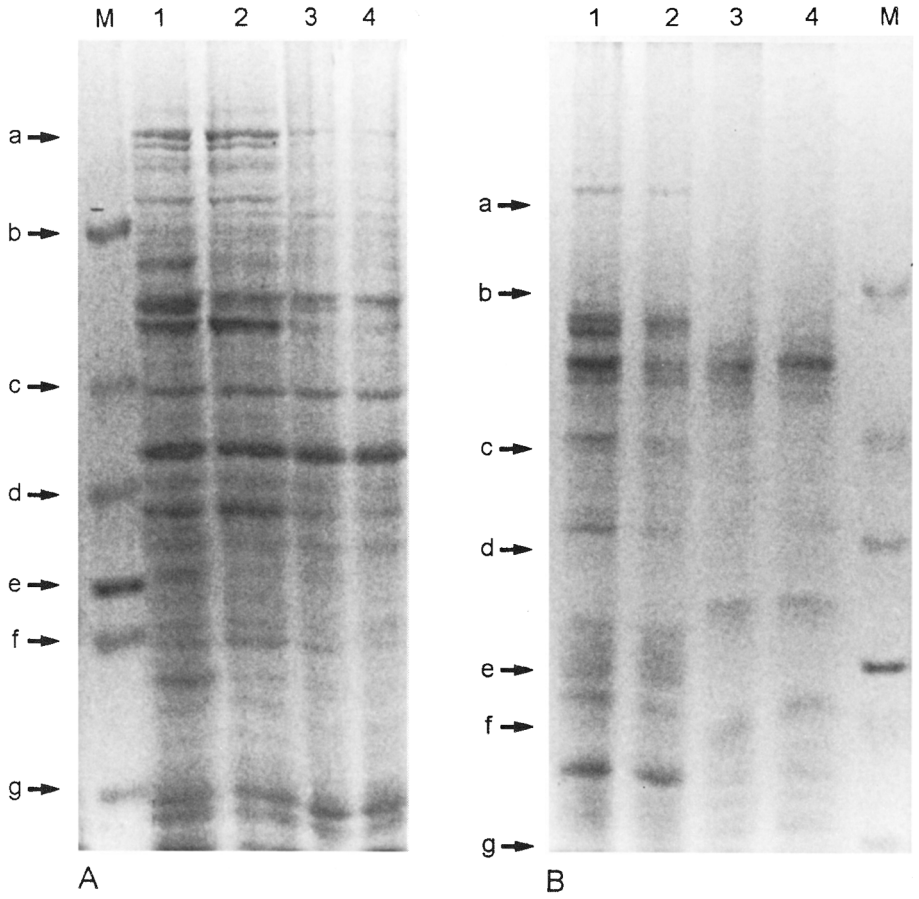

Fig. 6. Analysis of albumin (*A*) and globulin (*B*) fractions from seeds of *Ebenus cretica* following SDS-PAGE. *A*: *lines 1* and *2* are albumin fractions from several seeds of T-plant without pretreatment with diethyl ether under reducing/non-reducing electrophoresis conditions, respectively; *lines 3* and *4* are as in *1* and *2*, respectively, but with albumin fraction from several seeds of K-plant. *B*: *lines 1*, *2*, *3* and *4* are globulin fractions from several seeds of T-plant and K-plant under similar conditions as in *A*. The *arrows* indicate the molecular mass markers (M) as in Fig. 1.

taxonomic comparisons within the genus (Smith and Desborough 1987). In any case, the extraction of total seed protein is a more rapid and simple procedure than to obtain alkaline soluble glutelin or globulin fraction from seeds. According to the electrophoretic patterns of total extracted proteins, *Ebenus cretica* species of west Crete showed the ecotypes A, B and C in a ratio of about 70, 25 and 5 %, respectively. On the other hand, central Crete *E. cretica* species showed four ecotypes (A, B, C and D) in a ratio of about 56, 6, 32, and 6 %, respectively. In other words, in west Crete the predominant ecotypes are A and B, while in central Crete A and C.

According to their affinities, A, B, C and D ecotypes could be classified into two groups. One included A and B and the next C and D ecotypes (Table 2, Fig. 5). This classification based on the above electrophoretic pattern agrees with the phenotype classification of *E. cretica* plants (four classes with distinguishable phenotypes; results not shown). Thus, we agree with Kovarev *et al.* (1987) that the analysis of the seed total storage proteins should be used as a routine test in the identification of plant taxa and furthermore according to our results in screening seeds within phenotypes of the species of *E. cretica*.

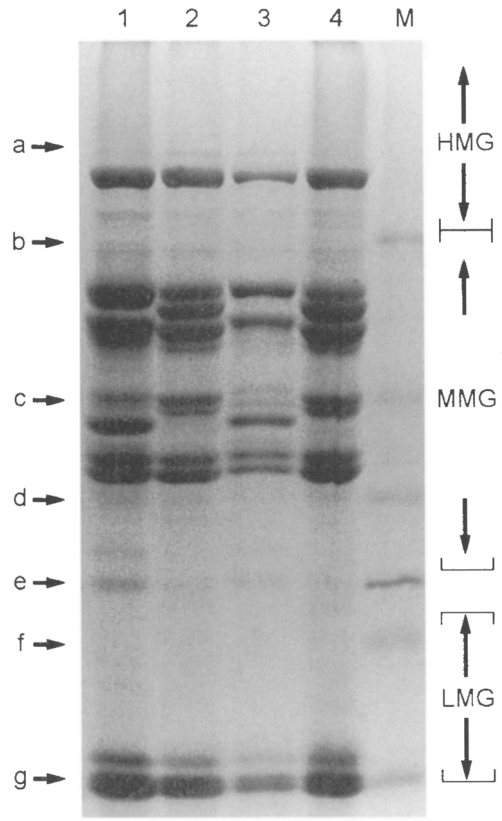

Fig. 7. Analysis of alkaline soluble glutelin fraction from seeds of *Ebenus cretica* following SDS-PAGE. *Lines 1* and *3* are alkaline soluble glutelin fraction from several seeds of T-plant without/with pretreatment with diethyl ether respectively; *lines 2* and *4* are as in *3* and *1*, respectively, but with alkaline soluble glutelin fraction from several seeds of K-plant. The *arrows* indicate the molecular mass markers (M) as in Fig. 1.

References

Autran, J.C. (ed.): Identification Variétale à Partir des Constitments Proteiques. Guide Pratique d'Analyses dans les Industries des Céréales. - I.N.R.A., Montpellier 1982.

Boulter, D., Thukman, D.A, Turner, B.L.: The use of disk electrophoresis of plant proteins in systematics. - Taxon **15**: 135-143, 1966.

Burgess, S.R., Shewry, P.R.: Identification of homologous globulins from embryos of wheat, barley, rye and oats. - J. exp. Bot. **185**: 1863-1871, 1986.

Casey, R., Domoney, C., Ellis, N.: Legume storage proteins and their genes. - Oxford Surv. Plant mol. Cell Biol. **3**: 1-95, 1986.

Collada, C., Caballero, R.G., Casado, R., Aragoncillo, C.: Different types of major storage seed proteins in *Fagaceae* species. - J. exp. Bot. **39**: 1751-1758, 1988.

Cooke, R.J.: Varietal identification of crop plants. - In : Skerritt, J.H., Appels, R. (ed.): New Diagnostics in Crop Sciences. Pp. 33-63. CAB International, Wallingford 1995.

Derbyshire, E., Wright, D.J., Boulter, D.: Legumin and vicilin storage proteins of legume seeds. - Phytochemistry **15**: 3-24, 1976.

Haider, A.S., El-Shanshoury, A.R., Haider, A.: Variation of storage proteins and isozymes within maize inbred lines. - Biol. Plant. **43**: 199-203, 2000.

Haider, A.S., El-Shanshoury, A.R.: Variability of storage proteins and esterase isoenzymes in *Vicia sativa* subspecies. - Biol. Plant. **43**: 205-209, 2000.

Kovarev, V.G., Gravriljuk, I.P., Gubareva, N.K.: Storage proteins in the identification of species, cultivars and lines. - Seed Sci. Technol. **15**: 675-678, 1987.

Krochko, J.E., Bewley, J.D.: Identification and characterization of the seed storage proteins from alfalfa (*Medicago sativa*). - J. exp. Bot. **41**: 505-514, 1990.

Ladizinsky, G. Hymowitz, T.: Seed protein electrophoresis in taxonomic and evolutionary studies. - Theor. appl. Genet. **54**: 145-151, 1979.

Laemmli, V.K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. - Nature **227**: 680-685, 1970.

Matveef, M.: Contribution à une méthode rapide de dosage des proteines dans les céreales et lent derivés. - Ann. Serv. bot. agron. Tunisie **30**: 119-142, 1957.

Minamide, L.S., Bamburg, J.R.: A filter paper dye-binding assay for quantitative determination of protein without interference. - Anal. Biochem. **190**: 66-70, 1990.

Moustakas, M., Symeonidis, L., Coucoli, H.: Seed protein electrophoresis in *Agropyron junceum* (L.) P.B. complex. - Ann. Bot. **57**: 35-40, 1986.

Nash, A.M., Kwolek, W.F., Wolf, W.J.: Extraction of soybean proteins with aqueous 2-mercaptoethanol. - Cereal Chem.

51: 220-227, 1974.

Nielsen, N.C.: The chemistry of legume storage proteins. - Phil. Trans. roy. Soc. London **304**: 287-296, 1984.

Smith, H.H., Hamill, D.E., Weaver, E.A., Thompson, K.S.: Multiple molecular forms of peroxidases and esterases among *Nicotiana* species and amphiploids. - J. Hered. **61**: 203-212, 1970.

Smith, J.A., Desborough, S. L.: The endosperm seed protein solin: biochemical characterization, induction by ABA and species-specific subunits. - Theor. appl. Genet. **74**: 739-745, 1987.

Vladova, R., Pandeva, R., Petcolicheva, K.: Seed storage proteins in *Capsicum annuum* cultivars. - Biol. Plant. **43**: 291-295, 2000.

Wrigley, C.W., Autran, J.C., Bushuk, W.: Identification of cereal varieties by gel electrophoresis of the grain proteins. - In: Pomeranz, Y. (ed.): Advances in Cereal Science and Technology. Vol. 5. Pp. 211-258. American Association of Cereal Chemists, St. Paul 1982.

Yupsanis, T., Moustakas, M., Karakoli, S.: Seed protein electrophoresis for varietal identification in rice (*Oryza sativa* L.). - J. Agron. Crop Sci. **168**: 95-99, 1992.

Yupsanis, T., Moustakas, M.: Relationship between quality, color of glum and gliadin electrophoregrams in *Durum* wheat. - Plant Breed. **101**: 30-35, 1988.

Yupsanis, T., Shewry, P.R.: Protein phosphorylation and protein patterns in developing endosperms of barley. - Plant Breed. **111**: 73-77, 1993.

Yupsanis, T.: [Identification and technological screening of wheat varieties by electrophoresis of gliadins.]. - Agr. Res. **7**: 157-167, 1983. [In Greek.]