Seed storage proteins in cultivars and subspecies of alfalfa (*Medicago sativa* L.)

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Abstract

Seed storage proteins were analysed in 27 varieties of alfalfa (*Medicago sativa* L.); these included five subspecies (*glomerata, caerulea, falcata, hemicycla, praefalcata*), seven of the nine sources of *Medicago* germplasm introduced into North America and a sample of additional cultivars. The protein patterns were remarkably consistent for all of these taxa. One-dimensional and two-dimensional gel electrophoresis revealed only minor differences in polypeptide composition within each of the three major classes of storage protein (7S globulin, 11S globulin, 2S albumin). The slight variations that were found provided no information on either parentage or evolutionary relationships amongst these particular taxa. Nonetheless, persistent and reproducible heterogeneity of some minor polypeptides of 11S globulin (medicagin) may be useful under other circumstances for cultivar identification in alfalfa. Both subfamilies (I and II) of the 11S globulin were strongly expressed in all of the cultivars and subspecies examined. It was concluded that this structural divergence within the 11S storage protein family predated the evolution of the *M. sativa* L. species complex. Most of the variability in storage proteins was quantitative. However, even this variability was reduced when data were standardized with respect to seed dry weights. The consistent similarities in qualitative and quantitative expression of seed storage proteins amongst all of these taxa suggest a high degree of uniformity in both seed physiology and genetics within the alfalfa species complex.

Keywords: alfalfa, *Medicago sativa* L., seed storage proteins, cultivars and subspecies

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Introduction

The *Medicago sativa* species complex is an artificial classification comprising all alfalfa cultivars and essentially all of the wild types of *Medicago* that have contributed germplasm to the cultivars. These taxa are able to interbreed with varying degrees of success. Because alfalfa was cultivated before recorded history, its taxonomic origin and evolution are difficult to trace. Several classifications have been proposed (Gunn *et al.*, 1978; Lesins and Lesins, 1979; Small, 1984). Gunn *et al.* (1978) divided the *M. sativa* complex into nine subspecies on the basis of morphological characteristics, Lesins and Lesins (1979) have maintained the rank of species for interbreeding taxa in recognition of their divergent evolution, and Small (1984) has reduced the complexity of classification by recognizing only four subspecies (one of which has two varieties) and two common hybrids.

The relationships of the major taxa of the *M. sativa* L. complex are similar for all classifications (Gunn *et al.*, 1978; Lesins and Lesins, 1979; Small, 1984) although precise rankings may be different. *M. sativa* L. ssp. *glomerata* is a diploid with yellow flowers, and pods are tightly coiled and covered with glandular hairs (Lesins, 1976). It is native to the Caucasus and may represent the ancestral progenitor of the *sativa* and *falcata* lines (Lesins and Lesins, 1979). Separation of a population of *M. glomerata* north and south by a connected Black–Caspian Sea during an ancient transgression may have been the impetus for separate adaptation to differing climates (Lesins and Lesins, 1979). *M. sativa* ssp. *sativa* is an autotetraploid plant characterized by purple flowers with nondehiscent spiral legumes (Small, 1984). The diploid form is ssp. *caerulea*. These taxa probably evolved under warm, dry, semi-desert conditions around the Caspian Sea (Lesins and Lesins, 1979). The brightly coloured flowers suggest competition for insect pollinators and the spiral coils an adaptation for seed dispersal by pods rolling along the ground (Lesins and Lesins, 1979). *M. sativa* ssp. *falcata* is a tetraploid (sometimes diploid) plant with yellow flowers and dehiscent

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falcate-shaped legumes (Small, 1984). These taxa are cold- and heat-tolerant and seem to have evolved in the more northern latitudes (Lesins and Lesins, 1979). According to Lesins (1976), the early hybridization of *M. sativa* ssp. *sativa* and *M. sativa* ssp. *falcata*, resulting in stress-tolerant and vigorous plants, probably contributed to the cultivation of alfalfa throughout much of the temperate zone. Naturally occurring hybrids between these two subspecies have given rise to other varieties (e.g. ssp. x *hemicycla* (diploid) cf. Gunn *et al*., 1978; Lesins and Lesins, 1979; and ssp. x *varia* (tetraploid) cf. Small, 1984; Lesins and Lesins, 1979). *M. sativa* spp. *glutinosa* (cf. spp. *praefalcata* Gunn *et al*., 1978; Quiros and Bauchan, 1988) is a tetraploid with loosely coiled pods with glandular hairs (Lesins, 1976) and probably also is derived from the *M. glomerata* ancestor (Quiros and Bauchan, 1988).

The earliest cultivation of alfalfa (or lucerne) as a forage crop probably occurred about 7000 BC. From its origin in Vavilov's 'Near Eastern Centre' (Iran, Turkey, Caucasus), alfalfa spread to Greece and Rome about 2400 years ago (Ivanov, 1977). It may have been introduced into Greece from Media during the Persian wars in about 470 BC. From there it probably spread across North Africa to Spain, then eastward through Europe and into Russia, and also into China and India. In the 16th century it was introduced into Mexico and Peru by the Spanish. After several introductions, alfalfa was established in North America in the mid-1700s (Gunn *et al*., 1978).

There are no species of *Medicago* native to the New World. Most of the genetic diversity found in alfalfa cultivars growing in North America can be traced to nine distinct sources of germplasm that were introduced between 1850 and 1947 (Barnes *et al*., 1977; Table 1). These germplasm sources (or landraces) interbreed readily and contain varying amounts of *M. sativa* and *M. falcata* germplasm. They have developed distinctive biochemical and morphological characteristics as a result of isolation and differences in selective pressures over several centuries.

The major seed storage proteins in alfalfa are a 7S vicilin-like globulin (alfin), an 11S legumin-like globulin (medicagin) and a 2S albumin (LMW) (Krochko and Bewley, 1990; Coulter and Bewley, 1990). These proteins comprise 10%, 30% and 20%, respectively, of the total accumulated protein in mature seeds of *M. sativa* cv. Excalibur (Krochko *et al*., 1994). The 7S globulin is a trimeric protein consisting of variously sized polypeptides (α_1–α_6; 50, 38, 34, 20, 16, 14 kDa), produced by post-translational proteolysis of a family of 50 kDa subunit precursors (Krochko and Bewley, 1990). Subunits of the 11S globulin (60–70 kDa) consist of disulphide-bonded acidic (A_1, A_2, A_3, $A_{5/9}$, $A_{6/10}$; 49–39 kDa) and basic ($B_{1/2}$, B_3; 24–20 kDa) polypeptides. These subunits (AB) are classified as belonging to either subfamily I or II on the basis of the size of the B (basic) polypeptide and the presence (or absence) of an additional disulphide bond (Krochko and Bewley, 1990; Krochko *et al*., 1992). Subunits of subfamily I ($A_5B_{1/2}$, $A_6B_{1/2}$, $A_9B_{1/2}$, $A_{10}B_{1/2}$) have an additional intramolecular disulphide bond and show less size variability than subunits of subfamily II (A_1B_3, A_2B_3, A_3B_3, A_4B_3, A_7B_3) (Krochko and Bewley,

Table 1. Geographical origins of alfalfa germplasm lines introduced into North America between 1850 and 1947 (summarized from Barnes *et al*., 1977; Quiros and Bauchan, 1988). The germplasm sources are listed in order of decreasing hardiness and increasing *M. sativa* content

Germplasm source[a]	Geographical origins[a]	*falcata*/*sativa* content[b]
M. falcata	Russia, Siberia	*falcata*
Ladak	Ladakh province of Kashmir in northern India	*sativa* and *falcata*
M. x *varia*	Originated in western Europe as naturally occurring hybrids of *M. falcata* and *M. sativa* strains	*sativa* and *falcata*
Flemish	Northern France	*sativa* and *falcata*
Chilean (Spanish)	Introduced into Mexico and Peru by the Spaniards in the 16th century	*sativa* and *falcata*
African	Egypt or Arabia	*sativa* and *falcata*
Turkistan	Originated in southern Russia, Afghanistan, Iran and Turkey	*sativa*
Peruvian	Peru	only *sativa*
Indian	India	only *sativa* (non-dormant)

[a] Barnes *et al.* (1977).
[b] Quiros and Bauchan (1988).

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1990). The 2S albumin comprises a family of much smaller, acidic disulphide-bonded polypeptides (Coulter and Bewley, 1990). Extensive study has confirmed that the 2S storage protein of alfalfa is distinct from most of the other well-characterized 2S seed storage proteins (Coulter and Bewley, 1990).

Homologues of legumin-like (11S) and vicilin-like (7S) globulins are widespread within the plant kingdom (Borroto and Dure, 1987). There is substantial interspecific conservation, and related proteins have been identified in some monocots and gymnosperms (Borroto and Dure, 1987; Misra and Greene, 1994). Yet intraspecific variability also occurs. Breeding experiments have confirmed that there are quantitative and qualitative differences in storage protein subunits within several legume species, e.g. pea, bean, soybean (Hall *et al.*, 1977; Thomson and Schroeder, 1978; Thomson *et al.*, 1978; Casey, 1979; Utsumi *et al.*, 1980; Brown *et al.*, 1981; Staswick and Nielsen, 1983).

Seeds of the cultivar Excalibur were examined initially (and somewhat arbitrarily) as a standard of comparison to mark the development and maturation of alfalfa somatic embryos (Krochko *et al.*, 1992; Pramanik *et al.*, 1992; Krochko *et al.*, 1994). It was of interest to determine the similarity of these protein profiles for the 11S, 7S and 2S storage proteins across the range of taxa comprising the *M. sativa* L. species complex. We have compared 27 varieties of alfalfa for the amounts and types of storage proteins present in samples of mature seed. Polypeptides were displayed using one-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). In addition, the 11S seed storage proteins were analysed by two-dimensional SDS-PAGE to separate and clearly delineate their subunits. The similarities and differences between polypeptides of the 2S, 7S and 11S storage proteins are discussed in terms of the genetic diversity of these characters among the varieties of alfalfa examined.

Materials and methods

Seed storage proteins were analysed in 27 varieties of alfalfa (*Medicago sativa* L.). These included five subspecies (*glomerata, caerulea, falcata, hemicycla, praefalcata*), seven of the nine sources of *Medicago* germplasm introduced into North America (Tables 1 and 2: Anik/Peace, DuPuits/Vertus, Hairy Peruvian, Ladak, Lahonton, Moapa/Sonora, Sirsa #9), and 12 additional cultivars (Apica, Beaver, Caliverde 65, Citation, CUF 101, Excalibur, Rambler, Rangelander, Regen S, Spreador II, Vernal, 120).

Seeds of *M. sativa* L. subspecies (*glomerata, caerulea, falcata, hemicycla* and *praefalcata*) were obtained from

Table 2. Distribution of parental germplasm in selected alfalfa cultivars. Percent source contribution according to Barnes *et al.* (1977). Where accurate figures were not available, the authors have estimated the contribution as major (+++) or minor (+) on the basis of available information in the literature.

	Per cent source parental germplasm								
Cultivar	*M. falcata*	Ladak	*M. x varia*	Flemish	Chilean (Spanish)	African	Turkistan	Peruvian	Indian
Anik	**100**								
Apica		2	7	87	2		2		
Beaver	2	55	23				20		
Caliverde 65					72		28		
Citation	7	6	26	55	5		1		
CUFIOI					+	+++	+		++
DuPuits				**100**					
Excalibur	++	+	++	+++	+				
Hairy Peruvian								**100**	
Ladak		**100**							
Lahonton							**100**		
Moapa						**100**			
Peace	**100**								
Rambler	45	45	10						
Rangelander	+++	+	+++						
Regen S			+	+++					
Sirsa #9									**100**
Sonora						**100**			
Spreador II	28	28	27		3	14			
Vernal	16	17	50		17				
Vertus				**100**					
120	++	+	+++	+++	+		+		+

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the North Central Regional Plant Introduction Station, Iowa State University, Ames, Iowa. Cultivated varieties were obtained from seed stocks held at the Crop Science (now Plant Agriculture) Department, University of Guelph.

Samples of 25 seeds were analysed in triplicate for each of the cultivars. Seeds were scarified and imbibed at 4°C overnight. This treatment made subsequent dissections and extractions more efficient and yet was brief enough to prevent protein hydrolysis (Krochko, unpublished). The whole embryo was dissected away from the seed coat and the endosperm, frozen in liquid nitrogen and lyophilized to dryness. Samples were weighed to provide dry weight (DW) values and then extracted for protein.

Storage proteins were fractionated by means of three sequential extractions (Krochko and Bewley, 1990; Krochko *et al.*, 1992): low salt (0.2 M NaCl, pH 7.0; S-1 fraction protein), high salt (1.0 M NaCl, pH 7.0; S-2 fraction protein) and Laemmli's buffer (2% SDS, 10% glycerol, 62.5 mM Tris–HCl, pH 6.8; S-3 fraction protein). The first fraction (S-1) contained the majority of the soluble proteins including the 7S globulin (alfin) and 2S albumin, while the 11S globulin (medicagin) was the predominant protein in the second (S-2) and third (S-3) fractions.

The S-1 and S-2 fraction proteins were precipitated with 80% acetone, or alternatively, using the sodium deoxycholate–TCA procedure described by Peterson (1983) and redissolved in Laemmli's buffer lacking β-mercaptoethanol. Protein determinations were based on a BSA standard using the BCA protein assay (bicinchoninic acid assay; Pierce, Rockford, IL) (Smith *et al.*, 1985). For one-dimensional polyacrylamide gel electrophoresis (1-D PAGE), the S-1, S-2 and S-3 fraction proteins prepared with Laemmli's buffer (see above) were diluted to a standard protein concentration with additional Laemmli's buffer. β-Mercaptoethanol (ME; 5% v/v) was added to the S-2 and S-3 samples to reduce disulphide bonds (Laemmli, 1970; Krochko and Bewley, 1990). For two-dimensional analysis of the 11S globulins (2-D PAGE), proteins of the S-2 fraction were electrophoresed initially in the absence of a reducing agent. A vertical strip cut from this gel was incubated in Laemmli's buffer with 5% ME for 15 min and then laid across the top of a second slab gel for electrophoresis in the second dimension (Krochko and Bewley, 1990). Under these conditions polypeptides covalently linked by intermolecular disulphide bonds are displaced from the diagonal during the second-dimension electrophoresis. The relative rates of migration of proteins without disulphide bonds are unaffected by these treatments, and these lie along the diagonal of the second dimension gel. Proteins were stained using Coomassie BBR.

Total protein in embryos from mature seeds was determined for each of the cultivars. Total extractable protein was calculated by summing protein values for S-1, S-2 and S-3 fractions for individual samples for each of the cultivars.

Results

It was of interest to determine the generality of the protein pattern previously observed for *M. sativa* cv. Excalibur (Krochko and Bewley, 1990; Krochko *et al.*, 1990) with respect to subspecies and cultivars of the *M. sativa* L. complex. Seed storage proteins of 22 cultivars and five subspecies were analysed quantitively and qualitatively using 1-D and 2-D electrophoresis. S-1 and S-2 (and in some cases S-3 fraction proteins) were compared for the five subspecies (*glomerata, caerulea, falcata, hemicycla, praefalcata*), seven (of nine) recognized North American germplasm sources (Barnes *et al.*, 1977) and a sample of additional alfalfa cultivars.

The polypeptide patterns of the S-1, S-2 and S-3 fraction proteins following one-dimensional electrophoresis were similar in all of the cultivars (Fig. 1) and subspecies examined (Fig. 2). The 2S storage protein, the α_1, α_2 and α_3 polypeptides of the 7S globulin, as well as HMW_2, one of the minor storage proteins, were prominent in all of the S-1 samples examined (Figs 1 and 2). In the S-2 fractions the patterns of reduced acidic and basic polypeptides of the 11S storage protein were very similar to those described previously for cv. Excalibur (Krochko and Bewley, 1990) (Figs 1 and 2). For each of the cultivars, additional 11S protein was solubilized using Laemmli's buffer (S-3 fraction). However, the pattern of acidic and basic polypeptides was not substantially different amongst the cultivars (Fig. 1). There were some minor cultivar-specific variations in some of the acidic polypeptides of the 11S globulin. For example, Rangelander had an additional smaller acidic polypeptide (A_8) absent from Excalibur (Fig. 1) (Krochko *et al.*, 1990). Cultivar Anik (Fig. 1, Lane v) had more A_1 than Excalibur (Fig. 1, Lane a), while Regen S (Lane l) and Citation (Lane m) both had strong A_7 and A_8 polypeptide bands (Fig. 1).

The 11S globulin in alfalfa is comprised of a family of individually distinct subunits, each composed of an acidic polypeptide (A_1, A_2, A_3, $A_{5/6}$, $A_{9/10}$; molecular mass (M_r) = 49–39 kDa) linked via disulphide bond(s) to a basic polypeptide (B_1, B_2, B_3; M_r = 24, 23 and 20 kDa, respectively) (Krochko and Bewley, 1990). This pairing is highly specific, and the vertical alignment of the A and B polypeptides below the diagonal on the 2-D gels clearly shows pairing of the A_1, A_2 and A_3 polypeptides with B_3 and the $A_{5/6}$, $A_{9/10}$ polypeptides with $B_{1/2}$ (Krochko and Bewley, 1990) (Figs 3 and 4).

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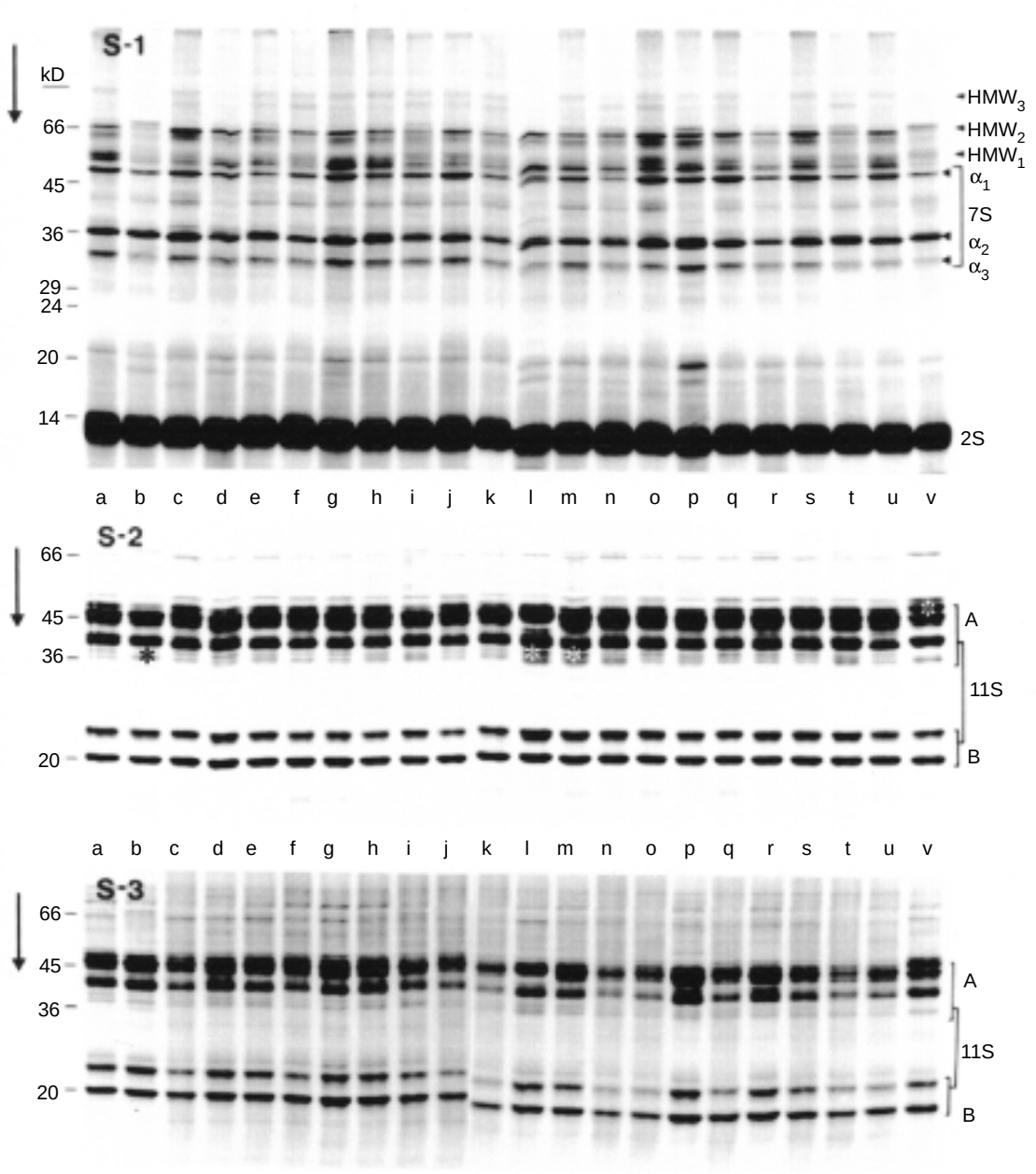

Figure 1. One-dimensional gel electrophoresis (SDS-PAGE) of storage proteins extracted from mature embryos from 22 cultivars of *Medicago sativa*. Isolated embryos were extracted sequentially with standard buffers: S-1 fraction (0.2 M NaCl, pH 7.0), S-2 fraction (1.0 M NaCl, pH 7.0), S-3 fraction (Laemmli's buffer; 2% SDS, 10% glycerol, 62.5 mM Tris–HCl, pH 6.8). The S-1 samples were prepared for electrophoresis in Laemmli's buffer without a reducing agent, while the S-2 and S-3 samples were incubated with 5% β-mercaptoethanol. Proteins were loaded at 30 μg/lane for the S-1 fraction, 15 μg/lane for the S-2 fraction and 20 μg/lane for the S-3 fraction and detected after electrophoresis with Coomassie BBR. The major storage proteins (2S, 7S, 11S), the A (acidic) and B (basic) polypeptides of the 11S globulin, the α_{1-3} polypeptides of the 7S globulin, as well as three minor storage proteins (HMW_1, HMW_2, HMW_3) are indicated. Asterisks in Lanes b, l, m and v of the S-2 gel indicate differences between these and other cultivars. Standard molecular mass markers are given. Cultivar designations are as below.

a: Excalibur
b: Rangelander
c: Lahonton
d: Vertus
e: 120
f: Moapa
g: CUF 101
h: Ladak
i: Spreador II
j: Hairy Peruvian
k: Vernal
l: Regen S
m: Citation
n: Beaver
o: Sirsa #9
p: Peace
q: Caliverde 65
r: Apica
s: DuPuits
t: Rambler
u: Sonora
v: Anik

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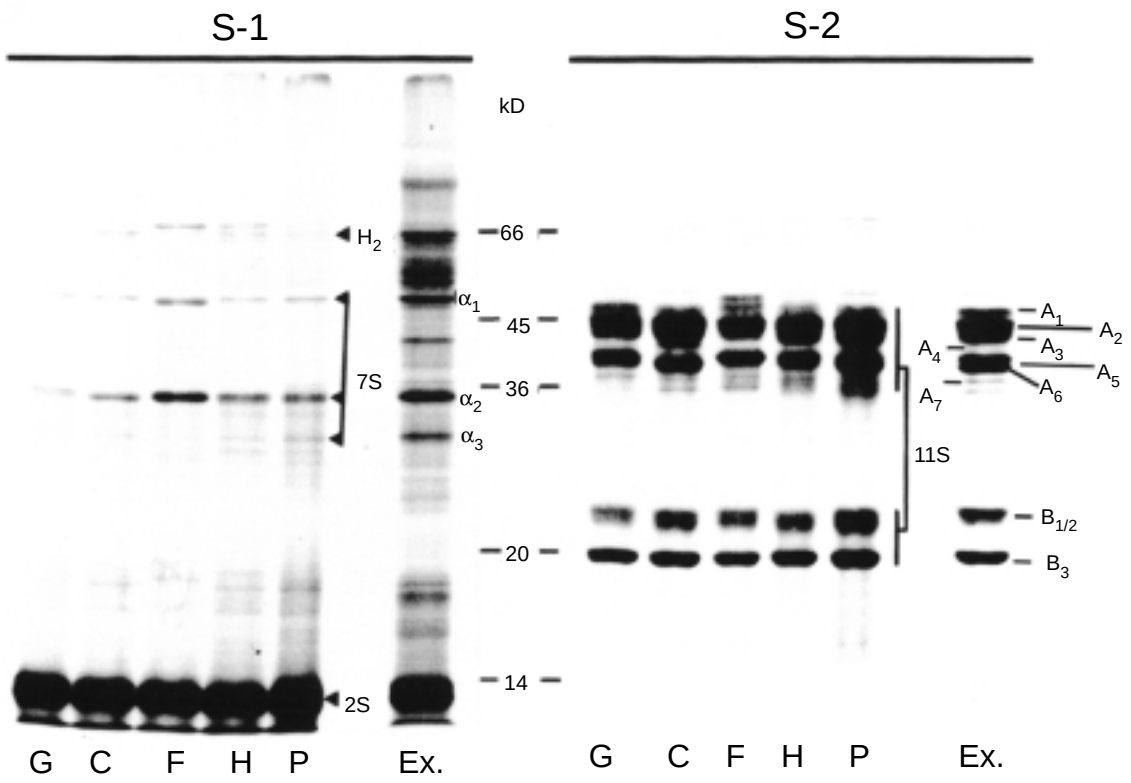

Figure 2. One-dimensional electrophoresis of S-1 and S-2 fraction proteins extracted from five subspecies of *M. sativa*: (G), ssp. *glomerata*; (C), ssp. *caerulea*; (F), ssp. *falcata*; (H), ssp. *hemicycla*; (P), ssp. *praefalcata*. SDS-PAGE: S-1 fraction (Laemmli's buffer), S-2 fraction (Laemmli's buffer + ME). The major storage proteins (2S, 7S, 11S) and their polypeptides are indicated as well as HMW_2 (H_2). The cultivar Excalibur (Ex.) is shown for comparison. Standard molecular mass markers are given.

Two 11S subfamilies were recognizable on the basis of the B polypeptide (B_3 or $B_{1/2}$) (Krochko and Bewley, 1990). The proportional representation of the two 11S subfamilies, subfamily I ($A_{5/9}B_{1/2}$, $A_{6/10}B_{1/2}$) and subfamily II (A_1B_3, A_2B_3, A_3B_3, A_4B_3, A_7B_3), varied only slightly among the cultivars and subspecies (Figs 1 and 2).

S-2 fraction proteins from 19 cultivars and five subspecies were subjected to two-dimensional electrophoresis (SDS/SDS-PAGE + ME) (Figs 3 and 4; Excalibur and Rangelander are not shown here) (cf. Krochko and Bewley, 1990; Krochko *et al*., 1990). In all of the cultivars and subspecies examined, the predominant subunits were those containing the A_1, A_2, A_3, $A_{5/9}$ or $A_{6/10}$ acidic polypeptides (Figs 3 and 4) (Krochko and Bewley, 1990). However, to a greater or lesser extent, the cultivars all contained some additional smaller acidic polypeptides. Within and among all of the cultivars, there was always more variability in size of the acidic polypeptide than in the basic component of the subunit. For example, acidic polypeptides smaller than $A_{6/10}$ were frequently seen on the gels, and the size, number and amounts of these varied among the cultivars examined (Fig. 3). In addition, there was more variability in molecular size of the acidic polypeptides within subfamily II (linked to B_3) than in those in subfamily I (linked to $B_{1/2}$) (Figs 3 and 4). This can be seen by aligning the A and B polypeptides as described earlier. Basic polypeptides different in size from $B_{1/2}$ and B_3 were seldom seen. Thus, in the 11S storage protein the basic polypeptide fragments have been more rigidly conserved than the acidic ones during the evolution of the alfalfa species complex.

The total amount of protein extractable from each set of 25 embryos varied about two-fold among the cultivars tested (Fig. 5A). Embryo dry weights also showed considerable variation between cultivars (Fig. 5B). However, when total protein was expressed on a dry weight basis, the variability among the cultivars was reduced substantially (Fig. 6A). The proportional representation of each of the contributing fractions (S-1, S-2, S-3) to total protein was calculated (Fig. 6B). The S-1 fraction protein accounted for about 35–55% of the total extractable protein; the other fractions (S-2 plus S-3) from 45 to 65% (Fig. 6B). The contribution of

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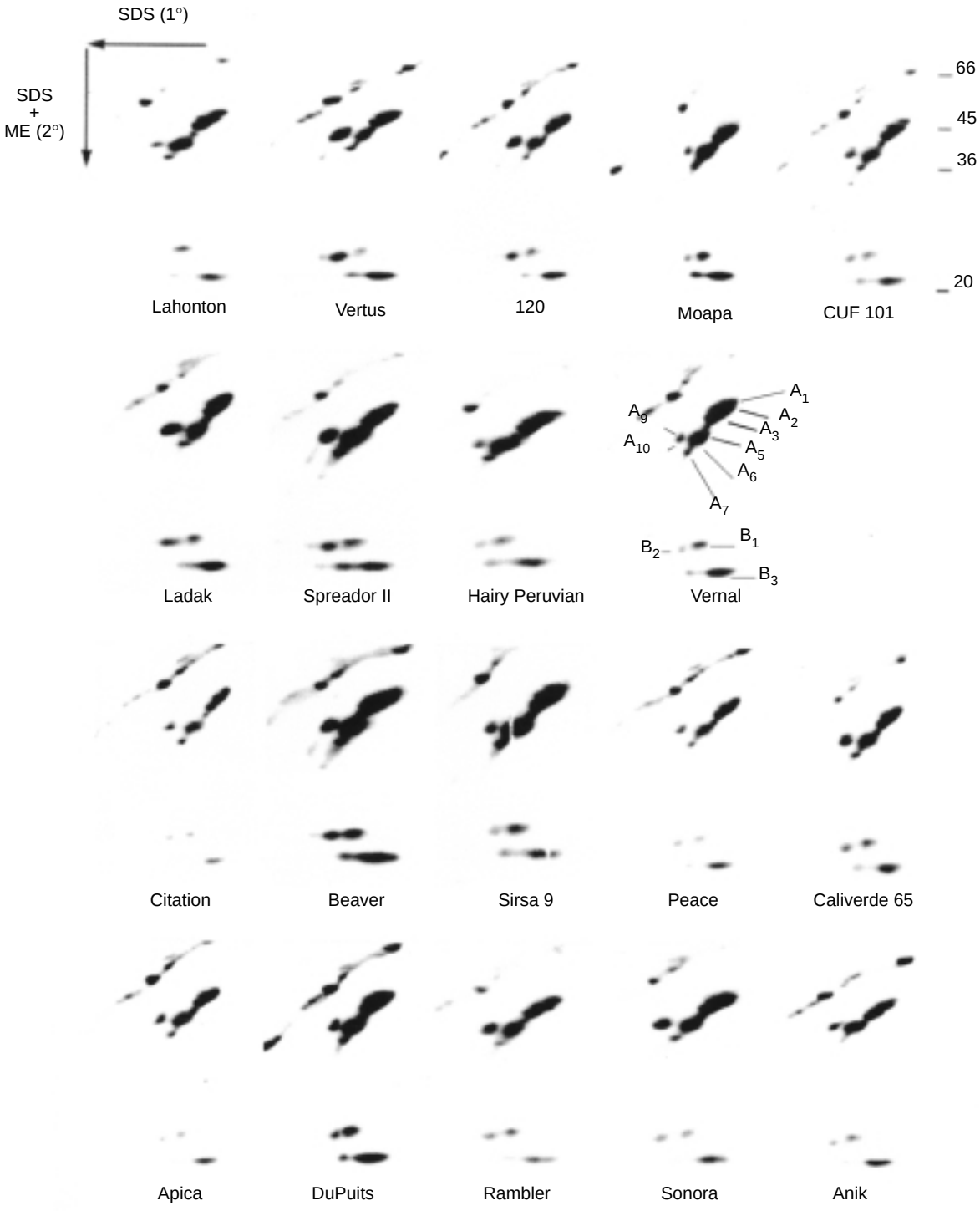

Figure 3. Two-dimensional gel electrophoresis of S-2 fraction proteins from 19 cultivars of alfalfa. First dimension (1°): non-reduced (Laemmli's buffer), second dimension (2°): reduced (Laemmli's buffer + ME). Acidic (A) and basic (B) polypeptides of the 11S protein are indicated (for more detail see Krochko and Bewley, 1990). Standard molecular mass markers are shown.

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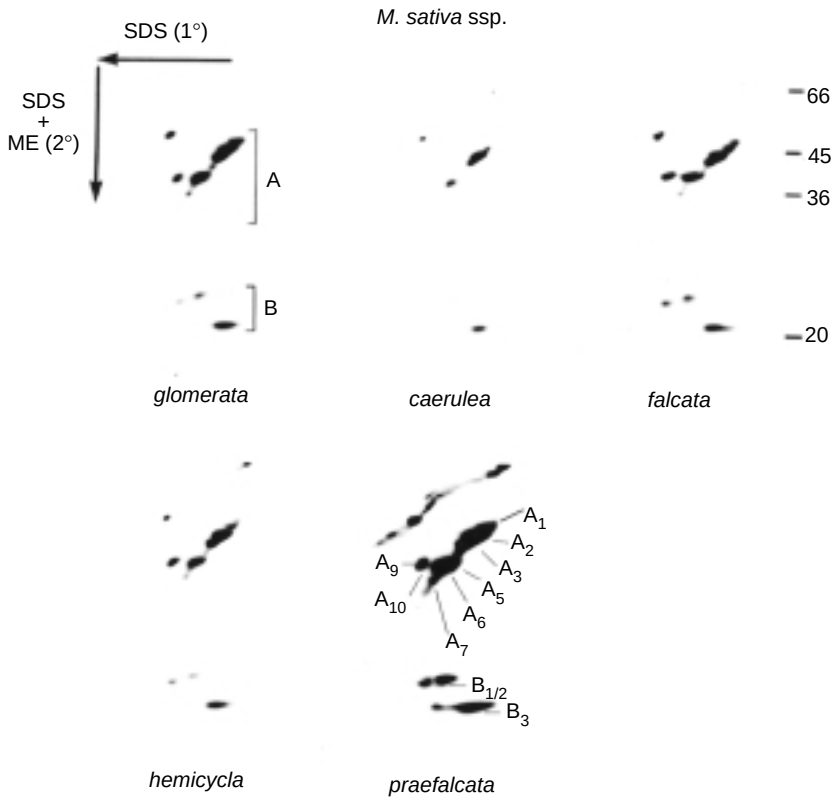

Figure 4. Two-dimensional gel electrophoresis of S-2 fraction proteins from five subspecies of the *M. sativa* species complex: ssp. *glomerata*, ssp. *caerulea*, ssp. *falcata*, *hemicycla*, *praefalcata*. Gel electrophoresis as in Fig. 3. Standard molecular mass markers are shown, as are the acidic (A) and basic (B) subunits.

the S-3 fraction was always much smaller than that of the S-2 fraction (Figs 5A and 6B). When the relative contributions of the two major protein fractions (S-1 and S-2) for each of the cultivars were expressed as a ratio (S-1/S-2), these values ranged from 0.8 to 2 (Fig. 6C). The majority of the alfalfa cultivars expressed ratios between 1 and 1.5.

Discussion

Variability in seed storage proteins in taxa within the *M. sativa* L. complex was investigated by analysis of subspecies and cultivars. On the basis of one-dimensional SDS-PAGE, all cultivars showed almost equivalent expression patterns for polypeptides of the 2S albumin and 7S globulin (Fig. 1). Although the 11S globulin is characterized by considerably more polypeptide heterogeneity, there were no major deviations in any of the subspecies or cultivars examined (Figs 1–4) from the pattern of polypeptides described previously for *M. sativa* using the cultivar Excalibur as a standard (Krochko and Bewley, 1990; Krochko *et al.*, 1990). These patterns were not influenced by the ploidy level of the embryo. The subspecies *hemicycla, glomerata* and *caerulea* are diploid, *sativa* is tetraploid, and *falcata* can be either (Quiros and Bauchan, 1988).

Seed proteins in other species have been used successfully to probe evolutionary relationships (Ladizinsky and Hymowitz, 1979; Singh *et al.*, 1991; Tomooka *et al.*, 1992). However, in the *M. sativa* species complex, the polypeptide pattern of the 11S globulin is remarkably uniform and provided no information on either parentage or relationships among the cultivars. For example, varieties containing substantial *falcata* germplasm (Anik, Peace, Rambler, Rangelander) were no more similar to each other than to other alfalfa cultivars (Figs 1 and 3). The cultivar Excalibur was no more similar to its parental genotypes (DuPuits, Vertus) than it was to any of the other cultivars tested. No clear trends were seen when sets of cultivars were grouped on the basis of the amounts of *falcata* or *sativa* germplasm they contained and proteins compared (Table 1).

Divergence within the 11S globulin to form two subfamilies (I and II) predates the evolution of the *M. sativa* L. complex; this dichotomy occurs in all of the

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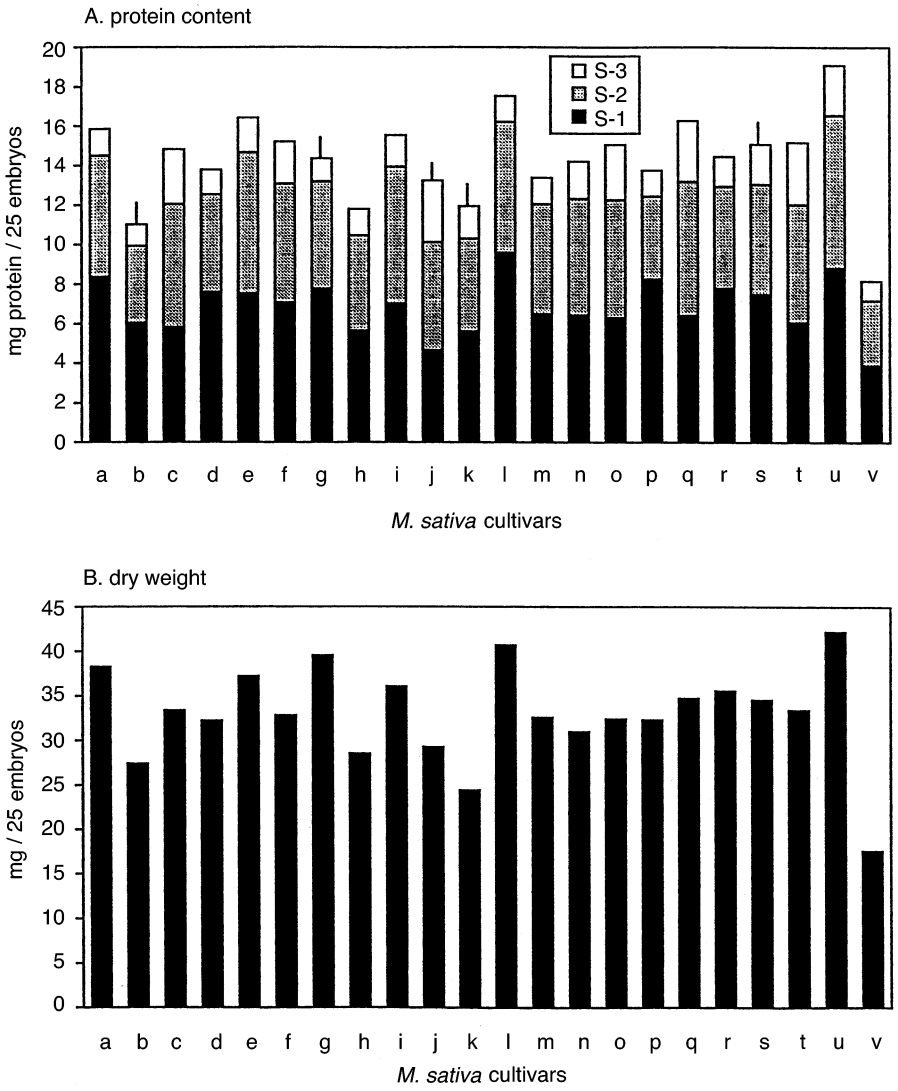

Figure 5. Protein content (A) and dry weight (B) of embryos isolated from mature seeds of *M. sativa* cultivars. Total protein values are the sum of component fractions for each seed sample (+ SE for the total). Values plotted are the means of three separate determinations of 25 embryos. Values in (B) are the dry weight per 25 embryos after lyophilization. Cultivar designations as in Fig. 1.

samples tested and also has been described in other legumes (Horstmann, 1983; Nielsen, 1985) and sunflower (Vonder Haar *et al.*, 1988).

Most of the variability in storage protein fractions among cultivars was primarily of a quantitative nature (Fig. 5A). Yet, when the data are normalized on a DW basis (Fig. 6A), even this variability is reduced. The ratio of S-1/S-2 protein is indicative of the (7S + 2S)/11S protein ratio, and this ratio is between 1 and 1.5 for most of the cultivars. These S-1/S-2 ratios have been used diagnostically to describe the relative contributions of the 7S and 2S (S-1 fraction) and 11S (S-2 fraction) storage proteins in developing seeds and somatic embryos (Krochko *et al.*, 1992, 1994). In developing alfalfa seeds the 7S protein is synthesized somewhat earlier than the peak of 11S globulin accumulation, although in mature seeds the 11S and 2S storage proteins predominate (Krochko *et al.*, 1992). In alfalfa somatic embryos grown on standard Boi2Y media, the storage protein patterns are abnormal compared with zygotic embryos, having substantial 7S but very little 2S or 11S storage protein (Krochko *et al.*, 1992, 1994). Thus, abnormally high S-1/S-2 values may reflect developmental and/or nutritional

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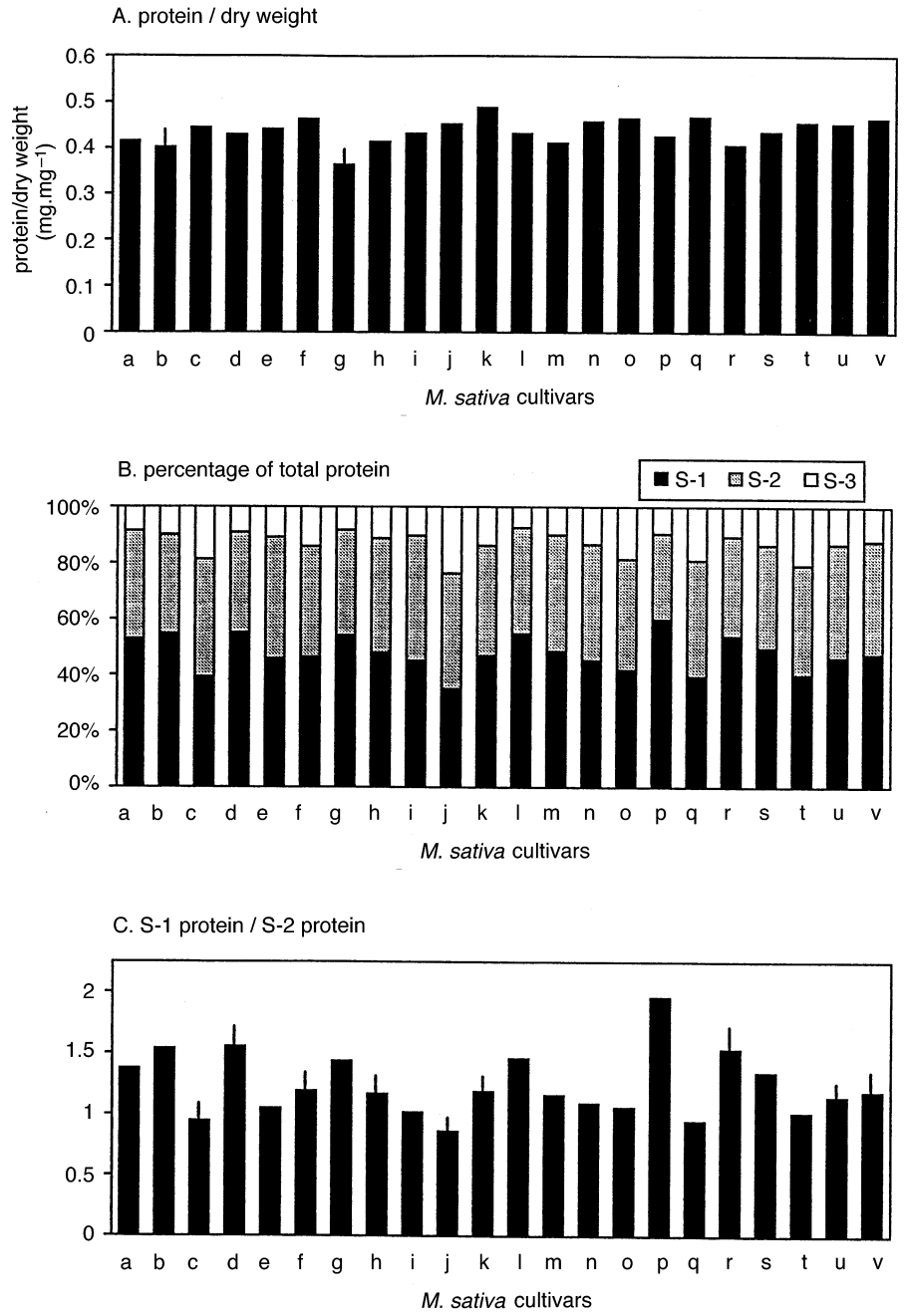

Figure 6. Seed protein content of alfalfa cultivars. (A) Protein per unit dry weight. (B) Proportional representation of S-1, S-2 and S-3 fraction proteins in cultivars of alfalfa. S-1, S-2 and S-3 fraction proteins are expressed in terms of their percentage contribution to total extractable protein. (C) Ratio of S-1 fraction protein to S-2 fraction protein (S-1/S-2). Values plotted are the means of three separate determinations (± SE). Cultivar designations as in Figs 1 and 5.

imbalances resulting in impaired 11S globulin accumulation, and in the case of alfalfa somatic embryos, this protein imbalance can be rectified by nutrient supplementation of the maturation media (Krochko *et al.*, 1992; Lai and McKersie, 1993; Sreedhar and Bewley, 1998). Considering that the seed samples used for each of the alfalfa cultivars examined were obtained from commercially available seed lots grown under a wide range of conditions and diverse geographical regions, it is still somewhat

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surprising that there was not a greater variability in the proportional expression of individual seed storage proteins as indicated by the S-1/S-2 ratios in these cultivars (Fig. 6C). The only significant qualitative differences in seed proteins revealed by the present study were in some of the minor acidic polypeptides of the 11S globulin, which may be useful for cultivar identification, and some of the higher-molecular-weight and poorly-characterized seed proteins (HMW_1, HMW_2, HMW_3) (Figs 1 and 3).

Cultivated alfalfa does not set seed well unless cross-pollinated, and in many areas requires the assistance of pollinating leaf-cutter bees to produce adequate seed yields (Barnes *et al.*, 1977). New varieties are developed by maintaining maximum heterozygosity (Rumbaugh *et al.*, 1988). Consequently each variety is composed of closely related heterozygotic biotypes and may be expected to be highly polymorphic. There are several explanations and arguments for the lack of variability in the major seed proteins between cultivars and subspecies of alfalfa. One possibility is that the basic genetic background from which cultivated alfalfa was derived was either limited or has suffered some genetic erosion. Alternatively, it may be due to the absence of strong selective pressures or to structural constraints related to function, which prevent or limit the kinds of alterations that can occur in seed storage proteins. Following synthesis, storage proteins undergo a series of modifications as they are transported to, and appropriately packaged within, the protein bodies (Kermode and Bewley, 1999). In general, any changes to the amino acid sequences of the subunits that would interfere with these functions would not be incorporated into the breeding population as a whole. There are regions of the 11S globulin subunits where flexibility in amino acid composition is allowed and regions where the sequence requirements are rigid (Argos *et al.*, 1985; Shotwell *et al.*, 1988). Heterogeneity in subunits of the 11S globulin in alfalfa cultivars is confined almost exclusively to changes in the number and size of the acidic polypeptides of subfamily II (Fig. 3), while the acidic polypeptides of subfamily I and all basic polypeptides are highly conserved within this species complex.

Given that the germplasm sources (landraces) are relatively recent separations, it was not surprising that there are no marked differences in polypeptide profiles between them. However, the lack of substantial differences between the ancestral types (subspecies) and the modern cultivars clearly suggested that the rate of evolution or changes in these globulin storage proteins have been slow compared with other phenotypic characters. The high degree of similarity among these taxa in the qualitative and quantitative expression of seed proteins suggests a high degree of uniformity in the seed physiology and genetics of alfalfa.

Protein fractions were obtained from the bulk extraction of random pools of 25 seeds for each cultivar. However, because of the substantial genetic heterozygosity maintained within alfalfa cultivars, protein profiles from individual seeds within a cultivar may actually show more variability than has been indicated here. For example, in an RFLP-based comparison of alfalfa accessions, there was more variability when DNA from individual seeds was used than when DNA pools from several individuals were compared. Pooling DNA from several plants obscured individual differences, and the accessions appeared more homogeneous than they were otherwise presumed to have been (Kidwell *et al.*, 1994). A complete analysis of the inherent variability in storage proteins in the alfalfa species complex will require the analysis of individual seeds within selected cultivars.

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