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Genetic variation for isozyme genes and proteins in Spanish primitive cultivars and wild subspecies of *Lens*

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Summary

An analysis of the variability for genes encoding seven isozyme systems and storage proteins in a collection of cultivated and wild accessions of *Lens* is reported. The collection, which is part of the Spanish INIA Genebank, contains the ssp. *culinaris, orientalis, odemensis, nigricans* and *ervoides,* and presents a high degree of genetic diversity both within and between the accessions. A total of 25 loci were examined; of these, 18 were polymorphic (the 7 genes encoding storage proteins, and the following isozyme loci: *Acp-1, Acp-2, Cpx-1, Cpx-2, Aat-p, Aat-m, Lap-1, Mdh-2, Mdh-3, Mdh-4* and *6pgd-p*) and 7 were monomorphic (*Aat-mb, Aat-c, Mdh-1, Mdh-5, 6pgd-2, Pgm-c* and *Pgm-p*). The phylogenetic relationships between subspecies were analyzed using the allelic frequencies. The study suggests that *orientalis* and *odemensis* share more biochemical characters than the other subspecies, and that those subspecies keep an intermediate position between *Lens culinaris* and *Lens nigricans.*

Introduction

Knowledge of genetic diversity in crop gene pools is central to the development of effective germplasm conservation in genebanks to help plant breeding strategies. To this end, considerable efforts have been underway to collect and conserve cereals and legumes in Spain over the last 15 years. However, the genetic variation of the collected material and its evaluation using information from field plots still requires analysis and study. The morphological and agronomical characterization is being investigated.

Modern genetic studies of genus *Lens* have only recently begun (Muehlbauer & Slinkar, 1981; Zamir & Ladizinsky, 1984; Muehlbauer et al., 1989). The use of electrophoresis provides valuable information on the range of protein variation within different populations, identifies new genes and alleles that can differentiate individuals and populations, and helps us to understand the original genomic variation between different subspecies (Hayward, 1985). The present work is concerned with an analysis of variation in the genes encoding seven isozyme systems and storage proteins in a collection of cultivated and wild accessions of *Lens* that is maintained in the Spanish Genebank of the INIA.

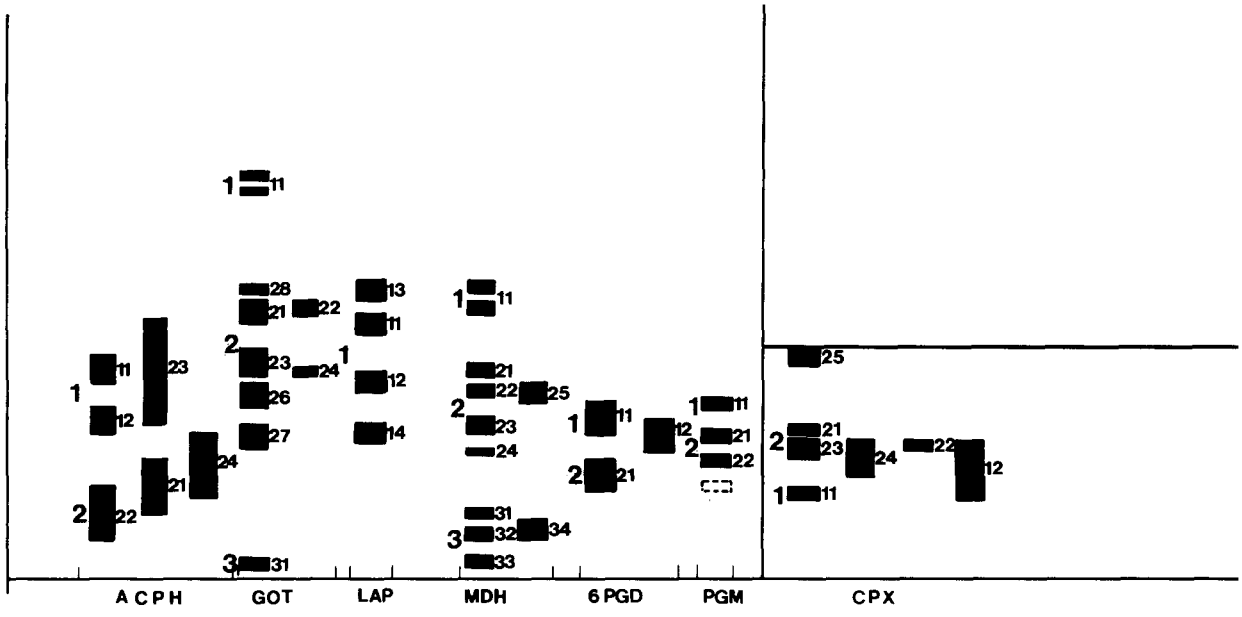

Fig. 1. Electrophoretical mobility variants observed in the isozyme analysis.

Materials and methods

Plant materials

We studied 34 accessions of *Lens culinaris* ssp. *culinaris*, 5 of *Lens culinaris* ssp. *orientalis*, 1 of *Lens culinaris* ssp. *odemensis*, 3 of *Lens nigricans* ssp. *nigricans* and 4 of *Lens nigricans* ssp. *ervoides*.

Isozyme analysis

Seed samples were germinated under controlled environmental conditions and leaves from 15-day-old seedlings were used to get crude extracts. A minimum of 10 plants for each accession were analyzed following the suggestions of Nielsen (1984) for isozmye polymorphism studies in self-pollinating species. Pieces of leaves from each plant were excised and ground in a mortar with 0.25 M sodium acetate pH 5.0 in the proportion of 1–2 mg/ml. The crude extract obtained was soaked up with a paper wick (Whatman 3 MM) and placed into the gel. Horizontal starch (Connaught 10% w/v) gel electrophoresis was used for the analysis of acid phosphatases (ACPH, E.C. 3.1.3.2), cathodal peroxidases (CPX, E.C. 1.11.1.7), glutamate oxaloacetate transaminases (GOT, E.C. 2.6.1.1), leucinamino peptidases (LAP, E.C. 3.4.11.1), malate dehydrogenases (MDH, E.C. 1.1.1.37), 6-phosphogluconate dehydrogenases (6-PGD, E.C. 1.1.1.44), and phosphoglucose mutases (PGM, E.C. 2.7.5.1). For the analysis of ACPH, CPX, MDH and 6-PGD isozymes, gels were run at 185 V for 5–6 hours with 0.005 M histidine-HCl acid pH 7.0 gel buffer and 0.043 M tris-citric acid pH 7.0 electrode buffer. Electrophoretic conditions for the rest of the enzymatic systems consisted of 400 V for 3–4 hours with 0.15 M tris-boric acid pH 8.0 gel buffer and 0.2 M tris-boric acid pH 8.5 electrode buffer. The gels, 1 cm thick, were cut into 2 mm thick slices and then each slice was used to reveal the activity of each enzyme system. Enzyme staining methods have been reported previously by other authors (Shaw & Koen, 1968; Brewer & Sing, 1970; Rao & Rao, 1980).

Storage protein analysis

Seeds were excised separating a piece of the cotyledons to analyze the storage proteins. These were extracted by grinding the piece with 20 μl/mg of 2%

sodium dodecyl sulphate (SDS), 10% glycerol, 4% 2-mercaptoethanol in 0.0625 M tris-HCl acid pH 6.8 buffer, for 2 hours. The extracts were kept at room temperature for 2 hours, and afterwards incubated in a water-bath for 2 min at 100° C, and then centrifuged at 6.000 rpm for 10 min. Seed protein subunits were fractionated by SDS-polyacrylamide gel electrophoresis as described by Payne et al. (1981). The gels were run at 8 mA for 16 hours at room temperature.

Results and discussion

We studied the phenotypes for each isozyme system and storage proteins (Figs 1 and 2). The identity of individual bands in the isozyme gels makes it possible to attribute allelic products to specific loci, and hence, the loci can be compared across populations and subspecies (Table 1).

Acph

Two active regions were revealed in the zymogram phenotypes. The fast-moving group of phosphatases (ACPH-1) presented phenotypes with two bands (11 and 12), a single band or no bands in ssp. *culinaris*. The slow-moving zone (ACPH-2) contained one band which showed approximately double intensity of each band in region ACPH-1 in ssp. *culinaris*. This band migrated to two different positions (21 and 22). An intensely stained band was found in the region ACPH-1 in ssp. *ervoides* (23). In addition, an intermediate band (24) appeared in two accessions of *Lens nigricans* ssp. *nigricans*. Weeden & Marx (1984) described a similar electrophoretical pattern in *Pisum sativum*, and found two genes which are responsible for the isozymes manifested in each region of the zymogram. Bands 11 and 12 could be the active products of two different alleles (*Acp-11* and *Acp-12*) of the gene *Acp-1*, if one assumes that the absence of a band in this region is explained by the existence of a null allele (*Acp-10*). On the other hand, bands 21 and 22 could be the products of different alleles of the *Acp-2* gene, found in cultivated lentils of the ssp. *culinaris*

Table 1. Allelic frequencies for the isozyme genes in the five ssp. of *Lens*

Genes	*culinaris*	*ervoides*	*odemensis*	*orientalis*	*nigricans*
Acp-11	12.6	0	0	0	0
Acp-12	31.8	0	0	0	0
Acp-10	55.6	100	100	100	100
Acp-21	12.9	25	0	100	33.3
Acp-22	87.1	0	100	0	0
Acp-23	0	75	0	0	0
Acp-24	0	0	0	0	66.6
Cpx-11	89.4	0	100	60	25
Cpx-12	0	25	0	0	0
Cpx-10	10.6	75	0	40	75
Cpx-21	2.3	25	100	0	0
Cpx-22	35.3	25	0	40	40
Cpx-23	58.8	0	0	60	60
Cpx-24	3.6	25	0	0	0
Cpx-25	0	25	0	0	0
Aat-mb	100	100	100	100	100
Aat-p1	15	25	0	0	0
Aat-p2	37.6	50	100	100	100
Aat-p3	25.9	25	0	0	0
Aat-p4	21.5	0	0	0	0
Aat-m1	0.3	0	0	0	0
Aat-m2	57.1	0	0	20	33.3
Aat-m0	42.6	100	100	80	66.6
Aat-c	100	100	100	100	100
Lap-11	69.7	0	100	20	33.3
Lap-12	29.4	50	0	80	0
Lap-13	0.8	25	0	0	33.3
Lap-14	0	25	0	0	33.3
Mdh-1	100	100	100	100	100
Mdh-21	100	100	100	100	33.3
Mdh-22	0	0	0	0	66.6
Mdh-31	100	100	0	100	33.3
Mdh-32	0	0	100	0	0
Mdh-30	0	0	0	0	66.6
Mdh-41	32.1	50	0	25	33.3
Mdh-42	0	0	100	0	0
Mdh-40	67.9	50	0	75	66.6
Mdh-51	100	100	100	100	100
6pgd-p1	89.4	75	100	20	100
6pgd-p2	7.9	25	0	80	0
6pgd-p0	2.6	0	0	0	0
6pgd-2	100	100	100	100	100
Pgm-c	100	100	100	100	100
Pgm-p	100	100	100	100	100

(*Acp-21* and *Acp-22*), whereas bands 23 and 24 could be the products expressed by other homoalleles present in wild ssp. *ervoides* and *nigricans* (*Acp-23* and *Acp-24*).

CPX

Two major independent regions could be distinguished in the zymograms of the *Lens* subspecies. The most cathodal region (CPX-1) showed one band, although plants lacking expression in this region were frequently observed in some accessions. Therefore, the presence or absence of this band in the populations is presumably due to the variability for the active and null alleles of the gene *Cpx-1* (*Cpx-11* and *Cpx-10*, respectively). The less cathodal region (CPX-2) showed one band per plant on the four different forms, all of which had similar mobilities but different staining intensities (*Cpx-2* gene with the alleles *Cpx-21*, *Cpx-22*, *Cpx-23* and *Cpx-24*). Polymorphism for this band both within and between populations was observed in the ssp. *culinaris*. One accession of *Lens nigricans* ssp. *ervoides*, collected in Israel, showed a different pattern of cathodal peroxidases: abroad slow-moving band described above (alleles *Cpx-12* and *Cpx-25*) in each region instead of the patterns of the other wild and cultivated accessions.

GOT

Three regions are clearly seen in the phenotypes of all subspecies: GOT-1, GOT-2, and GOT-3. No mobility variants were found for regions GOT-1 and GOT-3. GOT-1 appears on zymograms as a fast-moving doublet with a lower activity relative to the other two regions. Based on previous results in peas (Weeden & Marx, 1984), Weeden & Emmo (1985) postulated that GOT-1 isozymes had a microbody origin (locus Aat-mb). The intermediate broad zone (GOT-2) shows one-, two- or three-banded phenotypes as the result of combining different bands distributed in two regions. *Aat-p*, which codes for the intermediate-moving GOT isozymes, have also been previously reported (Muehlbauer et al., 1989). The group of bands 21-22-23-24 observed here presumably represent the active allelomorphs of this gene (*Aat-p1*, *Aat-p2*, *Aat-p3* and *Aat-p4*, respectively). The lower migrating subregion of GOT-2 showed a single band (26-27), or no bands. These phenotypes may represent the expression of either active or null alleles for the gene *Aat-m* (*Aat-m1*, *Aat-m2* and *Aat-m0*). The gene was reported to be strongly associated with the mitochondrial pellet (Muehlbauer et al., 1989). GOT-3 always presents a single slow band which presumably represents the isozyme of cytosolic origin designated *Aat-c* (Muehlbauer et al., 1989).

LAP

LAP activity showed a single band with four mobility positions. These are the presumptive products of different alleles of the locus *Lap-1*. The allele *Lap-11* was the most frequent among the cultivated lentils and presented an intermediate rate of migration.

MDH

The patterns of MDH revealed three regions in the leaf tissue of all the *Lens* subspecies. Region MDH-1 exhibited a two-banded phenotype which would be coded by the monomorphic locus *Mdh-1*. Region MDH-2 shows a set of four bands (21-22-23-24) that are invariably present in the cultivated *Lens*. Two accessions of ssp. *nigricans* showed a different phenotype (21-25). This region must be coded for by at least one locus designated as Mdh-2. The alleles Mdh-21 and Mdh-22 should explain the differences between *nigricans* and the remaining subspecies. Region MDH-3 showed a three-banded phenotype (31-32-33) incultivated lentils. Band 33, near the origin, was constant in both cultivated and wild subspecies.

6-PGD

This system exhibits a maximum number of two

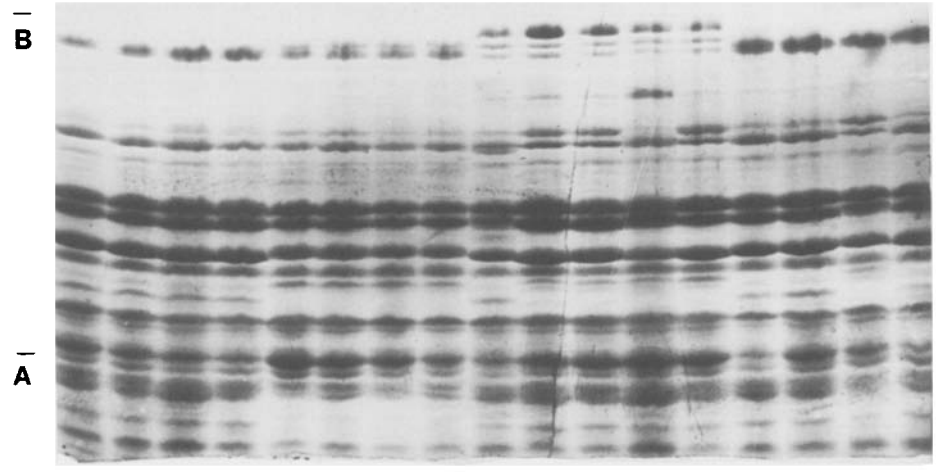

Fig. 2. Examples of the electrophorenograms obtained by SDS-PAGE of seed storage proteins of different accessions of cultivated lentils.

bands and three different phenotypes. One band (21) was common to all the accessions and should be the product of the monomorphic gene *6-pgd-2*. On the other hand, two isozymes for 6-PGD in *Lens*, one which was found in the cytosol and the second in the plastid (coded by *6-pgd-p*) were previously described (Muehlbauer et al., 1989). The latter corresponds well with our region 6-PGD1, the most anodal zone, which displayed variation and exhibited one band in two alternative positions. This result can be explained by assuming the existence of two active alleles (*6pgd-p1* and *6pgd-p2*).

PGM

Two areas of activity were distinguishable on gels stained for PGM. The phenotype was uniform and revealed three active bands, one for the region PGM-1 (fast-moving) and two for the region PGM-2 (slow-moving). Zamir & Ladizinsky (1984) observed two regions in the zymograms of PGM in hybrids of *Lens culinaris* ssp. *culinaris* × *L. culinaris* ssp. *orientalis*, in which PGM-2 varied between the subspecies and segregated in the F2. They proposed the existence of two genes that would code for these isozymes, *Pgm-c* and *Pgm-p*, and would respectively belong to either cytosolic or the plastidic fractions.

Storage proteins

SDS-PAGE electrophoregram of seed storage proteins found in all materials analyzed are shown in Figs 2 and 3. Molecular weight markers expressed in kilodaltons (kDa) are indicated on the left hand side of the SDS-pattern. The electrophoregram can be divided in two regions depending on the molecular weight of the polypeptide groups: A (molecular weight lower than 29 kDa), and B (molecular weight higher than 29 kDa). Region B showed variability in and between populations whereas region A was monomorphic. The region A phenotype revealed a maximum number of eight bands.

The band of the lowest molecular weight in region B (around 29 kDa) migrated to two alternative positions. Of the 25 bands comprising this region, the pairs of bands 4-41 and 5-51 (aprox. 45 kDa) and 12-121 and 13-131 (approx. 66 kDa) showed alternate variations of intensity with a 'plus-minus' pattern of relative staining intensity. These results could suggest certain allelic alternatives. However, without establishing the formal genetics of this proteins it is not known how many bands relate to different loci and different alleles.

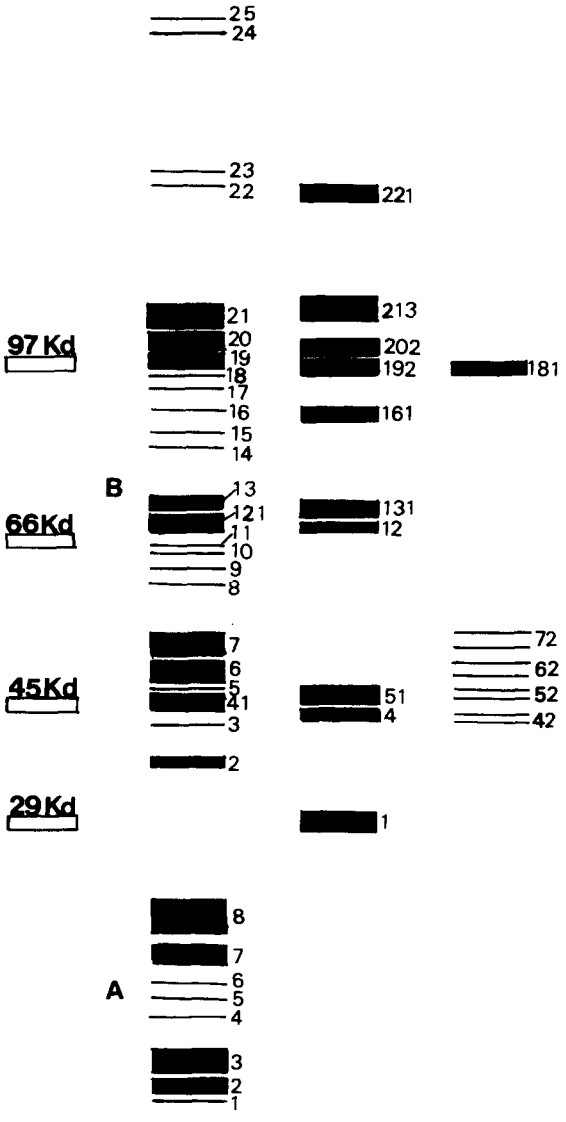

Fig. 3. Diagram of the alternative bands found in the SDS-PAGE fractionation patterns of total seed proteins extracted from the collection of cultivated and wild lentils.

Analysis of variation

The Spanish lentil germplasm collection examined represents a high level of genetic diversity. The variation which exists among phenotypes, loci and alleles was not uniform. Intravarietal diversity was higher in the cultivated populations than in the wild subspecies. Table 2 gives the data for the number of phenotypes observed, estimated mean number of loci and alleles and the percentage of uniform systems in both cultivated and wild accessions.

Table 2. Phenotypic and loci variability for the different systems in cultivated and wild subspecies of *Lens*

Systems	Num. patterns	Num. loci		% uniform accessions	
		Polym. (alleles)	Monom.	cult.	wild
Proteins	26	7 (2.1)	0	11.8	100
CPX	8	2 (4)	0	8.8	84.6
ACPH	9	2 (3.5)	0	17.6	100
MDH	5	3 (2.7)	2	32.3	100
GOT	9	2 (3.5)	2	38.2	100
LAP	4	1 (4)	0	50.0	100
6-PGD	3	1 (3)	1	88.3	100
PGM	1	0	2	100	100

The analysis for the 7 isozyme systems was carried out in the same samples within each population. This permitted associations between different isozyme phenotypes to be analysed. A multi-dimensional contingency table was made to compare the expected and observed values for each kind of association. The results are shown in Table 3. The expected values were estimated on the basis of the observed frequencies for each separate phenotypic system (maximum likelihood hypothesis). The total number of associations was 201, which is 28.79% of the associations theoretically possible. To determine the extension of the associations over groups of accessions, we estimated the expected values for the wild and cultivated populations, and the macrosperm and microsperm subgroups within cultivated forms. Comparison between observed

Table 3. The number of phenotypic associations for the isozyme systems in the groups of cultivated and wild subspecies of *Lens*

Groups of accessions	n° Associations observed	% respect to expected
Total	201	0.2879
Wild	15	0.0509
Cultivated	186	0.3662
-macrosperm	117	0.3066
-microsperm	89	0.2016

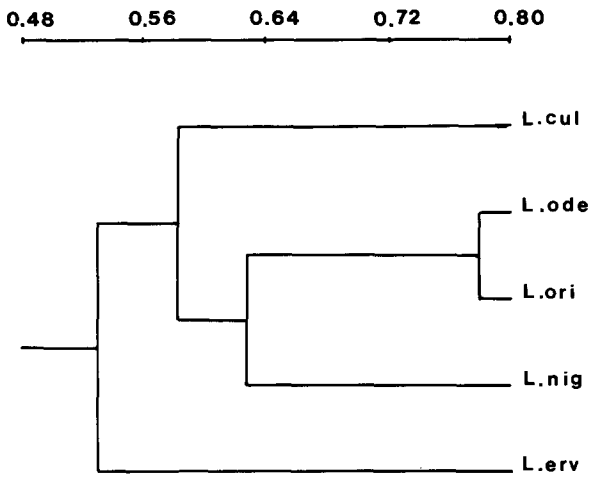

Fig. 4. Phylogenetic tree inferred from the isozyme analysis.

data and theoretical values revealed only a significant association between phenotypes with the allele *6pgd-p2* and the microsperm subgroup in the cultivated lentils.

Ladizinsky et al. (1984) reexamined the taxonomy of genus *Lens*, included in the tribe *Vicieae* of the family *Fabaceae*. The genus consists of two species and five subspecies: *Lens culinaris* Medik (ssp. *culinaris, orientalis* and *odemensis*) and *Lens nigricans* (ssp. *nigricans* and *ervoides*). The phylogenetic relationships between subspecies should perhaps be assessed by comparing the allele frequencies of isozymes in all accessions grouped by subspecies criteria. The cluster analysis dendrogram (Fig. 4) suggests that *orientalis* and *odemensis* share more biochemical phenotypes than the other subspecies. Moreover, the mentioned subspecies seem to keep an intermediate position between the two species *Lens culinaris* and *Lens nigricans*. These results support the phylogenetic position proposed by Havey & Muehlbauer (1989) who studied the genetic variability within and between the subspecies of genus *Lens* with RFLPs and demonstrated that estimates of gene variation based on RFLPs tended to be greater than estimates from isozymes.

References

Brever, C.J. & C.F. Sing, 1970. An introduction to isozyme techniques. Academic Press, New York.

Hayward, M.D., 1985. Isozymes as genetic markers for characterization of variability. Collection, characterization and utilization of genetic resources of temperate forage grass and clover. p. 54–57. In: B.F Tyler (Ed). IBPGR, Rome, Italy.

Havey, M.J. & F.J. Muehlbauer, 1989. Variability for restriction fragment lengths and phylogenies in lentil. Theor. Appl. Genet. 77: 839–843.

Ladizinsky, G., D. Brown & F.J. Muehlbauer, 1984. The biological species of the genus *Lens*. Bot. Gaz. 145: 253–261.

Muehlbauer, F.J. & A. Slinkard, 1981. Genetic and breeding methodology. In: C. Webb & G. Hawtin (Eds). Lentil Commonwealth Agricultural Bureau. London.

Muehlbauer, F.J., N.F. Weeden & D.L. Hoffman, 1989. Inheritance and linkage relationships of morphological and isozyme loci in lentil (*Lens* Miller). J. Hered. 80: 298–303.

Nielsen, G., 1984. The use of isozymes as probes to identify and label plant varieties and cultivars. p. 1–32 In: M.C. Razzati, J.G. Scandalios & G.S. Whitt (Eds). Isozymes: current topics in biological and medical research. Vol. 12: Academic Press.

Payne, P.I., K.G. Corfield, L.M. Holt & J.A. Blackman, 1981. Correlations between inheritance of certain high-molecular weight subunits of glutenin and bread making quality in progenies of six crosses of bread wheat. J. Sci. Food Agric. 32: 51–60.

Rao, I.N. & N.V.P. Rao, 1980. Evidence for duplicate genes coding for 6-phosphogluconate dehydrogenase in rye. Z. Pflanzenzüchtg. 89: 115–120.

Shaw, C.R. & A.L. Koen, 1968. Starch gel electrophoresis of isozymes. In: I. Smith (Ed). Chromatographic and electrophoretic techniques. Interscience, New York.

Weeden, N.F. & A.C. Emmo, 1985. Isozyme characterization of Kentucky bluegrass cultivars. Can. J. Plant Sci. 65: 985–994.

Weeden, N.F. & G.A. Marx, 1984. Chromosomal location of twelve isozyme loci in *Pisum sativum*. J. Hered. 73: 365–370.

Zamir, D. & G. Ladizinsky, 1984. Genetics of allozyme variants and linkage groups in lentil. Euphytica 33: 329–336.