B A
S E
Biotechnol. Agron. Soc. Environ. 1999 **3** (1), 10–27

Genetic control of isozymes in the primary gene pool *Phaseolus lunatus* L.

Irié Zoro Bi [1], Alain Maquet [2], Bernard Wathelet [3], Jean-Pierre Baudoin [2]

[1] Université d'Abobo. Adjamé, UFRSN, D2 BP 801 Abidjan 02 (Côte d'Ivoire).
[2] Unité de Phytotechnie des Régions intertropicales. Faculté universitaire des Sciences agronomiques de Gembloux. Passage des Déportés, 2. B–5030 Gembloux (Belgique). E-mail: phytotrop@fsagx.ac.be.
[3] Unité de Chimie biologique et industrielle. Faculté universitaire des Sciences agronomiques de Gembloux.

Received 20 May 1998, accepted 6 November 1998.

Suitable electrophoretic separation methods of 34 isozymes from 17 enzyme systems resolved in *Phaseolus lunatus* L. (Lima bean) were developed. Data from the migration of the staining zones indicated that three loci control EST, GPI, IDH and MDH, while two loci control ACO, ADH, DIA, G6PDH, PER, PGDH, and PGM. The difference between the stained zones intensity on gels assayed for GDH and SOD suggest that each of these enzymes is also controlled by two loci. A single locus controls END, β-GLU, LAP and SKDH. Based on the observed isozyme banding patterns, we inferred the quaternary structure of 11 enzyme systems. DIAisozymes were identified as tetrameric, ADH, fEST, GPI, MDH and PGDH as dimeric, and ACO, cEST, END, G6PDH, PGM and SKDH as monomeric. Allozyme polymorphisms of the resolved loci were estimated at both species and population levels. At species level, 74 percent of the analysed loci were polymorphic while 44 percent were polymorphic at population level.
Keywords. *Phaseolus lunatus* L., Lima bean, starch gel electrophoresis, enzyme systems, isozyme, allozyme, polymorphisms, linkage.

Déterminisme génétique des isozymes dans le pool génétique primaire de *Phaseolus lunatus* L. Des méthodes fiables de séparation électrophorétique de 34 isozymes issus de 17 systèmes enzymatiques ont été mises au point chez *Phaseolus lunatus* L. (haricot de Lima). La migration des zones d'activité enzymatique sur gel indique que trois loci contrôlent l'expression de EST, GPI, IDH et MDH, deux loci contrôlent l'expression de ACO, ADH, DIA, G6PDH, PER, PGDH et PGM. La différence entre l'intensité des zones de coloration pour l'activité enzymatique des GDH et SOD suggère que deux loci contrôlent également l'expression de chacun des deux systèmes enzymatiques. Un seul locus contrôle l'expression des END, β-GLU, LAP et SKDH. À partir des patrons électrophorétiques observés, la structure quaternaire de 11 systèmes enzymatiques a été inférée. Ainsi, les DIA ont été identifiés comme des enzymes tétramériques, ADH, fEST GPI, MDH et PGDH comme des enzymes dimériques, et ACO, cEST, END, G6PDH, PGM et SKDH comme des enzymes monomériques. Les polymorphismes allozymiques exprimés par les loci mis au point ont été évalués aussi bien au niveau de l'espèce qu'au niveau population. Au niveau de l'espèce, 74 pour cent des loci analysés étaient polymorphes alors que 44 pour cent étaient polymorphes au niveau population.
Mots-clés. *Phaseolus lunatus* L., haricot de Lima, électrophorèse sur gel d'amidon, système enzymatique, isozyme, allozyme, polymorphisme, liaison génétique.

1. INTRODUCTION

The ability to observe allelic variation at isozyme loci revolutionized the research in the field of biochemical genetics, population genetics and evolution (Weeden, Wendel, 1989; Crawford, 1990; Schaal *et al.*, 1991; Weir, 1996, p. 3). This variation, designated allozyme polymorphism, is useful to examine genetic processes at every stage of the life cycle and to ascertain genetic diversity in both plant and animal species. Nowadays, the technique is relatively straightforward and methods have been developed for many kinds of plant species (Hamrick, Godt, 1990). However, isozymes and their mode of inheritance have been studied only for a limited number of cultivated taxa, such as maize (Goodman *et al.*, 1980b), wheat (Jaaska, 1983), tomato (Rick, 1983), or common bean (Vallejos *et al.*, 1992; Freyre *et al.*, 1996). Lima bean (*Phaseolus lunatus* L.) is one of the plant species, for which little information is available on isozymes (Weeden *et al.*, 1989; Maquet, 1995). Up to now, a well-developed linkage map including biochemical or molecular

markers such as qualitative and quantitative trait loci, loci encoding storage proteins, isozymes, or restriction fragment length polymorphisms (RFLPs) are not yet available for the Lima bean. Such linkage maps are useful to provide guidelines for gene bank management and also to develop marker-based selection in plant breeding programmes (Kazan *et al.*, 1993). Inevitably, in the first instance, linkage map studies using molecular markers focus on two interdependent considerations that promote the efficient development of the target markers: selection of the electrophoresis methodology and genetic interpretation (Wendel, Weeden, 1989). An in-depth study of allozyme polymorphisms in Lima bean was initiated by Maquet *et al*. (1996). These authors postulated the genetic control and the quaternary structure of the analysed enzyme systems on the basis of those reported for *Phaseolus vulgaris* (Koenig, Gepts, 1989b). However, changes in genetic control of enzymatic systems can occur among species of the same genus. For example in the genus *Clarkia*, Soltis *et al*. (1987) observed two phosphoglucomutase (PGM, E.C. 5.4.2.2) isozymes in *C. concinna*, three in *C. breweri*, whereas they identified four PGM isozymes in *C. arcuata.* In the genus *Phaseolus*, using primary leaves and lithium-borate buffer, Schinkel and Gepts (1989) identified three leucine aminopeptidase (LAP, E.C. 3.4.11.1) isozymes in *P. acutifolius*, while only two were observed in *P. vulgaris* by Koenig and Gepts (1989a). Therefore, to infer the isozyme genetics of one species from those of another species could be misleading.

In order to take advantage of the isozyme markers potential for population genetic studies and plant breeding programmes, the knowledge of their inheritance is a prerequisite. Once the genetic control of enzyme systems is known, allozymes or isozymes can be designated more accurately.

One of the purposes of this paper was to describe thoroughly procedures currently used in our laboratory for horizontal starch gel electrophoresis in the primary gene pool *P. lunatus*, including preparation of extracts, electrophoretic buffer systems and specific staining assays for enzyme activity. It was intended as a complement to other descriptions that may be outdated or abbreviated (Wall, Wall, 1975; Bassiri, Adams, 1978; Weeden, 1984; Hussain *et al.*, 1988; Jaaska, Jaaska, 1988; Schinkel, Gepts, 1989; Weeden *et al.*, 1989). Another purpose is to analyse, gathering data from previous studies (Maquet, 1995; Zoro Bi *et al*., 1996; Zoro Bi *et al.*, 1997) and current results, the genetic aspects of allozyme variation in the indicated gene pool, including the quaternary structure of the resolved isozymes, the linkage relationships for certain loci and the polymorphisms at both species and population levels.

2. MATERIAL AND METHODS

2.1. Plant materials

Tissues from three plant organs (cotyledon, leaf and root) were used to perform resolution of the enzyme systems.

In order to identify polymorphic loci at species and population levels, we selected 17 accessions and three hybrid lines (F_2) obtained from the Centro Internacional de Agricultura Tropical (CIAT, Cali, Columbia) and six wild Lima bean populations from the Central Valley of Costa Rica (**Table 1**). The percentage of polymorphic loci (P) was estimated according to Hamrick and Godt (1990). Five to ten seeds were analysed per accession obtained from CIAT collection while five to 204 seeds from one to 37 plants selected in the six Costa Rican populations were examined.

Table 1. Sample size, biological status and origin of Lima bean accessions tested for allozyme polymorphisms — *Taille de l'échantillon, statut biologique et origine des introductions du haricot de Lima testées pour le polymorphisme des allozymes*.

Accession	Seed sample size	Biological status (**1**)	Origin
A45	5	H	-
A46	5	H	-
E28 (**2**)	10	Sil	Costa Rica
E76 (**2**)	58	Sil	Costa Rica
E88 (**2**)	151	Sil	Costa Rica
G20 (**2**)	10	Sil	Costa Rica
G25107	5	Lun	Brazil
G25221	10	Sil	Mexico
G25235	5	Lun	Argentina
G25298	5	Lun	Cuba
G25385	10	Lun	Costa Rica
G25388	5	Lun	Costa Rica
G25540	5	Lun	Argentina
G25551	5	Lun	Mexico
G25713	5	Sil	Mexico
G25881	5	Lun	Colombia
G25913	5	Sil	Peru
G25915	10	Sil	Peru
H95	10	H	-
HER30 (**2**)	5	Sil	Costa Rica
KM30 (**2**)	204	Sil	Costa Rica
S29705	5	Sil	Costa Rica
S31806	5	Wee	Peru
S32382	10	Lun	Ecuador
S32389	5	Sil	Ecuador
S32399	5	Sil	Ecuador

(**1**) H: hybrid line obtained from Ir. Gutierrez S. (CIAT, Cali, Colombia); Sil: wild form; Lun: landraces; Wee: Weedy form.
(**2**) Selected for polymorphism study at population level.

2.2. Enzyme assays

Analyses were carried out from four buffer systems (the discontinuous Lithium-borate/Tris-citrate, the discontinuous Citrate/Histidine, the continuous Histidine-citrate and the continuous Morpholine-citrate). For each buffer system, we tested several literature-cited pH values (Shaw, Prasad, 1970; Aebersold *et al.*, 1987; Wendel, Weeden, 1989; Murphy *et al.*, 1990; Kazan *et al.*, 1993), each value being combined with the three plant organs extracts and 31 enzyme systems (**Table 2**). Electrophoresis was performed using horizontal 10% starch-gel (Sigma # S-4501) containing 3% sucrose (Sigma # S-8501). Enzyme extraction was done by grinding on ice five-day old cotyledon tissues, young leaf or young root tissues in a potassium phosphate buffer, pH 7.0, containing 20% sucrose, 5% PVP-40, 0.1 M KH_2PO_4, 0.05% triton X-100 (Sigma # T-8532) and 14 mM 2-mercaptoethanol (Sigma # M-6250). The pH value was adjusted to 7.0 with a solution of 5 N NaOH. Triton X-100 and 2-mercaptoethanol were added just before use. Samples and extraction buffer were used in proportion (mg/mL) of 1/2 for cotyledon and leaf tissues and 1/1 for young root tissues. After extraction, samples were stored at – 20 °C until electrophoresis was carried out.

Table 2. Enzymatic systems assayed by electrophoresis of leaf, cotyledon and root tissues from Lima bean — *Systèmes enzymatiques testés par électrophorèse sur des tissus de feuille, cotylédon et racine du haricot de Lima.*

Enzyme	Abbreviation	IUBMB (**1**)	Section
Acid phosphatase	ACP	E.C. 3.1.3.2	Hydrolase
Aconitate hydratase	ACO	E.C. 4.2.1.3	Lyase
Alcohol dehydrogenase	ADH	E.C. 1.1.1.1	Oxidoreductase
α-Amylase	α-AMY	E.C. 3.2.1.1	Hydrolase
Ascorbate oxidase	ASCO	E.C. 1.10.3.3	Oxidoreductase
Aspartate aminotransferase	AAT	E.C. 2.6.1.1	Transferase
Diaphorase	DIA	E.C. 1.8.1.4	Oxidoreductase
Endopeptidase	END	E.C. 3.4.-.- (**2**)	Hydrolase
Esterases	EST	E.C. 3.1.1.- (**2**)	Hydrolase
Formate dehydrogenase	FDH	E.C. 1.2.1.2.	Oxidoreductase
Fumarate hydratase	FUM	E.C. 4.2.1.2	Aldolase
β-Galactosidase	β-GAL	E.C. 3.2.1.23	Hydrolase
Glucose-6-phosphate dehydrogenase	G6PDH	E.C. 1.1.1.49	Oxidoreductase
Glucose-6-phosphate isomerase	GPI	E.C. 5.3.1.9	Isomerase
Glutamate dehydrogenase	GDH	E.C. 1.4.1.2	Oxidoreductase
β-Glucosidase	β-GLU	E.C. 3.2.1.21	Hydrolase
Hexokinase	HEX	E.C. 2.7.1.1	Transferase
Isocitrate dehydrogenase	IDH	E.C. 1.1.1.42	Oxidoreductase
Lactate dehydrogenase	LDH	E.C. 1.1.1.27	Oxidoreductase
Leucine aminopeptidase	LAP	E.C. 3.4.11.1	Hydrolase
Malate dehydrogenase	MDH	E.C. 1.1.1.37	Oxidoreductase
Mannose-6- phosphate isomerase	MPI	E.C. 5.3.1.8	Isomerase
Peroxidase	PER	E.C. 1.11.1.7	Oxidoreductase
Phosphoglucomutase	PGM	E.C. 5.4.2.2	Isomerase
Phosphogluconate dehydrogenase	PGDH	E.C. 1.1.1.44	Oxidoreductase
Pyruvate kinase	PK	E.C. 2.7.1.40	Transferase
Shikimate dehydrogenase	SKDH	E.C. 1.1.1.25	Oxidoreductase
Succinate dehydrogenase	SUDH	E.C. 1.3.99.1	Oxidoreductase
Superoxide dismutase	SOD	E.C. 1.15.1.1	Oxidoreductase
Triose-phosphate isomerase	TPI	E.C. 5.3.1.1	Isomerase
Xanthine dehydrogenase	XDH	E.C. 1.1.1.204	Oxidoreductase

(**1**) Nomenclature of the International Union of Biochemistry and Molecular Biology (Webb, 1992).
(**2**) No specific enzyme.

To eliminate selectively overlapping bands from the cytoplasmic MDH, 8.3 % ascorbic acid (Sigma # A-7506) was added to the extraction buffer following the method of Goodman *et al.* (1980a). This method lowered the pH of the homogenisation solution to around pH 2.0.

Paper wicks (Whatman # 3) 0.5 cm × 1 cm were dipped into the raw extract, blotted on tissue paper to remove excess solution and inserted into the slice across the starch gel. The electrophoresis procedures followed the method described by Murphy *et al*. (1990).

To visualize the assayed enzyme systems activity, we tested histochemical staining reagents and procedures reported by Vallejos (1983), Hussain *et al*. (1988), Wendel and Weeden (1989) and Murphy *et al*. (1990).

Loci were labelled sequentially with those migrating closest to the anodal end designated as number 1. The first and the last stacks correspond to the accession G25221, a Mexican wild form, considered as a standard for our analysis. Allozymes from this genotype were designated 100 and all other allozymes were assessed according to their relative distance from the standard.

3. RESULTS AND DISCUSSION

3.1. Enzyme assays

Table 3 includes the electrophoresis buffers, electrophoretic conditions and plant organs for which the highest number of enzyme systems are observed. The selected histochemical staining methods are presented in **table 4**. From the 31 enzyme systems assayed, 17 showed suitable and reproducible resolution: ACO, ADH, DIA, END, EST, GDH, β-GLU, GPI, G6PDH, IDH, LAP, MDH, PER, PGDH, PGM, SKDH and SOD. The enzymatic systems ACP, α-AMY, ASCO, AAT, FDH, FUM, β-GAL, HEX, LDH, MPI, PK, SUDH, TPI and XDH did not express a good resolution in our conditions.

The electrophoretic patterns of the various enzyme systems were examined in extracts of cotyledon tissues since all of them expressed a good resolution with this organ. The observed zymogrammes will be presented separately.

Table 3. Factor combinations producing a reliable resolution of isozymes in three *P. lunatus* organs — *Combinaisons de facteurs produisant une résolution fiable des isozymes de trois organes de* P. lunatus.

Parameters/ organs	Buffer system I (1)	Buffer system II (2)	Buffer system III (3)
Voltage (constant)	10 V·cm^{-1}	4 V·cm^{-1}	10 V·cm^{-1}
Running time	5 h	17 h	5 h
Cotyledon	ADH, DIA, GPI, IDH, LAP, MDH, PGDH, PGM, SKDH, SOD	ACO, DIA, END, EST, G6PDH, GDH, PGM, SKDH, SOD	DIA, EST, β-GLU, PER
Leaf	ADH, MDH, PGM	G6PDH, SOD	PER
Root			PER

Note: The underlined enzyme systems are those selected in the conditions indicated for future evaluation.

(**1**) 0.065 M histidine-0.019 M citric acid, pH 6.1 (Kazan *et al.*, 1993) prepared by dissolving L-histidine (Sigma # H-8000) in distilled water and adjusting the pH with citric acid (Sigma # C-7129); the electrode buffer is the undiluted solution while gels are prepared with a 1:10 mixture of solution buffer system I:H_2O.

(**2**) 0.03 M lithium-0.19 M borate, pH 8.1/0.05 M Tris-0.008 M citrate, pH 8.4 (Murphy *et al.*, 1990) prepared as follows: stock solution A: a mixture of boric acid (Sigma # B-0252) and lithium hydroxide (Sigma # L4256), the pH is adjusted to 8.1; stock solution B: a mixture of Tris (Sigma # T-1503) and citric acid, the pH is adjusted to 8.4; the electrode buffer is the undiluted stock solution A while gels are prepared with a 1:9 mixture of stock solution A:B.

(**3**) 0.040 M citric acid-0.068 M morpholine, pH 6.1 (Wendel, Weeden, 1989) obtained by dissolving citric acid in distilled water, the pH value being adjusted to 6.1 with N-(3-Aminopropyl)-morpholine (Sigma # A-9028); the electrode buffer is the undiluted solution while gels are prepared with a 1:20 mixture of solution buffer system III:H_2O.

3.2. Monomorphic enzymes

Glutamate dehydrogenase (GDH) and superoxide dismutase (SOD) produced two monomorphic zones of activity with different intensities (**Figures 1a** and **1c** respectively). It was assumed that each zone is specified by one gene: *Gdh-1* and *Gdh-2* controlling GDH isozymes and *Sod-1* and *Sod-2* that determined SOD isozymes. Peroxidase (PER) displayed four to six staining bands according to tested genotypes (**Figure 1b**). However, four bands from which two migrated to the anodal side relative to the origin were reproducible. These results indicated that at least two genes controlled PER isozymes in Lima bean. It should be noted that PER activity was very sensitive to both concentration of chemicals and sample storage duration. Best staining zones intensity was obtained when sample extraction and electrophoresis were carried out the same day.

Table 4. Stain recipes and chemicals required for isozymes activity staining in *P. lunatus* organ tissues —

Enzyme	**Chemicals and stains involved (1)**	**Quantity**	**Reference (2)**
ACO (**3**)	0.2 M Tris-HCl, pH 8.0	100 mL	Modified
	0.43 M cis-aconitic acid (A-3412), pH 8.0	2 mL	from I
	50 units/mL IDH (I-2516)-0.25 M Glycine (G-8898), pH 7.4	1 mL	
	10 mg/mL β-NADP (N-0505)	1 mL	
	10 mg/mL MTT (M-2128)	1 mL	
	5 mg/mL PMS (P-9625)	1 mL	
ADH (**3**)	0.2 M Tris-HCl, pH 8.0	100 mL	I
	95% ethanol	5 mL	
	20 mg/mL β-NAD (N-0632)	0.5 mL	
	10 mg/mL MTT	1 mL	
	5 mg/mL PMS	1 mL	
DIA (**3**)	0.1 M Tris-HCl, pH 8.0	100 mL	III
	Menadione sodium bisulfite (M-5750)	50 mg	
	10 mg/mL β-NADH (N-8129)	2 mL	
	10 mg/mL MTT	1 mL	
END (**4**)	0.2 M Tris-0.2 M Malate, pH 5.5	100 mL	II
	0.1 M $MgCl_2 \cdot 6H_2O$	3.5 mL	
	BANA (B-4750) dissolved in 2 mLN,N-dimethylformamide (D-4254)	25 mg	
	Fast Black K salt (F-7253)	25 mg	
cEST (**4**) (**5**)	0.1 M Na-phosphate, pH 6.0	100 mL	III
	α-Naphtyl acetate (N-8505) dissolved in 2 mL acetone	25 mg	
	β-Naphtyl acetate (N-6875) dissolved in 2 mL acetone	25 mg	
	Fast Garnet GBC (F-0875)	50 mg	
fEST (**6**) (**7**)	Solution A:		III
	0.05 M Na-acetate buffer, pH 5.0	50 mL	
	Agar (A-7002)	400 mg	
	Solution B:		
	0.05 M Na-acetate buffer, pH 5.0	50 mL	
	4-Methylumbelliferyl acetate (M-0883) dissolved in 2 mL acetone	10 mg	
GDH (**3**)	0.1 M Tris-HCl, pH 8.0	100 mL	Modified
	L-Glutamic acid (G-1626)	250 mg	from III
	20 mg/mL β-NAD	2 mL	
	10 mg/mL MTT	1 mL	
	5 mg/mL PMS	1 mL	
β-GLU (**4**)	0.05 M Na-phosphate, pH 6.5	100 mL	III
	6-Bromo-2-naphtyl β-D-glucopyranoside (B-7877)	50 mg	
	PVP-40	1 g	
	Fast Blue BB salt (F-3378)	50 mg	
GPI (**3**)	0.1 M Tris-HCl, pH 8.0	100 mL	II
	0.1 M $MgCl_2 \cdot 6H_2O$	1 mL	
	D-Fructose 6-phosphate, Na_2 salt (F-3627)	10 mg	
	50 units/mL G6PDH (G-5885)-2 µg/mLBovine Serum Albumin		
	(Ac-BSA, B-8894)-0.005 M Glycine, pH 8.0	0.2 mL	
	10 mg/mL β-NADP	1 mL	
	10 mg/mL MTT	0.6 mL	
	5 mg/mL PMS	1 mL	
G6PDH (**3**)	0.1 M Tris-HCl, pH 8.0	100 mL	III
	0.1 M $MgCl_2 \cdot 6H_2O$	2.5 mL	
	50 mg/mL D-Glucose 6-phosphate, Na_2 salt (G-7250)	2 mL	
	10 mg/mL β-NADP	2 mL	
	10 mg/mL MTT	1 mL	
	5 mg/mL PMS	1 mL	
IDH (**3**)	0.2 M Tris-HCl, pH 8.0	100 mL	I
	0.1 M $MgCl_2 \cdot 6H_2O$	3 mL	
	0.1 mg/mL DL-Isocitric acid (I-1252)	0.8 mL	
	10 mg/mL β-NADP	1 mL	

Table 4. *Recettes de révélation et composés chimiques requis pour l'activité des isozymes de* P. lunatus.

Enzyme	Chemicals and stains involved (**1**)	Quantity	Reference (**2**)
	10 mg/mLMTT	0.5 mL	
	5 mg/mLPMS	1 mL	
LAP (**4**)	0.1 M KH_2PO_4 buffer, pH 6.0	100 mL	II
	0.1 M $MgCl_2 \cdot 6H_2O$	5 mL	
	L-leucyl-β-naphtylamide·HCl (L-0376) dissolved in 2 mL N,N-dimethylformamide	20 mg	
	Fast Black K salt (F-7253)	20 mg	
MDH (**3**)	0.1 M Tris Malate, pH 7.2	100 mL	II
	50 mg/mLDL-Malic acid (M-0750)	0.4 mL	
	20 mg/mL β-NAD	1 mL	
	10 mg/mLMTT	0.8 mL	
	5 mg/mLPMS	1 mL	
PER (**4**)	0.05 M Na-acetate buffer, pH 5.0	100 mL	III
	0.1 M $CaCl_2$	5 mL	
	3-Amino-9-ethylcarbazole (A-5754) dissolved in 2 mL N,N-dimethylformamide	25 mg	
	3% H_2O_2	0.25 mL	
PGDH (**3**)	0.2 M Tris-HCl, pH 8.0	100 mL	I
	0.1 M $MgCl_2 \cdot 6H_2O$	5 mL	
	20 mg/mL6-phosphogluconic acid, Na_3 salt (P-7877)	0.8 mL	
	10 mg/mL β-NADP	0.8 mL	
	10 mg/mLMTT	1 mL	
	5 mg/mLPMS	1 mL	
PGM (**3**)	0.1 M Tris-HCl, pH 8.0	100 mL	
	0.1 M $MgCl_2 \cdot 6H_2O$	5 mL	
	α-D-Glucose-1-phosphate, Na_2 salt (G-7000)	2 mL	
	50 units/mL G6PDH-2 µg/mLAc-BSA-0.005 M Glycine, pH 8.0	0.8 mL	
	10 mg/mL β-NADP	0.8 mL	
	10 mg/mLMTT	0.8 mL	
	5 mg/mLPMS	0.8 mL	
SKDH (**3**)	0.1 M Tris-HCl, pH 8.5	100 mL	III
	Shikimic acid (S-5375)	50 mg	
	10 mg/mL β-NADP	2 mL	
	10 mg/mLMTT	1 mL	
	5 mg/mLPMS	1 mL	
SOD (**8**)	Solution A:		IV
	0.05 M Na-phosphate, pH 7.5	100 mL	
	10 mg/mLMTT	1 mL	
	Solution B:		
	0.05 M Na-phosphate, pH 7.5	100 mL	
	TEMED (T-3133)	0.2 mL	
	Riboflavin (R-4500)	1 mg	

(**1**) References given for the chemicals are those of Sigma products. (**2**) I: Murphy *et al.* (1990); II: Hussain *et al.* (1988); III: Wendel and Weeden (1989); IV: Vallejos (1983). (**3**) Incubate at 37° C in the dark until sufficient bands appear, fix bands using 40% glycerol for about 45 min and conserve gels in 50% ethyl alcohol. (**4**) Incubate at 37° C in the dark until sufficient bands appear, fix bands using an ethanol:acetic acid:water 25:7:68 solution for about 1 h and conserve gels in 50% ethyl alcohol. (**5**) Colorimetric esterase. (**6**) Fluorimetric esterase. (**7**) Bring solution A to boil. Cool to 60 °C. Gently mix in solution B and pour on the gel. Allow agar to set and view under long wave UV (370 nm). (**8**) Soak the gel in solution A in the dark at 37° C for about 20 min. Then, drain of solution A and pour in solution B and incubate under illumination until achromatic bands are distinguishable. Fix bands using 40% glycerol for about 45 min and conserve gels in water.

3.3. Polymorphic enzymes

3.3.1. Aconitate hydratase. Two zones of activity considered as products of two loci (*Aco-1* and *Aco-2*) were observed (**Figure 2**). At *Aco-1*, no polymorphism was observed while three alleles producing the allozyme phenotypes ACO-2^{91}, ACO-2^{100} and ACO-2^{112} were identified at *Aco-2*. Heterozygous genotypes possessed a double-banded allozyme phenotype (i. e. **figure 2**, lane 4), indicating the monomeric nature of this enzymatic system. Similar results have been reported in the genus *Sorghum* (Morden *et al.*, 1988) and in *Cicer arietinum* L. (Kazan *et al.*, 1993). These authors observed two aconitase loci, *Aco-1* and *Aco-2*. The fast migrating ACO-1 showed activity in the cytosolic fraction whereas ACO-2 was associated with the mitochondrial extracts. Experiments determining the subcellular location of the aconitase isozymes from Lima bean should clarify our results since the more polymorphic isozymes are usually reported as cytoplasmic products (Weeden, Gottlieb, 1980b).

3.3.2. Alcohol dehydrogenase. ADH zymogrammes are shown in **figure 3**. Gels stained for this enzyme system displayed three zones of activity for double homozygous genotypes. Both the band closest to the anode (ADH-1) and the one that migrated closest to the cathode (ADH-2) showed two allelic variants. The intermediate band migrated closely with both ADH-1 and ADH-2. This band could be the product of an intergenic reaction. Three isozyme phenotypes were observed for each locus (two alternative homozygous phenotypes and one heterozygous phenotype). The observed ADH banding patterns were similar to those found in *Pennisetum glaucum* (L.) R.Br (Banuett-Bourrillon, Hague, 1979), *Camellia* (Wendel, Parks, 1984), *Cicer arietinum* (Tuwafe *et al.*, 1988) as well as *P. coccineus*, and *P. vulgaris* L. (Wall, Wall, 1975). Segregation data obtained from the study of Zoro Bi *et al.* (1997) using four families analysed for *Adh-2* were in agreement with the expected 1:2:1 ratios. These results suggested that two loci (*Adh-1* and *Adh-2*) were coding for ADH isozymes expressed in *P. lunatus*. Each of the two loci showed two codominant alleles: *Adh-*1^{100} and *Adh-*1^{116} for *Adh-1* and *Adh-*2^{61} and *Adh-*2^{100} for *Adh-2*. Each of these genes coded for a functionally dimeric protein. In a double heterozygous genotype six of the nine bands observed represented four parental homodimers (two from *Adh-1* and two from *Adh-2*) and two intermediate heterodimers resulting from the random dimerisation of allelic products of both genes. The three remaining bands were the product of intergenic dimerisations. This pattern was in agreement with the dimeric quaternary structure of the ADH of maize suggested by Schwartz and Endo (1966). Single locus segregation analysis of *Adh-1* allelic variants and joint segregation tests of the two ADH loci are necessary to carry out the linkage analysis between them.

3.3.3. Diaphorase. Seven bands were observed on gels stained for DIA but five were characteristic for homozygous individuals (**Figure 4**, lane 2). Similar patterns were also observed when 2,6-Dichloroindophenol (Sigma # D-1878) was substituted in place of menadione sodium bisulfite (Sigma # M-5750). From the five reproducible bands, the most anodal and the most cathodal migrated independently. These bands were considered as the products of two genes, *Dia-1* and *Dia-2*. The three intermediate bands migrated closely with those from both *Dia-1* and *Dia-2*. We considered that these intermediate migrating bands represented the products of intergenic reactions. Four allozyme phenotypes were identified at *Dia-1* (null, DIA-1^{91}, DIA-1^{98} and DIA-1^{100}). At *Dia-2*, three allozyme phenotypes were detected (DIA-2^{100}, DIA-2^{173} and DIA-2^{200}). It should be noted that **figure 4** does not include DIA-2^{200}. Heterozygous individuals DIA-$1^{98/100}$ were observed but the relative migration rate of the allelic products from such individuals was so small that bands from interallelic reactions were not clearly identified. The results showed that *P. lunatus* carried two DIA genes (*Dia-1* and *Dia-2*) which produced polypeptide subunits that interacted to form the functional tetrameric DIA isozymes. These subunits combined freely to produce two intragenic homotetramers and three intergenic heterotetramers in homozygous plants so that homozygous genotypes produced 5 bands on gels. The same results were reported for barley (Brown *et al.*, 1978), *P. vulgaris* (Weeden, 1984; Sprecher, 1988; Brothers, Kelly, 1993) and *P. acutifolius* (Schinkel, Gepts, 1989).

3.3.4. Endopeptidase. Gels stained for END activity exhibited a single band for homozygous individuals and two bands for heterozygous individuals (**Figure 5**), suggesting that one locus (*End*) with three codominant alleles (End^{84}, End^{87} and End^{100}) control monomeric END enzymes in *P. lunatus*. Similar results were reported for soybean (Griffin, Palmer, 1987).

3.3.5. Esterase. Both colorimetric esterase and fluorimetric esterase (cEST and fEST) were assayed. Four zones of activity were detected on gel stained for cEST but only two revealed a reliable resolution (**Figure 6a**). We considered that these two staining zones represented products of two loci designated *cEst-2* and *cEst-4*. Polymorphisms were observed at both *cEst-2* and *cEst-4* loci. Two allelic variants that produced the two allozyme phenotypes cEST-2^{100} and cEST-2^{107} were detected at *cEst-2*. At *cEst-4*, the two

alleles *cEst-4*^{84} (not presented here) and *cEst-4*^{100} were identified. Heterozygous individuals with a two-banded isozyme phenotype (cEST-2$^{100/107}$) were detected at *cEst-2* locus, demonstrating the monomeric quaternary structure of cEST in Lima bean. Similar results were reported for several *Leguminosae*, for example *P. coccineus* and *P. vulgaris* (Wall, Wall, 1975) and *C. arietinum* (Kazan *et al.*, 1993).

fEST isozyme banding patterns are presented in **figure 6b**. Two zones of fEST activity were observed but only the most cathodal one was reproducible. This band was considered as the product of one locus (*fEst-2*), the non-reproducible band being from another locus (*fEst-1*). Two alleles were identified at *fEst-2*: *fEst-2*^{100} and *fEst-2*^{110}. Occasionally, bands representing the allozyme fEST-2^{100} were accompanied by faint bands slightly cathodal (**Figure 6b**, lane 3). These bands were considered as artefacts. fEST genes highlighted in *P. lunatus* encoded dimeric products. Such results were reported from several studies (Weeden, Marx, 1984; Aebersold *et al.*, 1987; Weeden, Wendel, 1989; Murphy *et al.*, 1990).

3.3.6. Glucose-6-phosphate isomerase. Four zones of activity were observed for GPI (**Figure 7**). The most anodal band (a) was polymorphic and co-migrated with band b. Absence of band c for some individuals (data not shown here), could be explained by a locus possessing a null allele. The most cathodal band (band d) was monomorphic. All these results suggested that GPI isozyme banding patterns from Lima bean represent products of three genes: *Gpi-1*, *Gpi-2* and *Gpi-3*. The two groups of GPI isozymes reported by Weeden and Lamb (1987) for apple and Weeden *et al.* (1989) for several *Leguminosae* including *P. lunatus* were identified in this study: those localised in the cytosol compartment (GPI-1 and GPI-2) and the plastidal isozyme (GPI-3). Both GPI-1 and GPI-2 appeared to be dimeric enzymes, generating a triple-banded phenotype, the intermediate band (band b) representing an intergenic heterodimer. Subunits of GPI-3 did not form active heterodimers either with GPI-1 or with GPI-2 subunits *in vivo*. Only *Gpi-1* was polymorphic generating two alleles: *Gpi-1*^{96} and *Gpi-1*^{100}. No heterozygous individuals possessing a triple-banded allozyme phenotype confirming the dimeric hypothesis were detected for identified loci. Nevertheless, it has been widely demonstrated that GPI isozymes are dimeric (Guries, Ledig, 1978; Harry, 1986; Weeden, Lamb, 1987).

3.3.7. Glucose-6-phosphate dehydrogenase. There were two polymorphic zones of activity on gels stained for G6PDH (**Figure 8**). Electrophoretic variants at the anodal staining zone segregated independently relative to those of the cathodal staining zone, suggesting that two loci control G6PDH isozymes: *G6pdh-1* and *G6pdh-2*. Two alleles were identified at *G6pdh-1*, producing the two allozyme phenotypes G6PDH-1^{95} and G6PDH-1^{100}. At *G6pdh-2*, three alleles that expressed the three allozyme phenotypes G6PDH-1^{95}, G6PDH-1^{100} and G6PDH-1^{110} were detected. At both loci, double-banded isozyme phenotypes representing heterozygous individuals were observed (i.e. **Figure 8**, lane 5 and lane 7). These results indicated that Lima bean G6PDH isozymes have a monomeric quaternary structure. According to Murphy *et al.* (1990), several quaternary structures have been reported for G6PDH. We observed that in Lima bean, G6PDH isozyme banding patterns appeared to be influenced by the physiological behaviour of the analysed seeds, namely their capacity to germinate. Thus G6PDH banding patterns were not so easy to score, and the hypothesis proposed might not be sufficient to explain the effective genetic inheritance mode of this enzyme system.

3.3.8. β-Glucosidase. Gels stained for β-GLU activity showed a single band with two electrophoretic variants (**Figure 9**). It was suggested that β-GLU enzyme system is controlled by one locus (β-*Glu*) with two codominant alleles expressing two allozymes: β-GLU^{100} and β-GLU^{125}. No heterozygous individuals were identified so that we could not suggest a hypothesis concerning the quaternary structure of this enzyme. Nevertheless, the dimeric nature of β-GLU was reported in maize (Stuber *et al.*, 1977). Like PER, β-GLU activity was very sensitive to concentration of chemicals and sample storage duration (see **figure 9**, lane 2 and lane 3 for illustration). Best staining zones intensity was obtained when sample extraction and electrophoresis were carried out the same day.

3.3.9. Isocitrate dehydrogenase. Three zones of activity were revealed, two zones with faint activity and one zone with strong activity (**Figure 10**). One of the two faint zones, the most anodal one showed a shadow band. From the three zones of activity, the two most anodal ones showed variation and were interpreted as products of two loci (*Idh-1* and *Idh-2*) each with two codominant alleles encoding allozymes phenotypes IDH-1^{100} and IDH-1^{120} for *Idh-1* (presented in Maquet, 1995) and IDH-2^{75} and IDH-2^{100} for *Idh-2*. Allozyme phenotype IDH-2^{75} overlapped the product of *Idh-3* (**Figure 10**, lanes 3, 4 and 5). The most cathodal migrating band was invariable and so far has been assumed to be determined by one locus, *Idh-3*. We could not highlight the quaternary structure of IDH isozymes but this enzyme system has been identified as dimeric

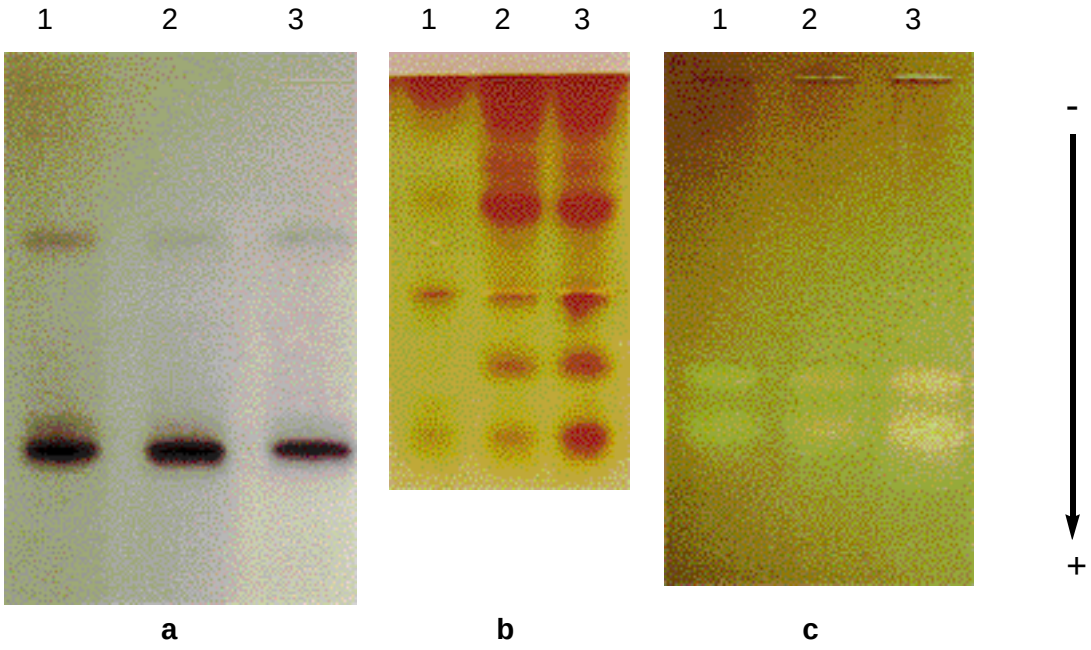


Figure 1. Zymogrammes of three monomorphic enzyme systems from Lima bean — *Zymogrammes de trois systèmes enzymatiques monomorphes du haricot de Lima.*
a. Glutamate dehydrogenase (GDH).
b. Peroxidase (PER).
c. Superoxide dismutase (SOD).

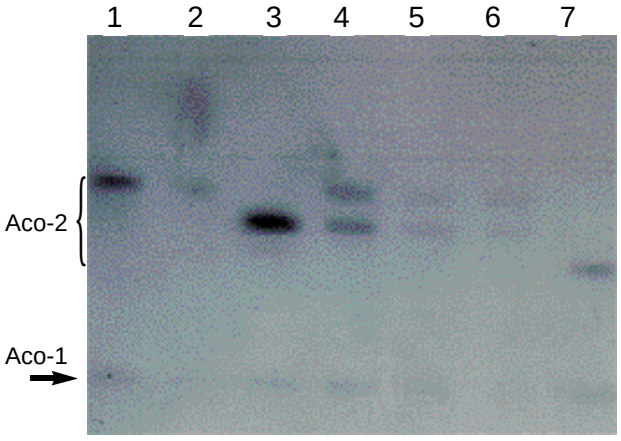


Figure 2. Aconitate hydratase (ACO) zymogrammes from Lima bean — *Zymogrammes de l'aconitate hydratase (ACO) du haricot de Lima*.
Lanes 1 and 2: ACO-$1^{100/100}$//ACO-$2^{91/91}$;
lane 3: ACO-$1^{100/100}$//ACO-$2^{100/100}$;
lanes 4, 5 and 6: ACO-$1^{100/100}$//ACO-$2^{91/100}$;
lane 7: ACO-$1^{100/100}$//ACO-$2^{112/112}$.

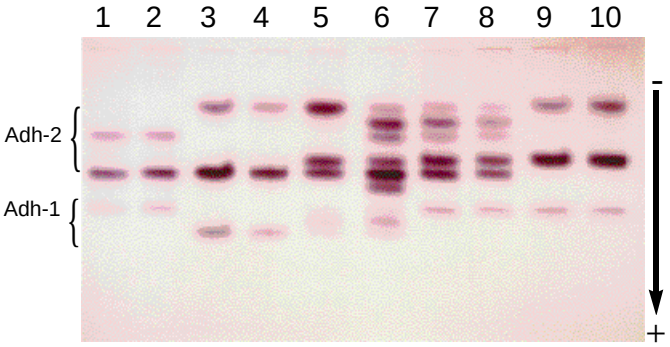


Figure 3. Alcohol dehydrogenase (ADH) zymogrammes from Lima bean (Zoro Bi *et al.*, 1997) — *Zymogrammes de l'alcool déshydrogénase (ADH) du haricot de Lima*.
Lanes 1 and 2: ADH-$1^{100/100}$//ADH-$2^{100/100}$;
lanes 3 and 4: ADH-$1^{116/116}$//ADH-$2^{61/61}$;
lane 5: ADH-$1^{100/116}$//ADH-$2^{61/61}$;
lane 6: ADH-$1^{100/116}$//ADH-$2^{61/100}$;
lanes 7 and 8: ADH-$1^{100/100}$//ADH-$2^{61/100}$;
lanes 9 and 10: ADH-$1^{100/100}$//ADH-$2^{61/61}$.

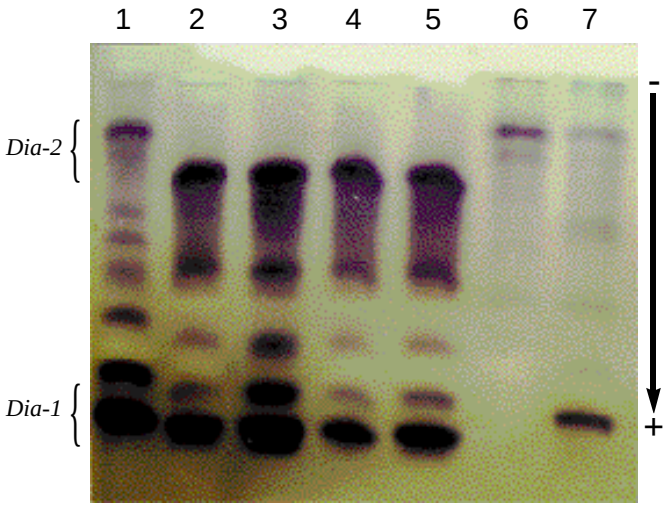


Figure 4. Diaphorase (DIA) zymogrammes from Lima bean — *Zymogrammes de la diaphorase (DIA) du haricot de Lima.*
Lane 1: DIA-1$^{98/98}$//DIA-2$^{100/100}$;
lanes 2, 3, 4 and 5: DIA-1$^{100/100}$//DIA-2$^{173/173}$;
lane 6: DIA-1$^{-/-}$//DIA-2$^{100/100}$;
lane 7: DIA-1$^{91/91}$//DIA-2$^{100/100}$.

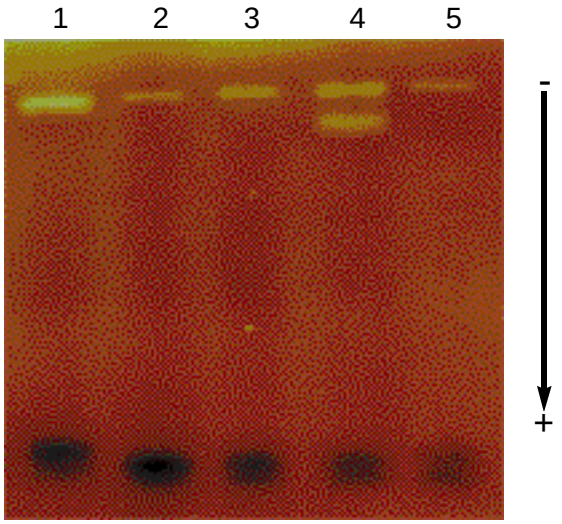


Figure 5. Endopeptidase (END) zymogrammes from Lima bean — *Zymogrammes de l'endopeptidase (END) du haricot de Lima*.
Lane 1: END$^{87/87}$;
lane 2: END$^{100/100}$;
lanes 3, 4, and 5: END$^{87/100}$.

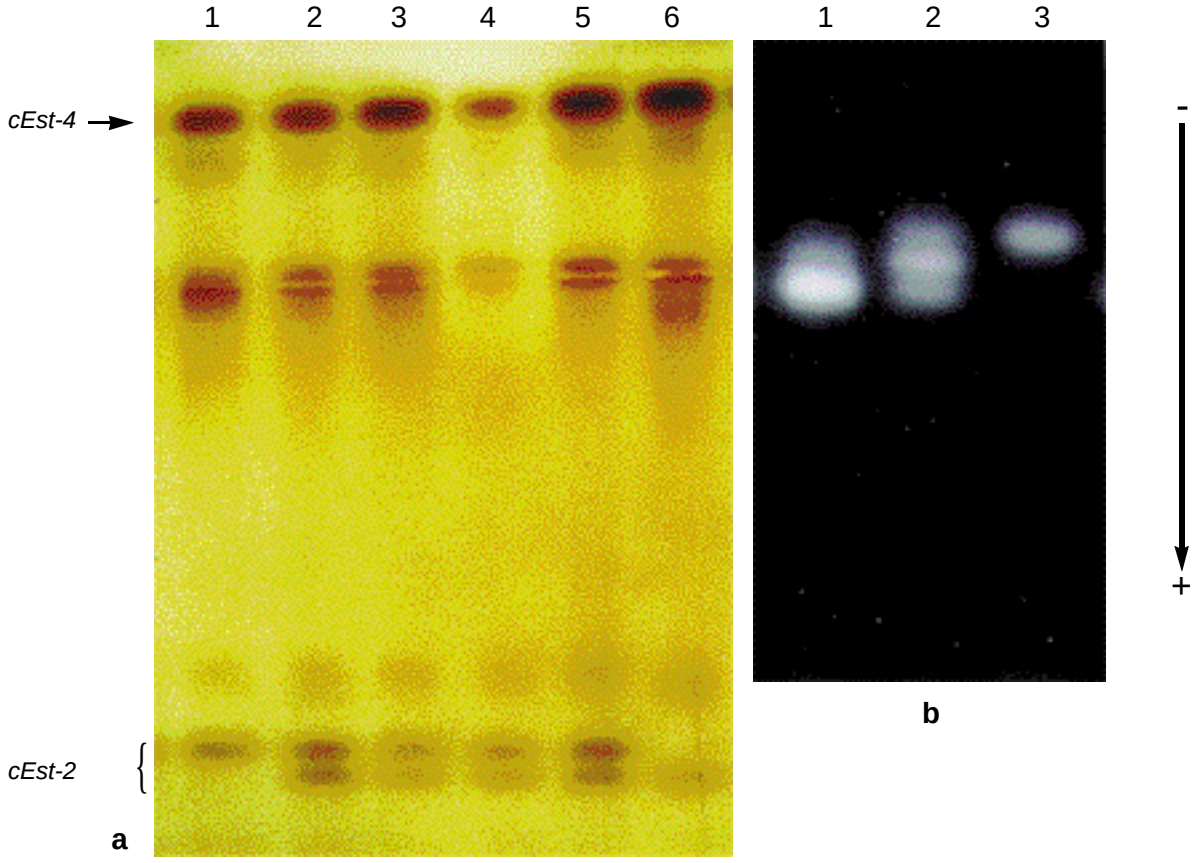


Figure 6. Esterase (EST) zymogrammes from Lima bean — *Zymogrammes de l'estérase (EST) du haricot de Lima*.
a. Colorimetric esterase (cEST).
Lane 1: cEST-2$^{100/100}$//cEST-4$^{100/100}$;
lanes 2, 3, 4 and 5: cEST-2$^{100/107}$//cEST-4$^{100/100}$;
lane 6: cEST-2$^{107/107}$//cEST-4$^{100/100}$.
b. Fluorimetric esterase (fEST).
Lane 1: fEST-2$^{110/110}$;
lane 2: fEST-2$^{100/110}$;
lane 3: fEST-2$^{100/100}$.

proteins (Guries, Ledig, 1978; Kiang, Gorman, 1985; Weeden, Lamb, 1987; Simonsen, Heneen, 1995).

3.3.10. Leucine aminopeptidase. Asingle polymorphic zone of activity was revealed for LAP (**Figure 11**). Four allelic variants generating four allozyme phenotypes were observed at *Lap* locus (but only three are presented in this paper). No heterozygous individuals were observed so LAP isozymes quaternary structure could not be inferred. Nevertheless, the dimeric nature of this enzyme system has been demonstrated with *Pisum sativum* (Scandalios, Espiritu, 1969) and *Abies balsamea* (Neale, Adams, 1981).

3.3.11. Malate dehydrogenase. MDH banding patterns are shown in **figure 12a**. Although several bands were observed, five of these bands were characteristic for homozygous individuals. From these five bands, some were weakly stained: bands 1a, 1b, and 2e (**Figure 12a**). The most cathodal band resulting from the third locus (*Mdh-3*) showed polymorphism generating two electrophoretic variants: MDH-3^{100} and MDH-3^{104} (presented in Maquet, 1995). The three intermediate bands migrated closely. One of these bands, the most anodal one, was considered as the product of *Mdh-2*, the two remaining bands being the products of other loci or shadow bands. *Mdh-1* locus showed two alleles (*Mdh-1*^{84} and *Mdh-1*^{100}) and two allozyme phenotypes: MDH-1^{84} and MDH-1^{100}. *Mdh-2* also showed two alleles (*Mdh-2*^{100} and *Mdh-2*^{140}) but three allozyme phenotypes: MDH-2^{100}, MDH-$2^{100/140}$, and MDH-2^{140}. The band representing the product of allele *Mdh-2*^{140} overlapped the one from the second allele (*Mdh-1*^{84}). Preliminary experiments using extraction buffer containing ascorbic acid suggested that isozymes from *Mdh-1* and *Mdh-2* have a cytoplasmic location, while isozymes from *Mdh-3* appeared to be localised in mitochondria. Samples homogenised in our standard extraction buffer showed both forms, while when homogenisation took place in a solution with a low pH, only the activity of allozymes from *Mdh-3* was observed (**Figure 12b**). The weak staining and nonreproducible bands, not interpreted in our study, might result from isozymes located in other cellular organelles such as peroxisomes and chloroplasts which were already reported by Gietl (1992). Such hypothesis is supported by the fact that isozymes located in separate compartments are usually encoded by different genes and tend to be distinctive in their electrophoretic mobility and biochemical properties, including kinetic parameters and inhibition by various metabolites (Newton, 1983). Two bands co-migrated with the one from *Mdh-2*. These could be shadow bands or the products of linked loci, so the dimeric nature of this enzyme was not clearly established. Nevertheless assuming the existence of two shadow bands, then 2b and 2c could be artefacts of 2a considered as a first homodimer (lanes 7 and 8 of **figure 12a**). In lanes 1 to 5 of **figure 12a** the 2c band has the same intensity as 2a in lanes 7 and 8 and could represent a second homodimer with 2d and 2e as artefacts. In the zymogramme of the heterozygous genotype (lane 6 of **figure 12a**) the second homodimer and the shadow band of the first homodimer are overlapping in band 2c. The dimeric quaternary structure of MDH is widely reported (Goodman *et al.*, 1980a; Weeden, Wendel, 1989; Murphy *et al.*, 1990). However, the absence of an intermediate band between the bands of the products of *Mdh-1* and *Mdh-2* (lanes 1 to 2 of **figure 12a**) suggested that there is no enzymatically active heterodimer, although both products seem to be located in the same cellular compartment (**Figure 12b**). Crosses between plants having the two alternative allelic variants of *Mdh-1* and their segregation analysis will provide more information on the genetic control of MDH isozymes in *P. lunatus*. *Mdh-2* locus segregation in five wild Lima bean families was carried out previously (Zoro Bi *et al.*, 1997). All families analysed for *Mdh-2* showed a significant deviation from a normal Mendelian ratio. These distorted ratios resulted from a deficiency of genotypes designated MDH-$2^{140/140}$. Such a distortion could be explained by two main factors: the gametophytic selection either through pollen competition or as a result of linkage to a self-incompatibility locus (Mulcahy, Mulcahy, 1983; Wendel, Parks, 1984) and the selective loss of some genotypes through zygotic abortion (Zamir, Tadmor, 1986). In cultivated Lima bean, Allard (1963) reported a gametophytic selection through pollen competition.

3.3.12. Phosphogluconate dehydrogenase. Two zones were revealed, one with faint activity and one with strong activity (**Figure 13**). Both anodal and cathodal zone expressed the variation expected for dimeric enzymes with triple-banded phenotype for heterozygous individuals (i.e. **Figures 13a** and **13b**, lane 3). Each of the two staining zones was interpreted as the product of one locus, *Pgdh-1* with two codominant alleles and *Pgdh-2* with four alleles. Similar results have been reported for *Pisum sativum* L. (Weeden, Marx, 1984), incense-cedar (Harry, 1986) and *C. arietinum* (Tuwafe *et al.*, 1988; Kazan *et al.*, 1993).

3.3.13. Phosphoglucomutase. Two zones of activity were detected on the gels stained for PGM (**Figure 14**, p. 26). In both of these zones, individuals showed

either a single or two bands. Two alleles were observed at *Pgm-1*: *Pgm-1*^{95} and *Pgm-1*^{100}. At *Pgm-2*, we identified three alleles designated as *Pgm-2*^{85}, *Pgm-2*^{100} and *Pgm-2*^{110}. Genotypes heterozygous at both loci possessed double-banded isozyme phenotypes. For PGM, no intergenic or interallelic products were detected. These observations are in accordance with the monomeric quaternary structure of PGM isozymes. *Pgm-2* locus segregation has been investigated in previous studies using four wild Lima bean families (Zoro Bi *et al.*, 1997). All families analysed for *Pgm-2* fitted with 1:2:1 ratios. These results confirmed that two loci (*Pgm-1* and *Pgm-2*) code for the PGM expressed in Lima bean. Similar PGM zymogrammes banding patterns were widely reported (Harry, 1986; Shore, Barrett, 1987; Weeden, Wendel, 1989).

3.3.14. Shikimate dehydrogenase. Two zones of activity which migrated closely were observed on gels stained for SKDH when samples from homozygous individuals were analysed (**Figure 15**, p. 26, lanes 1, 5, 6). Whether both zones were controlled by the same locus, *Skdh* (in this case one of the two bands could be a shadow band) or whether they represented two tightly linked loci (*Skdh-1* and *Skdh-2*) remained unknown. Two allelic variants producing allozyme phenotypes SKDH^{100} and SKDH^{110} were identified. Triple-banded isozyme phenotype was observed for heterozygous individuals (i.e. **Figure 15**, lanes 2, 3 and 4), due to the fact that the fast migrating band from a homozygous individual SKDH$^{100/100}$ and the slower migrating band from the alternative homozygous individuals (SKDH$^{110/110}$) overlapped. Such isozyme banding patterns were in accordance with a monomeric quaternary structure. We suggest that this enzyme system is determined by one locus (*Skdh*) or two tightly linked loci (*Skdh-1* and *Skdh-2*) with codominant alleles. Weeden and Gottlieb (1980a) highlighted two forms of SKDH in *Clarkia williamsonii* Lewis and Lewis: a chloroplastic and a cytosolic form. However the linkage relationships between loci expressing these two forms of isozyme was not investigated.

3.3.15. Polymorphism study. The number of loci for selected enzyme systems, locus designation, isozymes migration orientation, observed alleles and percentage of polymorphic loci (P) at both species and population levels are grouped in **table 5,** p. 24. Estimates of allozyme variation at both species (P = 74) and population (P = 44) levels are similar to those reported for 473 species and 468 populations (Hamrick, Godt, 1990). An analysis of linkage relationships between locus *Adh-2*, *Mdh-2* and *Pgm-2* has been investigated elsewhere (Zoro Bi *et al.*, 1997). The results indicated that these loci were independently inherited: the recombination fraction between locus pairs varies from 0.417 (for *Mdh-2*/ *Pgm-2*) to 0.443 (for *Adh-2*/*Mdh-2*).

4. CONCLUSIONS AND PROSPECTS

In this paper, emphasis was placed on starch gel electrophoresis of enzymes from simply prepared tissue homogenates, because these techniques are the most practical ones and are frequently applied in studies requiring scoring of several loci in large numbers of individuals. The described procedures were optimised for cost-effectiveness, ease of implementation and resolution. Other laboratories may effectively follow procedures that differ from ours.

Our data contribute to better understanding the genetic basis of isozyme polymorphisms in the primary gene pool of *P. lunatus*.

Approximately 74% of the 34 enzyme loci in Lima bean were determined as polymorphic. A total of 72 alleles were scored at these 34 loci. These allelic data will help to estimate the genetic variation within and among *P. lunatus* accessions or populations. However, further efforts should be devoted to analysing additional accessions and natural populations from the centres of diversity of this food legume.

Given the complex patterns of certain enzymes (i.e. DIA, MDH and G6PDH), genetic tests from segregation data and linkage relationships between polymorphic loci should be investigated to check current results (Bailey, 1961).

To evaluate more accurately Lima bean genetic resources, further techniques using nuclear markers, like microsatellites (Schaal *et al.*, 1991) should be helpful.

Acknowledgment

This study is financed by the Belgian Administration of Cooperation for Development (AGCD., Brussels, Belgium) and supervised by the International Plant Genetic Resources Institute (IPGRI, Rome, Italy). We thank Ir. Sandrine Henry (Laboratoire de Chimie générale et biologique, FUSAGx, Belgium) and Dr. Assié Lazare Komenan (Laboratoire de Zoologie générale et appliquée, FUSAGx, Belgium) for their technical assistance. We are grateful to Mrs Moreman (Bibliothèque Centrale, FUSAGx, Gembloux, Belgium) for checking the English style. Scholarship of the first author is provided by the Scientific Research Ministry of Ivory Coast.

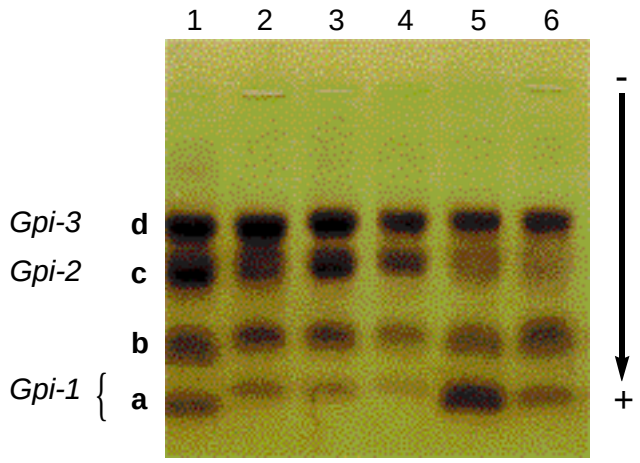


Figure 7. Glucose-6-phosphate isomerase (GPI) zymogrammes from Lima bean — *Zymogrammes de la glucose-6-phosphate isomérase (GPI) du haricot de Lima.*
Lanes 1, 5 and 6: GPI-1$^{100/100}$//GPI-2$^{100/100}$//GPI-3$^{100/100}$;
lanes 2, 3, and 4: GPI-1$^{96/96}$//GPI-2$^{100/100}$//GPI-3$^{100/100}$.

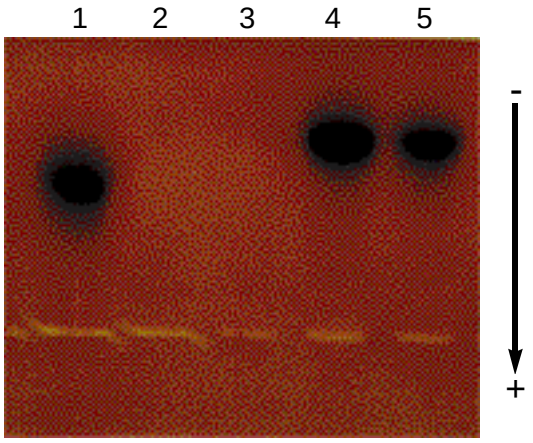
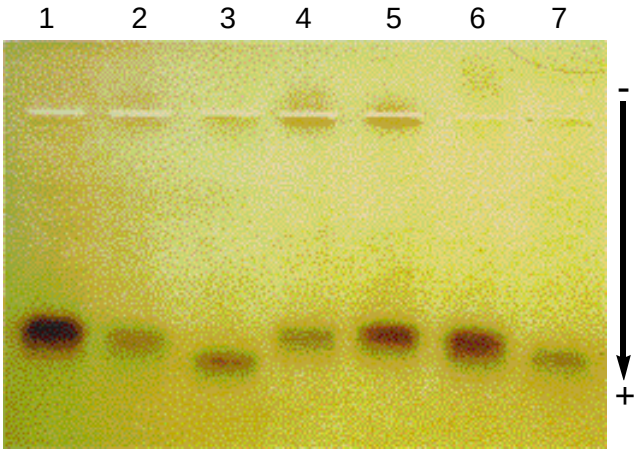


Figure 9. β-Glucosidase (β-GLU) zymogrammes from Lima bean — *Zymogrammes de la β-Glucosidase (β-GLU) du haricot de Lima*.
Lane 1: β-GLU $^{100/100}$;
lanes 2 and 3: -;
lanes 4 and 5: β-GLU $^{125/125}$.



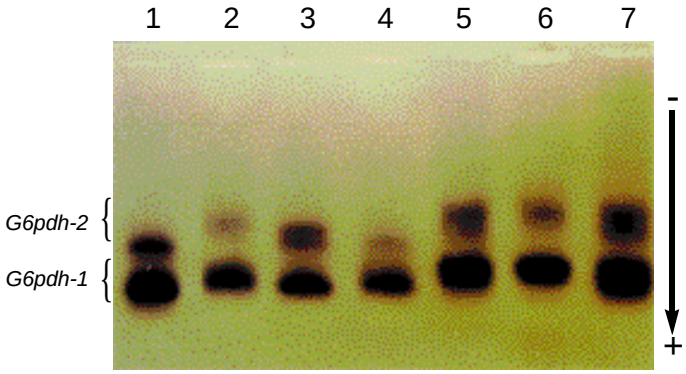


Figure 8. Glucose-6-phosphate dehydrogenase (G6PDH) zymogrammes from Lima bean — *Zymogrammes de la glucose-6-phosphate déshydrogénase (G6PDH) du haricot de Lima*.
Lanes 1 and 4: G6PDH-1$^{100/100}$//G6PDH-2$^{110/110}$;
lane 2: G6PDH-1$^{100/100}$//G6PDH-2$^{95/100}$;
lane 3: G6PDH-1$^{100/100}$//G6PDH-2$^{100/110}$;
lane 5: G6PDH-1$^{95/95}$//G6PDH-2$^{95/100}$;
lane 6: G6PDH-1$^{95/95}$//G6PDH-2$^{95/95}$;
lane 7: G6PDH-1$^{95/100}$//G6PDH-2$^{95/110}$.

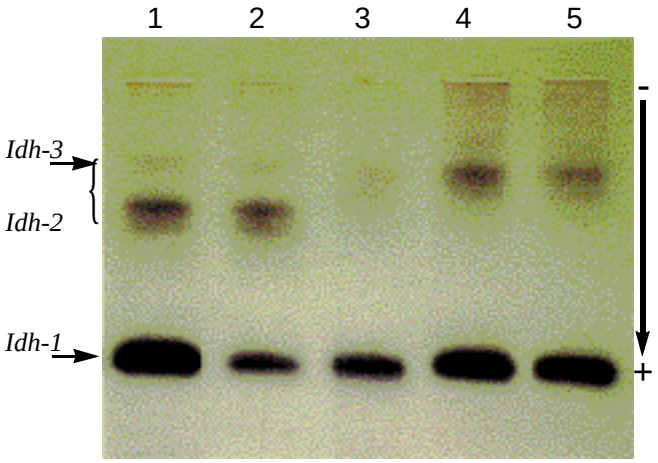


Figure 10. Isocitrate dehydrogenase (IDH) zymogrammes from Lima bean — *Zymogrammes de l'isocitrate déshydro - génase (IDH) du haricot de Lima.*
Lanes 1 and 2: IDH-1$^{100/100}$//IDH-2$^{100/100}$//IDH-3$^{100/100}$;
lanes 3, 4 and 5: IDH-1$^{100/100}$//IDH-2$^{75/75}$//IDH-3$^{100/100}$.

Figure 11. Leucine aminopeptidase (LAP) zymogrammes from Lima bean — *Zymogrammes de la leucine aminopeptidase (LAP) du haricot de Lima.*
Lane 1: LAP$^{94/94}$;
lanes 2, 4, 5 and 6: LAP$^{100/100}$;
lanes 3 and 7: LAP$^{108/108}$.

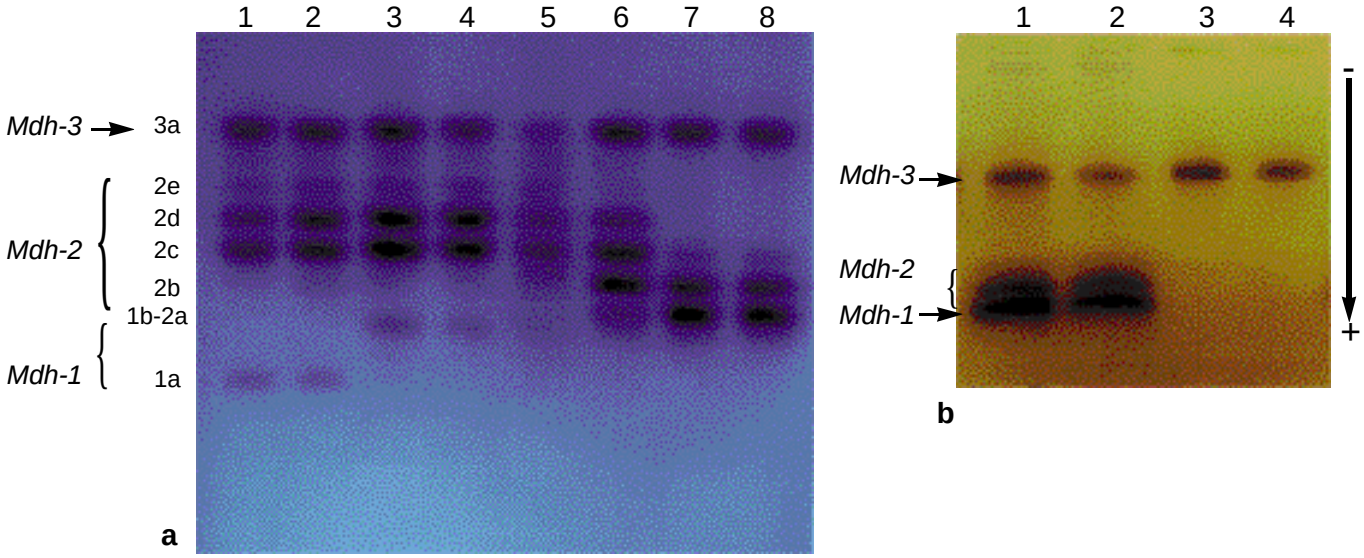


Figure 12. Malate dehydrogenase (MDH) zymogrammes from Lima bean (Zoro Bi *et al*., 1997) — *Zymogrammes de la malate déshydrogénase (MDH) du haricot de Lima*.

a. Zymogrammes obtained using the standard extraction buffer (Potassium phosphate, pH 7.0) — *Zymogrammes obtenus en utilisant le tampon d'extraction standard (Potassium phosphate, pH 7.0).*

Lanes 1 and 2: MDH-$1^{100/100}$//MDH-$2^{100/100}$// MDH-$3^{100/100}$;
lanes 3, 4, and 5: MDH-$1^{84/84}$//MDH-$2^{100/100}$// MDH-$3^{100/100}$;
lane 6: MDH-$1^{84/84}$//MDH-$2^{100/140}$// MDH-$3^{100/100}$;
lanes 7 and 8: MDH-$1^{84/84}$//MDH-$2^{140/140}$// MDH-$3^{100/100}$.

b. Zymogrammes showing the effect of ascorbic acid on MDH isozymes — *Zymogrammes montrant l'effet de l'acide ascorbique sur les isozymes de MDH*.

Lanes 1 and 2: samples homogenised in the standard extraction buffer;
lanes 3 and 4: the same samples homogenised in ascorbic acid solution (pH 2.0). The cytoplasmic products (MDH-1 and MDH-2) are inactivated by the ascorbic acid solution.

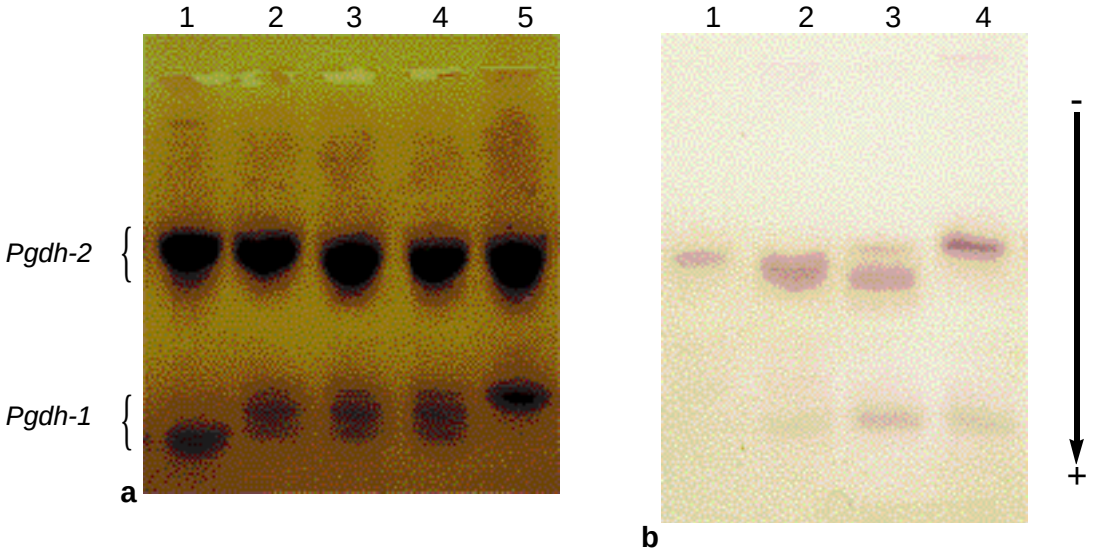


Figure 13. Phosphogluconate dehydrogenase (PGDH) zymogrammes from Lima bean — *Zymogrammes de la phospho - gluconate déshydrogénase (PGDH) du haricot de Lima*.

a. Lane 1: PGDH-$1^{100/100}$//PGDH-$2^{100/100}$;
lanes 2, 3 and 4: PGDH-$1^{86/100}$//PGDH-$2^{100/100}$;
lane 5: PGDH-$1^{86/86}$//PGDH-$2^{100/100}$.

b. Lane 1: PGDH-$1^{86/86}$//PGDH-$2^{109/109}$;
lane 2: PGDH-$1^{100/100}$//PGDH-$2^{119/119}$;
lane 3: PGDH-$1^{100/100}$//PGDH-$2^{100/135}$;
lane 4: PGDH-$1^{100/100}$//PGDH-$2^{100/100}$.

Table 5. Locus and allele designations, migration orientation and polymorphisms of enzymatic systems resolved from the cotyledonary tissues of *P. lunatus — Désignation des loci et allèles, sens de la migration des isozymes et polymorphismes des systèmes enzymatiques révélés à partir des tissus cotylédonnaires de* P. lunatus.

Enzyme	Locus number	Locus designation	Migration orientation (1)	Observed alleles	Polymorphism	
					Species	Population
ACO	2	*Aco-1*	A	100	No	No
		Aco-2	A	91; 100; 112	Yes	No
ADH	2	*Adh-1*	A	100; 116	Yes	Yes
		Adh-2	A	61; 100	Yes	Yes
DIA	2	*Dia-1*	A	null; 91; 98; 100	Yes	Yes
		Dia-2	A	100; 173; 200	Yes	No
END	1	*End*	A	84; 87; 100	Yes	No
EST	3	*cEst-2*	A	100; 107	Yes	Yes
		cEst-4	C	84; 100	Yes	No
		fEst-2	A	100; 110	Yes	Yes
GDH	2	*Gdh-1*	A	100	No	No
		Gdh-2	A	100	No	No
β-GLU	1	β-*Glu*	C	100; 125	Yes	No
G6PDH	2	*G6pdh-1*	A	95; 100	Yes	Yes
		G6pdh-2	A	95; 100; 110	Yes	Yes
GPI	3	*Gpi-1*	A	96; 100	Yes	Yes
		Gpi-2	A	null; 100	Yes	Yes
		Gpi-3	A	100	No	No
IDH	3	*Idh-1*	A	100; 120	Yes	No
		Idh-2	A	75; 100	Yes	Yes
		Idh-3	A	100	No	No
LAP	1	*Lap*	A	94; 100; 108; 118	Yes	No
MDH	3	*Mdh-1*	A	84; 100	Yes	No
		Mdh-2	A	100; 140	Yes	Yes
		Mdh-3	A	100; 104	Yes	No
PER	2	*Per-1*	A	100	No	No
		Per-2	C	100	No	No
PGDH	2	*Pgdh-1*	A	86; 100	Yes	Yes
		Pgdh-2	A	100; 109; 119; 135	Yes	No
PGM	2	*Pgm-1*	A	95; 100	Yes	Yes
		Pgm-2	A	85; 100; 110	Yes	Yes
SKDH	1	*Skdh*	A	100; 110	Yes	Yes
SOD	2	*Sod-1*	A	100	No	No
		Sod-2	A	100	No	No
N = 17	N = 34	N = 34		N = 70	P = 73.53	P = 44.12

cEst-2: colorimetric esterase. *fEst-2*: fluorimetric esterase.
N: total number. P: percentage of polymorphic loci.
(**1**) Isozyme migrating to the anodal side A or to the cathodal side C relative to the origin.

Bibliography

Aebersold PB., Winans GA., Teel DJ., Milner GB., Utter FM. (1987). *Manual for starch gel electrophoresis: a method for the detection of genetic variation,* Rep. No. NMFS 61. Seattle, USA: National Oceanic and Atmospheric Administration (NOAA).

Allard RW. (1963). An additional gametophyte factor in the Lima bean. *Züchter* **33**, p. 212–216.

Bailey NTJ. (1961). *Introduction to the mathematical theory of genetic linkage*. London: Oxford University Press.

Banuett-Bourrillon F., Hague DR. (1979). Genetic analysis of alcohol dehydrogenase isozymes in pearl millet (*Pennisetum typhoides*). *Biochem. Genet.* **17** (5/6), p. 537–552.

Bassiri A., Adams MW. (1978). An electrophoretic survey of seedling isozymes in several *Phaseolus* species. *Euphytica* **27**, p. 447–459.

Brothers ME., Kelly JD. (1993). Allozyme evaluation of upright common bean genotypes. *Euphytica* **67**, p. 65–70.

Brown AHD., Zohary D., Nevo E. (1978). Outcrossing rates and heterozygosity in natural populations of *Hordeum spontaneum* Koch in Israel. *Heredity* **41** (1), p. 49–62.

Crawford DJ. (1990). *Plant molecular systematics: macromolecular approaches.* New York: Wiley

Freyre R., Johnson WC., Shirmohamadali A., Llaca V., Tsai SM., Pereira PA., Skroch P., Nienhuis J., Adam-Blondon A-F., Dron M., Tohme J., Gepts P. (1996). Correlation among RFLP maps in common bean. *Annu. Rep. Bean Improv. Coop.* **39**, p. 231–232.

Gietl C. (1992). Partitioning of malate dehydrogenase isoenzymes into glyoxysomes, mitochondria, and chloroplasts. *Plant Physiol.* **100**, p. 557–559.

Goodman MM., Stuber CW., Lee CN., Johnson FM. (1980a). Genetic control of malate dehydrogenase isozymes in maize. *Genetics* **94** (1), p. 153–168.

Goodman MM., Stuber CW., Newton K., Weissinger HH. (1980b). Linkage relationships of 19 enzyme loci in maize. *Genetics* **96** (3), p. 697–710.

Griffin JD., Palmer RG. (1987). Inheritance and linkage studies with five isozyme loci in soybean. *Crop Sci.* **27**, p. 885–892.

Guries RP., Ledig FT. (1978). Inheritance of some polymorphic isoenzymes in pitch pine (*Pinus rigida* Mill). *Heredity* **40** (1), p. 27–32.

Hamrick JL., Godt JW. (1990). Allozyme diversity in plant species. *In* Brown AHD., Clegg MT., Kalher AL., Weir BS. *Plant population genetics, breeding, and genetic resources*. p. 43-63. Sunderland, Massachusetts: Sinauer.

Harry DE. (1986). Inheritance and linkage of isozyme variants in incense-cedar. *J. Hered.* **77**, p. 261–266.

Hussain A., Bushuk W., Ramirez H., Roca W., eds. (1988). *A practical guide for electrophoretic analysis of isozymes and proteins in cassava, field bean and forage legumes,* Working document **40**. Cali, Colombia: Centro Internacional de Agricultura Tropical (CIAT).

Jaaska V. (1983). Secale and triticale. *In* Tanksley SD., Orton TJ. *Isozymes in plant genetics and breeding, Part B.*, p. 79–101. Amsterdam: Elsevier.

Jaaska V., Jaaska V. (1988). Isoenzyme variation in the genera *Phaseolus* and *Vigna* (*Fabaceae*) in relation to their systematics: aspartate aminotransferase and superoxide dismutase. *Plant Syst. Evol.* **159**, p. 145–159.

Kazan K., Muehlbauer FJ., Weeden NF., Ladizinsky G. (1993). Inheritance and linkage relationships of morphological and isozyme loci in Chickpea (*Cicer arietinum* L.). *Theor. Appl. Genet.* **86**, p. 417–426.

Kiang YT., Gorman MB. (1985). Inheritance of NADP-active isocitrate dehydrogenase isozymes in soybeans. *J. Hered.* **76**, p. 279–284.

Koenig R., Gepts P. (1989a). Allozyme diversity in wild *Phaseolus vulgaris*: further evidence for two major centers of genetic diversity. *Theor. Appl. Genet.* **78**, p. 809–817.

Koenig R., Gepts P. (1989b). Segregation and linkage of genes for seed proteins, isozymes, and morphological traits in common bean (*Phaseolus vulgaris*). *J. Hered.* **80**, p. 455–459.

Maquet A. (1995). *Étude de la diversité génétique de la légumineuse alimentaire* Phaseolus lunatus *L. par l'analyse de caractères morphophysiologiques et de marqueurs protéiques*. PhD Thesis, Fac. Univ. Sci. Agron., Gembloux, Belgium.

Maquet A., Zoro Bi I., Rocha OJ., Baudoin JP. (1996). Case studies on breeding systems and its consequences for germplasm conservation. 1. Isoenzyme diversity in wild Lima bean populations in Central Costa Rica. *Genet. Res. Crop Evol.* **43**, p. 309–318.

Morden CW., Doebley JF., Schertz KF. (1988). Genetic control and subcellular localization of aconitase isozymes in Sorghum. *J. Hered.* **79**, p. 294–299.

Mulcahy DL., Mulcahy GB. (1983). Gametophytic self-incompatibility reexamined. *Science* **220**, p. 1247–1251.

Murphy RW., Sites JW. Jr, Buth DG., Haufler CH. (1990). Proteins I: isozyme electrophoresis. *In* Hillis DM., Moritz C. *Molecular systematics*, p. 45–126. Sunderland, Massachusetts: Sinauer.

Neale DB., Adams WT. (1981). Inherance of isozyme variants in seed tissues of balsam fir (*Abies balsamea*). *Can. J. Bot.* **59**, p. 1285–1291.

Newton KJ. (1983). Genetics of mitochondrial isozymes. *In* Tanksley SD., Orton TJ. *Isozymes in plant genetics and breeding, part A.*, p. 157–174. Amsterdam: Elsevier.

Rick CM. (1983). Tomato (*Lycopersicon*). *In* Tanksley SD., Orton TJ. *Isozymes in plant genetics and breeding, Part B.*, p. 147–165. Amsterdam: Elsevier.

Scandalios JG., Espiritu LG. (1969). Mutant aminopeptidases of *Pisum sativum.* I. Development genetics and chemical characteristics. *Mol. Gen. Genet.* **105**, p. 101–112.

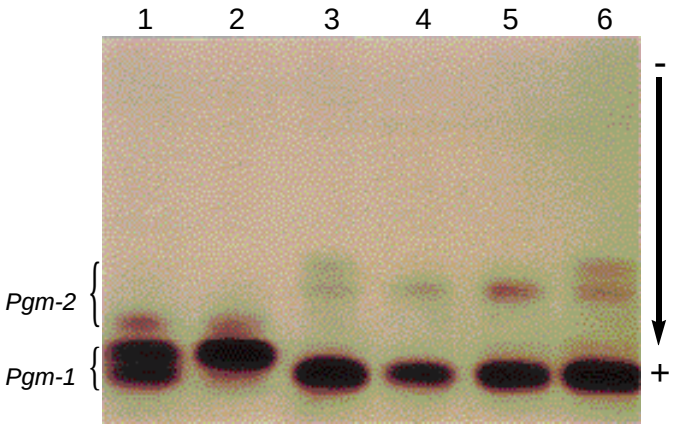


Figure 14. Phosphoglucomutase zymogrammes (PGM) from Lima bean (Zoro Bi *et al*., 1997) — *Zymogrammes de la phosphoglucomutase (PGM) du haricot de Lima*.
Lane 1: PGM-1$^{95/100}$//PGM-2$^{110/110}$;
lane 2: PGM-1$^{95/95}$//PGM-2$^{110/110}$;
lanes 3 and 6: PGM-1$^{100/100}$//PGM-2$^{85/100}$;
lanes 4 and 5: PGM-1$^{100/100}$//PGM-2$^{100/100}$.

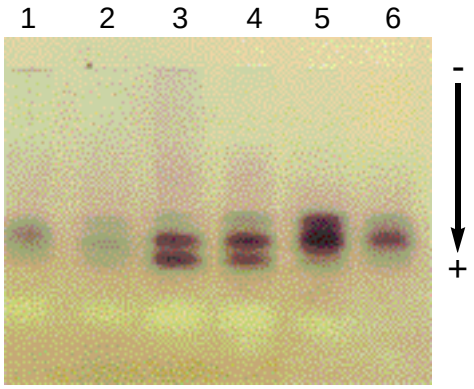


Figure 15. Shikimate dehydrogenase (SKDH) zymogrammes from Lima bean — *Zymogrammes de la shikimate déshydrogénase (SKDH) du haricot de Lima*.
Lanes 1, 5 and 6: SKDH$^{100/100}$;
lanes 2, 3 and 4: SKDH$^{100/110}$.

Schaal BA., LeverichWJ., Rogstad SH. (1991). A comparison of methods for assessing genetic variation in plant conservation biology. *In* Falk DA., Holsinger KE., eds. *Genetics and conservation of rare plants*, p. 123–134. Oxford, UK: Oxford University Press.

Schinkel C., Gepts P. (1989). Allozyme variability in the tepary bean, *Phaseolus acutifolius* A. Gray. *Plant Breed.* **102**, p. 182–195.

Schwartz D., Endo T. (1966). Alcohol dehydrogenase polymorphism in maize - simple and compound loci. *Genetics* **53**, p. 709–715.

Shaw CR., Prasad R. (1970). Starch gel electrophoresis of enzymes - a compilation of recipes. *Biochem. Genet.* **4**, p. 297–320.

Shore JS., Barrett SC. (1987). Inheritance of floral and isozyme polymorphisms in *Turnera ulmifolia* L. *J. Hered.* **78**, p. 44–48.

Simonsen V., Heneen WK. (1995). Inheritance of isozymes in *Brassica campestris* L. and genetic divergence among different species of Brassiceae. *Theor. Appl. Genet.* **91**, p. 353–360.

Soltis PS., Soltis DE., Gottlieb LD. (1987). Phospho-glucomutase gene duplications in *Clarkia* (Onagraceae) and their phylogenetic implications. *Evolution* **41** (3), p. 667–671.

Sprecher SL. (1988). New isozyme variants at two NADH diaphorase loci in dry beans: correlation to gene pools and commercial classes. *Annu. Rep. Bean Improv. Coop.* **31**, 92.

Stuber CW., Goodman MM., Johnson FM. (1977). Genetic control and racial variation of β-glucosidase isozymes in maize (*Zea mays* L.). *Biochem. Genet.* **15** (3/4), p. 383–394.

Tuwafe S., Kalher AL., Boe A., Ferguson M. (1988). Inheritance and geographical distribution of allozyme polymorphisms in chickpea (*Cicer arietinum* L.). *J. Hered.* **79**, p. 170–174.

Vallejos CE. (1983). Enzyme activity staining. *In* Tanksley SD., Orton TJ. p. 469–516. Amsterdam: Elsevier.

Vallejos CE., Sakiyama NS., Chase CD. (1992). A molecular marker-based linkage map of *Phaseolus vulgaris* L. *Genetics* **131**, p. 733–740.

Wall JR., Wall SW. (1975). Isozyme polymorphisms in the study of evolution in the *Phaseolus vulgaris* - *P. coccineus* complex of Mexico. *In* Markert CL. *Isozymes: IV. Genetics and evolution.* p. 287–305. New York: Academic Press.

Webb EC. (1992). *Enzyme nomenclature*. London: Academic Press.

Weeden NF. (1984). Distinguishing among white seeded bean cultivars by means of allozyme genotypes. *Euphytica* **33**, p. 199–208.

Weeden NF., Doyle JJ., Lavin M. (1989). Distribution and evolution of a glucosephosphate isomerase duplication in the *leguminosae*. *Evolution* **43** (8), p. 1637–1651.

Weeden NF., Gottlieb LD. (1980a). The genetics of chloroplast enzymes. *J. Hered.* **71**, p. 392–396.

Weeden NF., Gottlieb LD. (1980b). Isolation of cytoplasmic enzymes from pollen. *Plant Physiol.* **66**, p. 400–403.

Weeden NF., Lamb RC. (1987). Genetics and linkage analysis of 19 isozyme loci in apple. *J. Am. Soc. Hort. Sci.* **112** (5), p. 865–872.

Weeden NF., Marx GA. (1984). Chromosomal locations of twelve isozyme loci in *Pisum sativum*. *J. Hered.* **75**, p. 365–370.

Weeden NF., Wendel JF. (1989). Genetics of plant isozymes. *In* Soltis DE., Soltis PS. *Isozymes in plant biology*, p. 46–72. Portland, Oregon: Dioscorides Press.

Weir BS. (1996). *Genetic data analysis II: methods for discrete population genetic data*. Sunderland, Massachusetts: Sinauer.

Wendel JF., Parks CR. (1984). Distorted segregation and linkage of alcohol dehydrogenase genes in *Camellia japonica* L. (*Theaceae*). *Biochem. Genet.* **22** (7/8), p. 739–748.

Wendel JF., Weeden NF. (1989). Visualization and interpretation of plant isozymes. *In* Soltis DE., Soltis PS. *Isozymes in plant biology*, p. 5–44. Portland, Oregon: Dioscorides Press.

Zamir D., Tadmor Y. (1986). Unequal segregation of nuclear genes in plants. *Bot. Gaz.* **147** (3), p. 355–358.

Zoro Bi I., Maquet A., Wathelet B., Baudoin JP. (1996). New isozyme markers for the study of the gene pool *Phaseolus lunatus* L. *Annu. Rep. Bean Improv. Coop.* **39**, p. 247–248.

Zoro Bi I., Maquet A., Wathelet B., Baudoin JP. (1997). Genetic control of alcohol dehydrogenase, malate dehydrogenase, and phosphoglucomutase isozymes in Lima bean (*Phaseolus lunatus* L.). *Plant Breed.* **116** (2), p. 181–185.

(59 ref.)