

# Isozyme markers and genetic variability in three species of *Centrosema* (Leguminosae)

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## ABSTRACT

Isozyme patterns and their genetic control in three *Centrosema* species are described. Seven isozymatic systems (aspartate aminotransferase, glucose-6-phosphate isomerase, phosphoglucumutase, anodal peroxidase, malate dehydrogenase, 6-phosphoglucuronate dehydrogenase, and isocitrate dehydrogenase) were studied in 18 populations and several breeding lines of *C. acutifolium*, *C. brasilianum* and *C. pubescens*, using starch gel electrophoresis techniques. All systems, except glucose-6-phosphate isomerase, are described for the first time in these species. A total of 17 isozyme loci were scored; this represents the largest set of Mendelian loci known up to now in *Centrosema* species. Isozyme polymorphism and variability within and between populations and species were relatively high and allowed discrimination among species.

## INTRODUCTION

The genus *Centrosema* (Leguminosae) is a member of the subfamily Papilionoideae (National Research Council, 1979). It is included in the tribe Phaseolae, and contains at least 35 described species (Williams and Clements, 1990; Fantz, 1993). This genus is regarded as an important source of fodder in tropical and subtropical areas. All *Centrosema* species are native to America and most of them are distributed in savanna and forest habitats of tropical and subtropical regions of South and Central America. Brazil is the most important center of *Centrosema* diversity: 31 species occur naturally within its boundaries, and 10 of them occur exclusively in Brazil (Schultze-Kraft *et al.*, 1990).

Three *Centrosema* species are of special interest: *C. acutifolium* has good dry matter production and adapts to acid soils, *C. pubescens* is a good seed producer, and *C. brasilianum* has wide adaptive ability. *C. acutifolium* has a dispersed but relatively limited

distribution, mainly in lowlands, and it is capable of growing on acid soils of medium to low fertility. *C. pubescens* is a widely distributed species growing in scrub forest habitats from Mexico to tropical South America, and is also capable of growing on acid and medium fertility soils in lowlands and in mountain regions (up to 1,600 m above sea level). *C. brasilianum* has an intermediate distribution, mainly in Atlantic lowlands, in tropical areas such as the Brazilian "caatinga" (Schultze-Kraft *et al.*, 1990). Although these species have been relatively well studied from the agronomic point of view, basic genetic knowledge is still limited, making domestication and breeding programs difficult. For instance, chromosome numbers of  $2n = 18, 20$  and  $22$  have been described in species of the genus *Centrosema*, and in several cases, different numbers have been reported in a given species (see Miles *et al.*, 1990 for revision). The chromosome number of the three species studied in this paper was determined as  $2n = 22$  (Novaes and Penteadó, 1993). No single-gene Mendelian trait is known in any species of the genus (Miles *et al.*, 1990), with the exception of glucose phosphate isomerase (Weeden *et al.*, 1989).

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These species were assumed to be predominantly self-pollinating, in spite of field evidence of open fertilization based on morphological variability. Recently, Penteado *et al.* (1996) showed that the outcrossing rates ranged from the 15 to 47% among the populations of the three species of *Centrosema* mentioned, and therefore they were considered to have a mixed-mating system.

The use of molecular markers, isozymes in particular, in basic genetic studies and breeding is widely generalized in many crop, weed and wild plant species, and their advantages and limitations have been extensively discussed (Soltis and Soltis, 1989; Pérez de la Vega, 1993). In this work, isozyme patterns of three species of the genus *Centrosema* are described.

## MATERIAL AND METHODS

Three species of *Centrosema* (*C. acutifolium* Benth., *C. brasilianum* (L.) Benth. and *C. pubescens* Benth.) were studied. Seedlings from six populations (random samples of seeds produced by bulk field plantings) and several family arrays (progeny seeds of individual plants) per species were analyzed (Table I). Families were collected from breeding lines derived from the corresponding population and selected for phenotypic characters other than isozymes. All the populations were originally collected in Brazil. Populations have been multiplied, as part of the *Centrosema* active germplasm collection by the Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA) at the Centro Nacional de Pesquisa de Gado de Corte (CNPGC), in Campo Grande, Mato Grosso do Sul, Brazil, and by the Centro Internacional de Agricultura Tropical (CIAT) at Quilichao, Colombia (Table I). The breeding lines from which seed families were collected were developed in Campo Grande.

Seeds were germinated in a growth chamber under controlled temperature and photoperiod conditions. After three days on wet filter paper at 25°C in the dark, seedlings were transferred to vermiculite and kept under a photoperiod of 16 h of light at 25°C and 8 h dark at 20°C. After 10 days a cotyledonary leaf was excised from each seedling, crushed in

a drop of the extraction medium described by Roose and Gottlieb (1978), with minor modifications. The crude extracts were absorbed into paper wicks (Whatman 3 MM 0.4 x 1 cm), and electrophoresed in 10% horizontal starch gels. Each plant extract was analyzed in two different buffer systems: 1) Tris-borate acid, pH 8.9 (Tris 0.15 M, boric acid 0.036 M, EDTA Na<sub>2</sub> 0.001 M) as the gel buffer and pH 8.5 (Tris 0.2 M, boric acid 0.15 M, EDTA Na<sub>2</sub> 0.001 M) as the electrode buffer, a modification of Wendel and Weeden (1989), and 2) histidine 0.005 M adjusted to pH 7.0 with NaOH as the gel buffer and Tris-citrate, pH 7.0 (Tris 0.135 M, citric acid 0.043 M) as the electrode buffer (Hutchinson *et al.*, 1983). After electrophoresis, each gel, 1 cm thick, was sliced into 2-mm thick slabs and each slab was stained for a different enzymatic system. The slabs from the Tris-borate buffer system were stained for aspartate aminotransferase (AAT, EC 2.6.1.1, also known as glutamate-oxaloacetate transaminase or GOT), glucose-6-phosphate isomerase (GPI, EC 5.3.1.9), phosphoglucomutase (PGM, EC 2.7.5.1), and anodal peroxidase (PRX, EC 1.11.1.7); the slabs from the

Table I - *Centrosema* populations and families.

Species	BRA <sup>1</sup>	Populations <sup>2</sup>	N	Breeding lines <sup>3</sup>	Nf	N
<i>C. brasilianum</i>	007196	GC 442C*	55			
	007382	GC 489B**	112	GC 489-R8***	28	315
		GC 489C/CIAT5486	95			
	006025	GC 689C/CIAT5234	83			
	006025	CPAC 1219B	80			
	-	CIAT 5247C	68			
<i>C. pubescens</i>	017698	GC 102B	59			
	016586	GC 241B	54			
	017680	GC 383B	72			
	017764	GC 495B	57	GC 495-R6	9	86
		GC 495C/CIAT5203	33			
	-	CIAT 5642C	67			
<i>C. acutifolium</i>	013501	GC 350B	960	GC 350-R7	44	588
		GC 350C/CIAT15532	110			
	004090	GC 351B	46	GC 351-R5	29	330
		GC 351C/CIAT5118	70	GC 351-R10	23	237
	009229	GC 354B	75			
	-	CIAT 5568C	79			

<sup>1</sup> Accession number in the Banco Nacional de Germoplasma - Centro Nacional de Recursos Genéticos e Biotecnologia/EMBRAPA, Brasil.

<sup>2</sup> Accession number in the active germplasm collections: GC - Centro Nacional de Pesquisa de Gado de Corte; CPAC - Centro de Pesquisa de Ambito de Cerrados, EMBRAPA, Brasil; CIAT - Centro Internacional de Agricultura Tropical, Colombia.

<sup>3</sup> Breeding lines from which families were collected.

N = Number of individuals; Nf = number of families.

\*C indicates seeds collected in Quilichao, Colombia.

\*\*B indicates seeds collected in Campo Grande, Brazil.

\*\*\*R identifies breeding lines.

histidine buffer system were stained for malate dehydrogenase (MDH, EC 1.1.1.37), 6-phosphogluconate dehydrogenase (6PGD, EC 1.1.1.44), and isocitrate dehydrogenase (IDH, EC 1.1.1.42). The staining procedures were, with minor modifications, those described by Vallejos (1983). In addition to these systems, acid phosphatases were stained in a slab from the histidine buffer system, but due to its poor resolution data were not scored.

## RESULTS AND DISCUSSION

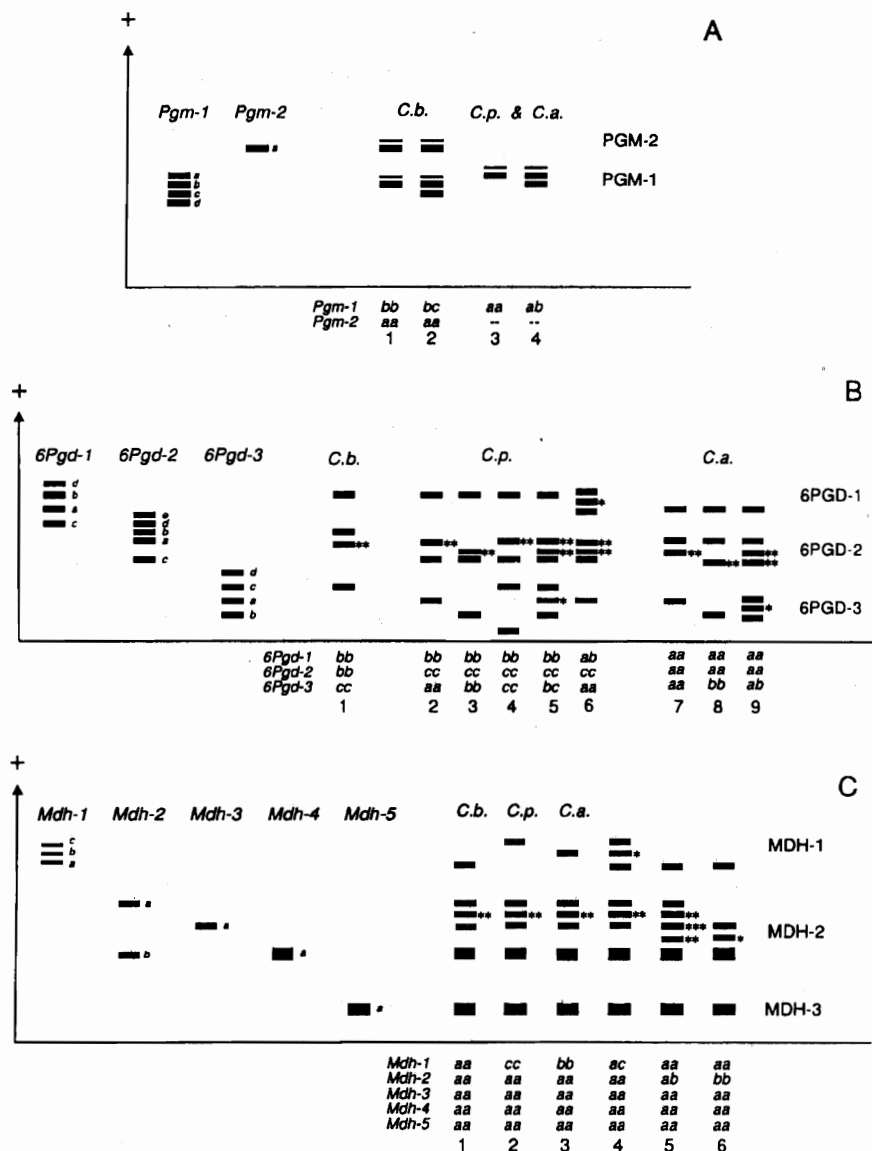
### Description and genetic control of isozyme patterns

Although *Centrosema* plants developed well under controlled conditions, in the climatic conditions of Leon, in the northern part of the Spanish Meseta, it was impossible to obtain adequate flowering in order to carry out crosses and study segregations. So, the probable mechanism of genetic control was deduced: 1) from the previous knowledge on the genetic control in other legume species and the monomeric or dimeric structure of the enzymes (Weeden and Wendel, 1989; Weeden *et al.*, 1989), 2) inferring homozygous genotypes as those observed in progeny arrays which bred true, 3) from the fact that heterozygous genotypes were observed in segregating progenies, either by selfing of heterozygous mother plants or by cross-pollination between plants, and 4) from the observation of isozyme seedling phenotypes in the general populations.

### Phosphoglucose mutase (PGM, EC 2.7.5.1)

Phosphoglucose mutase isozymes are reported to be monomeric. Two zones of activity, PGM-1 and PGM-2, were observed (Figure 1A). PGM-1 was observed in the three species

while PGM-2 was observed only in *C. brasilianum*. Thus this zone is suitable to distinguish this species from the other two species. In PGM-1 two kinds of patterns were observed: seedlings showing a single band and seedlings with two bands (a faint satellite band was always observed close to the fastest migrating band). These are the expected patterns for homozygous and heterozygous individuals, respectively, in monomeric isozyme systems. PGM-2 was monomorphic in *C. brasilianum*, always showing a single band, and its control was assigned to a locus with a single allele, *Pgm-2 a*.



**Figure 1** - Isozyme patterns observed in the three species of *Centrosema*: *C.b.*, *C. brasilianum*; *C.p.*, *C. pubescens*; *C.a.*, *C. acutifolium*. A, Phosphoglucose mutase; B, 6-phosphogluconate dehydrogenase; C, malate dehydrogenase. Loci and alleles and the corresponding bands are indicated on the left side of drawings. The patterns corresponding to some genotypes observed in *Centrosema* species are represented on the right. Genotypes are indicated under the horizontal line. \*Heterodimeric band of allelic subunits; \*\*heterodimeric band of non-allelic subunits; \*\*\*heterodimeric band of *Mdh-1 a* and *Mdh-2 b* products plus *Mdh-3 a* homodimer.

The hypothesis on the polymorphic PGM-1 was that it was controlled by a single locus, *Pgm-1*, and according to the mobility of electrophoretic bands with four alleles named *a* to *d*. This hypothesis was supported by the following facts: 1) 95 out of 96 families of *C. acutifolium* bred true for the single band pattern tentatively designated *Pgm-1 aa* (pattern 3 in Figure 1A). The remaining family had 13 seedlings with *Pgm-1 aa*, and two seedlings with the two-banded heterozygous *Pgm-1 ab* (pattern 4). These latter two seedlings were probably originated by cross-pollination. 2) In *C. brasilianum* 27 out of 28 families bred true for the single band pattern designated *Pgm-1 bb* (pattern 1), and the other family had 10 *Pgm-1 bb* seedlings and one two-banded *Pgm-1 bc* seedling (pattern 2); this seedling was also most likely originated by cross-pollination. 3) In *C. pubescens* two families bred true for *Pgm-1 aa* and seven segregated for *Pgm-1 aa* and *Pgm-1 ab*.

PGM-1 seems to be related to chloroplasts since it was not present in root or etiolated leaf extracts. It is probably located in plastids, but under the control of a nuclear locus, as it showed biparental inheritance, as indicated by the heterozygous genotypes. The existence of plastid-specific nuclear-encoded PGM isozymes has been described in other plant species (Weeden and Wendel, 1989).

## 6-Phosphogluconate dehydrogenase (6PGD, EC 1.1.1.44)

This system has been reported as dimeric and in *Centrosema* it has also shown a dimeric pattern, since heterozygous individuals showed heterodimeric bands which were formed both with subunits coded by the same locus and by different loci. The most common isozymatic pattern in each species was formed by four bands, one in each of the fastest and slowest migrating zones and two in the intermediate zone (Figure 1B). The hypothesis was that these patterns correspond to homozygous seedlings at the three loci controlling the 6PGD isozyme systems: *6Pgd-1* and *6Pgd-3* could, respectively, control the isozymes in the fastest and slowest migrating zones and *6Pgd-2* could control one of the intermediate bands. The band with an intermediate mobility between the two extreme bands may be the heterodimeric band formed with the subunits coded by *6Pgd-1* and *6Pgd-3*. This was deduced because changes in alleles (band mobility) at *6Pgd-1* or *6Pgd-3* determined changes in mobility in one of the intermediate zone bands without affecting the mobility of the other (see patterns 2, 3, and 4 of *C. pubescens* in Figure 1B). Seedlings considered heterozygous either at *6Pgd-1* or *6Pgd-3* showed three bands at the

**Table II** - Allelic frequencies of variable loci in the *Centrosema* populations and breeding lines.

Locus	<i>C. brasilianum</i>		<i>C. pubescens</i>		<i>C. acutifolium</i>	
	Pop.	B.l.	Pop.	B.l.	Pop.	B.l.
N	493	315	342	86	476	1155
<i>Aat-1 a</i>	-	-	0.031	-	0.446	0.788
<i>b</i>	0.950	1.000	-	-	-	-
<i>c</i>	0.050	-	0.969	1.000	0.554	0.212
<i>Aat-2 a</i>	0.993	1.000	1.000	1.000	1.000	1.000
<i>b</i>	0.007	-	-	-	-	-
<i>Aat-3 a</i>	-	-	0.188	0.093	0.155	0.241
<i>b</i>	-	-	0.703	0.825	0.706	0.722
<i>c</i>	-	-	0.109	0.081	0.139	0.037
<i>d</i>	1.000	1.000	-	-	-	-
<i>Gpi-1 a</i>	0.003	-	0.782	0.698	0.704	0.396
<i>b</i>	0.995	1.000	0.215	0.302	0.291	0.604
<i>c</i>	0.002	-	0.003	-	0.005	-
<i>Gpi-2 a</i>	0.020	-	0.015	-	0.842	0.985
<i>b</i>	0.007	-	0.982	1.000	0.044	-
<i>c</i>	0.382	0.003	0.003	-	-	-
<i>d</i>	0.022	-	-	-	-	-
<i>e</i>	0.093	-	-	-	0.008	-
<i>f</i>	0.056	-	-	-	0.099	-
<i>g</i>	0.419	0.997	-	-	0.006	0.005
<i>h</i>	-	-	-	-	-	0.010
<i>Gpi-3 a</i>	0.025	-	0.769	0.965	0.994	1.000
<i>b</i>	0.944	1.000	-	-	0.006	-
<i>c</i>	0.002	-	0.231	0.035	-	-
<i>d</i>	0.020	-	-	-	-	-
<i>e</i>	0.008	-	-	-	-	-
<i>Pgm-1 a</i>	0.009	-	0.973	0.797	0.990	0.999
<i>b</i>	0.667	0.997	0.027	0.203	0.010	0.001
<i>c</i>	0.267	0.003	-	-	-	-
<i>d</i>	0.057	-	-	-	-	-
<i>Prx-1 a</i>	0.485	0.283	0.223	0.047	0.615	0.102
<i>b</i>	0.515	0.717	0.777	0.953	0.385	0.898
<i>Mdh-1 a</i>	1.000	1.000	0.224	0.256	0.105	-
<i>b</i>	-	-	-	-	0.921	1.000
<i>c</i>	-	-	0.756	0.698	0.027	-
<i>Mdh-2 a</i>	0.999	1.000	0.921	0.988	0.995	0.999
<i>b</i>	0.001	-	0.079	0.012	0.005	0.001
<i>6Pgd-1 a</i>	0.032	-	-	0.006	0.971	1.000
<i>b</i>	0.946	1.000	1.000	0.994	0.019	-
<i>c</i>	0.020	-	-	-	0.007	-
<i>d</i>	0.002	-	-	-	0.003	-
<i>6Pgd-2 a</i>	0.002	-	-	-	0.921	1.000
<i>b</i>	0.996	0.987	-	-	0.007	-
<i>c</i>	-	-	1.000	1.000	0.004	-
<i>d</i>	0.002	0.013	-	-	0.026	-
<i>e</i>	-	-	-	-	0.042	-
<i>6Pgd-3 a</i>	0.008	0.007	0.594	0.959	0.638	0.493
<i>b</i>	0.034	-	0.206	-	0.313	0.507
<i>c</i>	0.956	0.993	0.200	0.041	0.049	-
<i>d</i>	0.002	-	-	-	-	-
<i>Idh-1 a</i>	0.519	-	0.958	0.965	0.825	0.985
<i>b</i>	0.256	-	0.042	0.035	0.168	0.015
<i>c</i>	0.224	1.000	-	-	-	-
<i>d</i>	-	-	-	-	0.007	-

N = Number of analyzed seedlings.

Pop. = Frequencies from populations considering all individuals as part of a single population.

B.l. = Frequencies from breeding lines; for *C. acutifolium* all lines indicated in Table III have been pooled as a single line.

corresponding zone and an additional band in the intermediate zone, which yielded a total of seven bands (patterns 5 and 6). The variability observed in these systems is summarized in Figure 1B and the alleles are listed in Table II.

The hypothesis of three loci controlling 6PGD isozymes and the formation of heterodimers with non-allelic subunits was supported by: 1) four banded patterns bred true, 11 families with a total of 115 seedlings in *C. acutifolium* (they included two different genotypes *aa aa aa* and *aa aa bb*, respectively, for loci *6Pgd-1*, *6Pgd-2* and *6Pgd-3*), 25 families and 279 seedlings in *C. brasilianum* (genotype *bb bb cc*), and five and 56 in *C. pubescens* (genotype *bb cc bb*) (Figure 1B); 2) segregating families for *6Pgd-3* in *C. acutifolium* and in *C. pubescens* produced some of the patterns shown in Figure 1B (patterns 7, 8 and 9, and 3, 4 and 5, respectively); 3) several heterozygous individuals for *6Pgd-1*, with three bands in the zone of faster mobility and the two corresponding heterodimers in the intermediate zone and some for *6Pgd-2* with characteristic three banded patterns in the intermediate zone and without affecting the two other zones, were observed in populations (Figure 1B, pattern 6). The heterodimeric bands between the products of *6Pgd-1* and *6Pgd-3* coincided in migration with bands determined by *6Pgd-2* in some genotypes.

The 6PGD was one of the most polymorphic systems and it was very useful in distinguishing the three *Centrosema* species.

### Malate dehydrogenase (MDH, EC 1.1.1.37)

The observations of MDH enzymes in *Centrosema* species coincide with the MDH dimeric pattern previously described for several other plant species (Weeden and Wendel, 1989), since heterodimeric bands among products of the same locus and also among products of different loci were observed. Three activity zones were observed (Figure 1C). The fastest zone, MDH-1, was polymorphic (single-banded and three-banded phenotypes) and under the control of locus *Mdh-1*, while the slowest one, MDH-3, was always monomorphic in the three species for a broad-diffuse band; this zone was not scored in genetic studies and it was considered to be controlled by locus *Mdh-5*. The MDH-3 zone was not observed when extracts of roots or etiolated plants were electrophoresed. In the intermediate zone, MDH-2, patterns with three, four or five bands were observed. It could be under the control of three loci: the monomorphic *Mdh-4* might determine the slowest migrating isozyme of this zone (it was always present in the three species), the also monomorphic

*Mdh-3* could code for a band of intermediate migration and present in all seedlings and species; finally, *Mdh-2* might present two alleles coding, respectively, the fastest isozyme (*Mdh-2 a*) of this zone and an isozyme (*Mdh-2 b*) with the same mobility as *Mdh-4 a* isozyme. Products of genes *Mdh-2* and *Mdh-3* could form non-allelic heterodimers. Isozyme patterns determined by the different genotypes at these five loci are shown in Figure 1C.

All the *C. acutifolium* and *C. brasilianum* families bred true for the corresponding single band pattern of *Mdh-1 bb* or *Mdh-1 aa*, respectively, and for the four-banded pattern determined by *Mdh-2 aa*, *Mdh-3 aa* and *Mdh-4 aa* (e.g., patterns 1 and 3). In *C. pubescens* some families segregated for *Mdh-1*, for instance *ac* with a three-banded phenotype (pattern 4) and *cc* with a single-banded phenotype (pattern 2), and some families for *Mdh-2* with four-band phenotypes in the MDH-2 zone (*Mdh-2 aa*, pattern 2), and five-band ones (*Mdh-2 ab*, pattern 5).

The most frequent *Mdh-1* allele in each species was different (Table II), and this locus was also useful to discriminate among populations of the three species.

### Glucose-6-phosphate isomerase (GPI, EC 5.3.1.9)

The GPI, EC 5.3.1.9, system and the presence of isozymes formed by non-allelic subunits in *Centrosema virginianum* and an unidentified species (*Centrosema* spp.) were previously reported by Weeden *et al.* (1989). In fact, GPI was the only system previously described in *Centrosema*.

Two zones of activity were distinguished in this system: GPI-1 and GPI-2. In zone GPI-1, patterns with a single band or with three bands were observed, being attributed, respectively, to homozygous and heterozygous genotypes of the *Gpi-1* locus. Three different alleles were observed in this locus according to electrophoretic mobility (Figure 2A). Zone GPI-2 was deduced to be under the control of two loci, *Gpi-2* and *Gpi-3*, whose products formed allelic and non-allelic heterodimers. Thus patterns with three bands (e.g., patterns 1, 3, and 6) are observed when both *Gpi-2* and *Gpi-3* are homozygous (the activity of these loci or their products must be different since band intensities were 1:4:4) and six bands when one of the two loci was homozygous and the other heterozygous (Figure 2A, pattern 5). Several alleles were distinguished in these two loci (Figure 2A and Table II). Although the mobility of the most frequent isozymes of each locus did not coincide, some of the less frequent did, and their assignment to *Gpi-2* or *Gpi-3* was deduced from the

relative intensity of the bands since *Gpi-2* seems to be less active.

All families of *C. brasilianum*, except one, bred true for the single banded pattern corresponding to *Gpi-1* *bb* and the three banded pattern corresponding to *Gpi-2* *gg* *Gpi-3* *bb* (pattern 2), these three alleles being relatively common in this species. In *C. acutifolium* segregation for *Gpi-1* genotypes was the rule with families showing three genotypes (*aa*, *ab* and *bb*) or two genotypes (*aa* and *ab*, or *ab* and *bb*). All families, except two families with infrequent alleles in *Gpi-2*, were fixed for the three banded pattern corresponding to the genotypes *Gpi-2* *aa* and *Gpi-3* *aa*, frequent in this species. In *C. pubescens* segregations for *Gpi-1* and alleles *a* and *b*, and for *Gpi-3* were observed. For this latter locus most seedlings were *Gpi-3* *aa* and showed three bands at zone GPI-2 and some, most likely produced by cross-pollination, were *Gpi-3* *ac* and showed six bands (pattern 5).

A lower activity of GPI isozymes was observed in root and etiolated leaf extracts in comparison with green leaf extracts, but none of the activity zones disappeared. This result might be due to a tissue- or physiological-dependent expression of these *Gpi* loci rather than to a chloroplastic location of any of these isozymes. Therefore, it was not possible to conclude if any of the nuclear *Gpi* loci observed encodes chloroplast-located enzymes.

### Aspartate aminotransferase (AAT, EC 2.6.1.1.)

The enzymes of this system also showed a dimeric pattern deduced by the presence of heterodimeric bands in the heterozygotes. Three zones of enzymatic activity, AAT-1, AAT-2 and AAT-3 (it was hypothesized that each one was controlled at a different locus, *Aat-1*, *Aat-2* and *Aat-3*, respectively), and no hybrid bands between products of non-allelic genes were observed (Figure 2B). This was deduced since the most frequent phenotypes showed three bands, and when two additional bands (heterozygous seedlings) appeared in any zone no changes occurred in the other two zones (for instance, see patterns 6-7 and 6-8).

Three alleles were observed at locus *Aat-1*, one of them (named *b*) specific to *C. brasilianum*. Alleles *a* and *c* were observed in the other two species, although allele *c* was practically exclusive to *C. pubescens* (Table II). Locus *Aat-2* was practically monomorphic and a

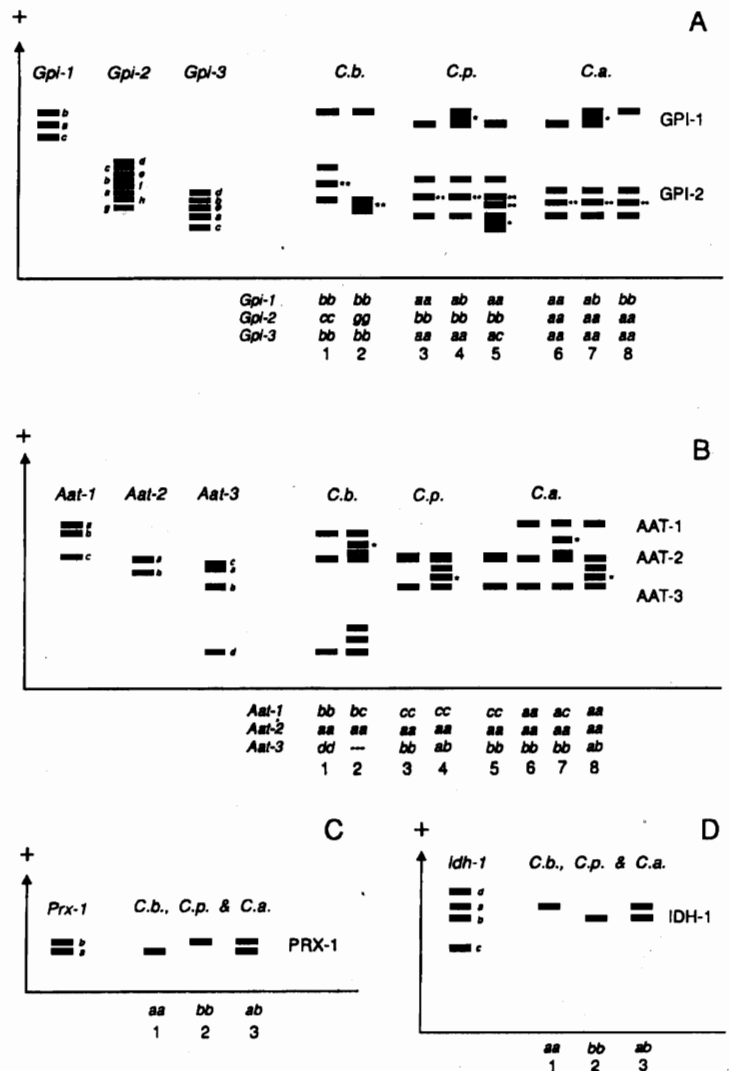


Figure 2 - Isozyme patterns of glucose phosphate isomerase, A; aspartate aminotransferase, B; peroxidase, C, and isocitrate dehydrogenase D (see legend of Figure 1, for details). Pattern 2 in B shows the three bands observed in zone AAT-3 of *C. brasilianum* root extracts.

second allele was observed only in a few individuals of a *C. brasilianum* population. The isozymes coded by *Aat-2* were not detectable in extracts of roots and etiolated seedlings, thus it could be related to the chloroplast. Three alleles (*a*, *b* and *c*) were identified at *Aat-3* in *C. acutifolium* and *C. pubescens*. The isozymes coded for these alleles showed migrations close to the products of locus *Aat-2*. The allele *Aat-3* *c* overlaps with the allele *Aat-2* *a*. In *C. brasilianum* none of these three AAT-3 bands was observed; a fixed band with mobility slower than any other AAT isozyme was observed instead (Figure 2B). This isozyme was attributed to locus *Aat-3* and allele *d*, though this hypothesis should be verified by interspecific hybridizations. Root and etiolated plantlet extracts of *C. brasilianum* showed a three-banded pattern in this region. This result suggests that addi-

tional loci could be implicated in the control of this isozyme zone in this species. The loci *Aat-1* and *Aat-3* showed specific patterns and were able to discriminate species.

In the progenies, it was observed that while all *C. brasilianum* families bred true for *Aat-1 bb Aat-2 aa Aat-3 dd* (pattern 1), many families of *C. acutifolium* segregated for *Aat-1* or *Aat-3* while all bred true for *Aat-2 aa* (patterns 6 to 8). Finally, in *C. pubescens* several families segregated for *Aat-3*, while they bred true for *Aat-1 cc Aat-2 aa* (patterns 3 and 4).

### Peroxidase (PRX, EC 1.11.1.7)

Only anodal peroxidases were studied. They showed a single zone of enzymatic activity and a monomeric pattern. A single locus, *Prx-1*, with 2 alleles common to the three species was hypothesized (Figure 2C). The absence of peroxidase bands was not observed, thus the presence of null alleles, relatively common in other species peroxidase systems, was not proved in *Centrosema*. Homozygotes showed a single band (patterns 1 and 2) and heterozygotes two bands (pattern 3). In the three species several families bred true for single-banded phenotypes while others segregated single-banded vs. two-banded phenotypes.

### Isocitrate dehydrogenase (IDH, EC 1.1.1.42)

In this system one zone was observed. This suggested the control by a single locus (*Idh-1*), with four alleles among the three species (Figure 2D). Two alleles (named *a* and *b*) were common to the three species; *c* was specific to *C. brasilianum* and the other appeared at a very low frequency in *C. acutifolium* (Table II). The enzyme, reported to be dimeric in several plant species (Weeden and Wendel, 1989), seemed to have a monomeric structure in *Centrosema* since the supposed heterozygotes presented a two-banded phenotype (pattern 3). Hypothetical homozygotes *Idh-1 aa* and *Idh-1 cc* (all *C. brasilianum* families showing the latter) bred true and several families segregated for genotypes with one (*Idh-1 aa* or *Idh-1 bb*) or two (*Idh-1 ab*) bands.

**Phosphatases:** Two zones of activity were observed in the acid phosphatase system. The fastest zone showed variability and the slowest a broad band, but the resolution was poor and data were not scored.

With the exception of isocitrate dehydrogenase, the deduced isozyme structure, monomeric or dimeric, of the *Centrosema* species agreed with the reported structure in other plant species. Peroxidases have been generally described as monomeric, although there are some reports of dimeric peroxidases, and null

alleles are relatively frequent in this system (García *et al.*, 1982; Weeden and Wendel, 1989). Peroxidases in *Centrosema* species had a monomeric structure, and the presence of null alleles was not proved, since no seedling lacked activity in this system. IDH was an exception, because this system is reported to be dimeric in other plant species, but it seems monomeric in *Centrosema*, since no hybrid band was observed in heterozygotes. A similar pattern, in which the band of intermediate mobility is not resolved in gels, has been described in other dimeric systems, for instance, for the *Mdh-2* locus in rye (*Secale cereale* L.) (Pérez de la Vega and Allard, 1984).

Most systems were controlled by two or more loci and most loci seemed to be common to the three *Centrosema* species. Some loci (*Aat-2*, *Pgm-1*, *Mdh-5*) seemed to be related to the chloroplast (as in other plant species, they should represent nuclear loci encoding enzymes located in the chloroplast) due to the changes observed in roots and etiolated *Centrosema* seedlings. Isozyme number and subcellular location may be variable because specific isozymes are not strongly expressed in certain tissues or species (Weeden and Wendel, 1989). AAT, PGM and GPI isozymes located in plastids have been previously described (see Weeden and Wendel, 1989). Weeden *et al.* (1989) observed in two species of *Centrosema* a three-banded phenotype of the cytosolic GPI enzymes, controlled by two loci, and detected the presence of a plastid enzyme. The results of AAT-3 isozymes in *C. brasilianum* are confusing since a higher number of isozymes were observed in etiolated seedlings; therefore more data are needed on the subcellular location of AAT-3 isozymes. On the other hand, MDH isozymes have not been previously related to the chloroplast (Weeden and Wendel, 1989), thus, further experiments on MDH-3 are necessary, such as the isolation of subcellular fractions, to prove this possible plastid location.

In addition to species-specific loci such as *Pgm-2*, some alleles were species-specific or nearly so (e.g., *6Pgd-2* alleles) or their frequency distribution among the *Centrosema* species was clearly different, e.g., *Aat-3*, *Gpi-2*, *Gpi-3* and *6Pgd-1* (Table II and Penteadó, 1994). Thus, isozyme allele frequencies proved to be a useful tool to distinguish one *Centrosema* species from another (Penteadó, 1994), although it was not possible to discriminate accessions within species.

### Genetic variation among populations and breeding lines

A relevant result, described in Table III, consisted of changes in genotypic frequencies, and hence in allelic frequencies, among the set of plants

Table III - Comparison of genotypic frequencies<sup>1</sup> between populations and breeding lines in *Centrosema*.

	<i>C. brasilianum</i>			<i>C. pubescens</i>			<i>C. acutifolium</i>		
	489R8	489B	495R6	495B	350R7	350B	351R5	351R10	351B
N	315	112	86	57	588	96	330	237	46
<i>Aat-1</i>									
<i>aa</i>	-	-	-	-	0.690	0.271	0.788	0.717	0.761
<i>ac</i>	-	-	-	-	0.133	0.281	0.118	0.131	0.174
<i>bb</i>	1.000	0.937	-	-	-	-	-	-	-
<i>bc</i>	-	0.054	-	-	-	-	-	-	-
<i>cc</i>	-	0.009	1.000	1.000	0.177	0.448	0.094	0.152	0.065
<i>Aat-3</i>									
<i>aa</i>	-	-	0.035	0.155	0.124	0.208	0.339	0.219	0.130
<i>ab</i>	-	-	0.116	0.067	0.078	0.052	0.054	0.055	0.022
<i>ac</i>	-	-	-	-	0.002	0.052	0.015	-	-
<i>bb</i>	-	-	0.744	0.689	0.743	0.500	0.530	0.713	0.717
<i>bc</i>	-	-	0.046	0.022	0.029	0.062	0.033	-	0.043
<i>cc</i>	-	-	0.058	0.067	0.024	0.125	0.027	0.013	0.087
<i>dd</i>	1.000	1.000	-	-	-	-	-	-	-
<i>Gpi-1</i>									
<i>aa</i>	-	-	0.547	0.649	0.175	0.729	0.182	0.236	0.783
<i>ab</i>	-	-	0.302	0.333	0.406	0.208	0.430	0.409	0.196
<i>bb</i>	1.000	1.000	0.151	0.118	0.418	0.062	0.388	0.354	0.022
<i>Gpi-2</i>									
<i>aa</i>	-	-	-	-	0.968	0.979	1.000	1.000	0.891
<i>bb</i>	-	-	1.000	1.000	-	-	-	-	-
<i>cc</i>	0.003	0.018	-	-	-	-	-	-	-
<i>gg</i>	0.997	0.982	-	-	0.010	-	-	-	0.065
Other	-	-	-	-	0.022	0.020	-	-	0.043
<i>Gpi-3</i>									
<i>aa</i>	-	-	0.930	1.000	1.000	0.969	1.000	1.000	1.000
<i>ab</i>	-	-	-	-	-	0.031	-	-	-
<i>ac</i>	-	-	0.070	-	-	-	-	-	-
<i>bb</i>	1.000	1.000	-	-	-	-	-	-	-
<i>Pgm-1</i>									
<i>aa</i>	-	-	0.593	1.000	0.997	0.958	1.000	1.000	1.000
<i>ab</i>	-	-	0.407	-	0.003	0.042	-	-	-
<i>bb</i>	0.997	0.964	-	-	-	-	-	-	-
<i>bc</i>	-	0.036	-	-	-	-	-	-	-
<i>cc</i>	0.003	-	-	-	-	-	-	-	-
<i>Prx-1</i>									
<i>aa</i>	0.003	0.840	0.012	-	0.053	0.064	0.003	-	1.000
<i>ab</i>	0.559	0.133	0.070	0.035	0.114	0.723	0.194	0.169	-
<i>bb</i>	0.438	0.027	0.919	0.965	0.833	0.213	0.803	0.831	-
<i>Mdh-1</i>									
<i>aa</i>	1.000	1.000	0.012	-	-	-	-	-	-
<i>ab</i>	-	-	0.035	-	-	-	-	-	-
<i>ac</i>	-	-	0.453	0.070	-	-	-	-	-
<i>bb</i>	-	-	-	-	1.000	1.000	1.000	1.000	1.000
<i>bc</i>	-	-	0.058	-	-	-	-	-	-
<i>cc</i>	-	-	0.442	0.930	-	-	-	-	-
<i>Mdh-2</i>									
<i>aa</i>	1.000	1.000	0.977	1.000	0.998	0.990	1.000	1.000	1.000
<i>ab</i>	-	-	0.023	-	-	-	-	-	-
<i>bb</i>	-	-	-	-	0.002	0.010	-	-	-
<i>6Pgd-1</i>									
<i>aa</i>	-	-	-	-	1.000	1.000	1.000	1.000	1.000
<i>ab</i>	-	-	0.012	-	-	-	-	-	-
<i>bb</i>	1.000	1.000	0.988	1.000	-	-	-	-	-
<i>6Pgd-2</i>									
<i>aa</i>	-	-	-	-	1.000	1.000	1.000	1.000	1.000
<i>bb</i>	0.987	1.000	-	-	-	-	-	-	-
<i>cc</i>	-	-	1.000	1.000	-	-	-	-	-
<i>dd</i>	0.013	-	-	-	-	-	-	-	-
<i>6Pgd-3</i>									
<i>aa</i>	-	-	0.930	0.912	0.196	0.531	0.691	0.350	0.783
<i>ab</i>	-	-	-	0.053	0.226	0.323	0.270	0.278	0.217
<i>ac</i>	0.013	-	0.058	-	-	-	-	-	-
<i>bb</i>	-	-	-	0.035	0.578	0.146	0.039	0.371	-
<i>bc</i>	-	-	-	-	-	-	-	-	-
<i>cc</i>	0.987	1.000	0.012	-	-	-	-	-	-
<i>Idh-1</i>									
<i>aa</i>	-	0.009	0.930	0.947	0.978	0.969	0.964	0.996	0.935
<i>ab</i>	-	-	0.070	0.018	0.017	-	0.021	-	0.022
<i>ac</i>	-	0.009	-	-	-	-	-	-	-
<i>bb</i>	-	-	-	0.035	0.005	0.031	0.015	0.004	0.043
<i>cc</i>	1.000	0.982	-	-	-	-	-	-	-

<sup>1</sup>All these populations and families were fixed for *Aat-2 aa*, *Mdh-3 aa* and *Mdh-4 aa*.

maintained as populations and the corresponding breeding lines (e.g., *Aat-1* or *Gpi-1* between *C. acutifolium* 350B and 350R7, or *Pgm-1* between *C. pubescens* 495B and 495R6). It is obvious that frequencies of the breeding lines can be partially biased due to the family structure of the sample studied here. Small sample size of maternal genotypes may have a great influence in the genotype frequencies of the progenies, an effect particularly important in species with a predominant self-pollinating mating system. Data on *Centrosema* (Penteado, 1994) indicate that self-pollination frequencies in all samples and species are higher than 50%, and sometimes as high as 85%. Even so, some of the changes are too extensive to be exclusively due to an effect of the family structure of the analyzed samples. On the other hand, taking into account that less than 10 generations have elapsed between the collection of the original samples in the wild and the samples studied here, some changes are too intense to be due to natural selection acting on isozymes (see, for instance, some changes in *Prx-1*). Therefore, we suppose that most likely these changes were caused by genetic drift in the breeding lines due to the small number of plants selected in each generation. Changes of similar intensity, probably partially originated by genetic drift, were observed among the populations maintained in Brazil and Colombia (Penteado *et al.*, 1996). Another factor determining the genetic structure of populations and breeding lines is outcrossing between non-completely isolated field plots, since *Centrosema* species were considered to be mainly self-pollinating. But cross pollination varies widely among populations and can be relatively high (Penteado, 1994; Penteado *et al.*, 1996). Probably the breeding process leads to a tendency to homozygosity and uniformity, but cross-pollination between adjacent plots can maintain some degree of heterozygosity. Our suggestion is that in these partially outcrossing *Centrosema* species the isolation between lines and populations should be reinforced in breeding programs and in the maintenance of germplasm, to avoid intercrossing.

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## RESUMO

Padrões isoenzimáticos e seus respectivos controles genéticos foram descritos para três espécies de *Centrosema*, *C.*

*acutifolium*, *C. brasilianum* e *C. pubescens*. As avaliações foram feitas utilizando-se a técnica de eletroforese horizontal em gel de amido e envolveram 18 populações em estado natural e várias outras selecionadas. Os sete sistemas isoenzimáticos analisados foram: aspartato aminotransferase, fosfoglicose isomerase, fosfoglicomutase, peroxidase anódica, malato desidrogenase, 6-fosfogliconato desidrogenase e isocitrato desidrogenase. Todos os sistemas, exceto fosfoglicose isomerase, são descritos pela primeira vez em *Centrosema*. Detectou-se um total de 17 loci isoenzimáticos, representando o maior conjunto de loci mendelianos conhecidos, até o momento, para o gênero *Centrosema*. O polimorfismo isoenzimático e a variabilidade genética observados entre e dentro de populações e espécies foram relativamente altos. Os padrões isoenzimáticos descritos são muito eficientes na discriminação das espécies estudadas.

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