

Isoenzymatic characterization of hybrids between *Leucaena leucocephala* and *Leucaena diversifolia* ssp. *diversifolia* grown in Rio Grande do Sul (Southern Brazil)

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ABSTRACT

Esterase (EST), superoxidedismutase (SOD) and malic enzyme (ME) isozyme patterns were analyzed in *Leucaena leucocephala* and tetraploid *L. diversifolia* ssp. *diversifolia* and in F2, F3, and F4 hybrid plants. No variability was detected in ME, all individuals presenting only one and the same band ($rm = 0.17$). For SOD clearly different patterns in *L. leucocephala* ($rm = 0.32, 0.40, 0.70$) vs. *L. diversifolia* ($rm = 0.12, 0.35, 0.39, 0.72$) were found. Hybrid plants presented combined patterns and some of them had a new band ($rm = 0.50$), absent in the parental species. In EST a great variation within species and hybrids was verified. Up to nine α ($rm = 0.30, 0.35, 0.40, 0.45, 0.50, 0.55, 0.62, 0.75$ and 0.90) and four β ($rm = 0.10, 0.20, 0.25, 0.28$) bands were detected; some hybrids had bands absent in the parental species. However, no distinctive EST patterns for the species could be established.

INTRODUCTION

Leucaena Benth is an important genus of multi-purpose trees, used for forage, green manure, wood production, handcraft human nutrition and erosion control (Brewbaker, 1989). The genus is native to Central America and was used by pre-Columbian people. Seventeen species were presented by Hughes (1993), but recent data recognize 23 species (Hughes, personal communication).

Due to its multiple uses and great germplasm diversity, this quick-growing, nitrogen fixing tree has been intensively studied in recent years. Nowadays it is widespread along the tropical belt of the world and,

especially among developing countries, this culture has great social importance.

The most widely used species is *Leucaena leucocephala* (Lam.) de Wit, a tetraploid ($2n = 104$) with two subspecies, *leucocephala* and *glabrata*. More than one hundred varieties are known, classified into three types: Hawaiian (shrubby), giant or Salvador (up to 20 m) and Peru (intermediate branched plants up to 15 m) (Brewbaker, 1980, 1987). The Cunningham variety is the most extensively grown cultivar. One of the problems with *L. leucocephala* cultivation is its narrow genetic base, since most of the material originated from one or a few self-pollinated progenies (Hughes, 1993). This species also has severe limitations due to lack of cold and drought tolerance and poor growth in acid soils.

Human influence on *L. leucocephala* evolution is considerable. There are even suggestions that this

species originated in domestication (Harris *et al.*, in press). Few genetic barriers to hybridization exist in the genus and crossability indexes are high enough to produce viable seeds (Sorensson, 1993). Therefore, one of the ways to overcome *L. leucocephala* limitations is hybridization with other species with desirable characteristics.

L. diversifolia (Schlecht.) Benth, a variable species, the most widespread one in its native range, has two subspecies: *ssp. diversifolia*, tetraploid ($2n = 104$) and *ssp. stenocarpa*, diploid ($2n = 52$), a widespread polymorphic taxon. Recent data tend to treat two subspecies as separate species (Hughes, personal communication). After *L. leucocephala*, this is the most widely known and planted species outside its native range (Hughes and Harris, 1995). Spontaneous hybrids between *L. leucocephala* and *L. diversifolia* have been found (Hughes, 1993).

Leucaena has a good potential to be used as a forage in Rio Grande do Sul (Southern Brazil), provided that soil tolerant and cold- and frost-tolerant material is developed. Crosses between *L. leucocephala* and *L. diversifolia ssp. stenocarpa* (diploid) have been made in a search for acid soil-tolerant lines (Hutton, 1984). Advanced generations of these hybrids were examined for chromosome number and meiotic behavior (Freitas *et al.*, 1988) and isozyme patterns (Schifino-Wittmann and Schlegel, 1990).

In order to select cold- and frost-tolerant plants, progenies from crosses between *L. leucocephala* and *L. diversifolia ssp. diversifolia* (tetraploid) have been evaluated in Rio Grande do Sul for morphological, physiological, and cytogenetical characteristics (Freitas *et al.*, 1991a,b, 1995).

MATERIAL AND METHODS

Plants of *Leucaena leucocephala* (5), including cv. Cunningham, *L. diversifolia ssp. diversifolia* (4) and *L. diversifolia ssp. stenocarpa* (2), *L. pulverulenta* (1) and a population of 53 F2, F3, and F4 hybrids of *L. leucocephala* (11/78-15 (K420)) \times *L. diversifolia ssp. diversifolia* (31 (K145)) were examined. Crosses were made by Dr. E.M. Hutton, who kindly supplied the seeds. These hybrids are the same studied by Freitas *et al.* (1991a,b, 1995) and are kept as a living collection at the Agronomic Experimental Station (EEA-UFRGS, Eldorado do Sul township). Seeds from these plants were collected and germinated in petri dishes with soaked filter paper after mechanical scarification. Seedlings were transplanted to pots with soil and grown in a greenhouse. Based on the results of Schifino-Wittmann and Schlegel (1990), young leaves were used, which gives the advantage of

allowing reexamination of the same individual. Electrophoretic procedures were also based on Schifino-Wittmann and Schlegel (1990), with some adaptations and the difference that we used 8% polyacrylamide gels and Scandalios buffer systems (Table I). Gels were run and analyzed on the same day. Relative mobility (rm) was calculated for each band and band patterns established for each individual.

The following enzymes were tested: aspartateaminotransferase (AAT), malatedehydrogenase (MDH), glucosedehydrogenase (GDH), superoxidedis-mutase (SOD), esterase (EST), and malic enzyme (ME). Among these, EST, SOD, and ME allowed the most precise determinations (good resolution and repeatability) of band patterns, and were analyzed in detail.

Table I - Composition of buffers, gels and staining solutions used for *Leucaena* tissue isoenzymes.

1) Extraction, gel and migration buffers (Scandalios, pH 8.3)			
Buffer A -	Lithium borate 0.24 M pH 8.3		
	Lithium hydroxide		1.20 g
	Boric acid		11.89 g
	H ₂ O		to 1000 ml
Buffer B -	Tris citrate 0.0592 M, pH 8.3		
	Tris		6.20 g
	Citric acid		1.60 g
	H ₂ O		to 1000 ml
	Extraction - Buffer A + B (1:9)		
	Migration - Buffer A		
2) Gel 8% - 70 ml Stock solution + 0.7 ml ammonium persulfate (10%)			
	Stock solution		
	Buffer solution A + B (1:9)		200 ml
	Acrylamide		15.20 g
	Bis-acrylamide		0.80 g
	TEMED		0.20 ml
3) Staining solutions			
EST	H ₂ O		40 ml
	Naphthyl acetate 1%		2 ml
	Naphthyl acetate 1%		3 ml
	Fast blue RR Salt		0.05 g
	Buffer C + D		60 ml
Buffer C		Buffer D	
NaH ₂ PO ₄	27.8 g	Na ₂ HPO ₄	53.63 g
H ₂ O	to 1000 ml	H ₂ O	to 1000 ml
SOD	0.2 M Tris HCl, pH 9.0		50 ml
	5 mg/ml MTT		1 ml
	5 mg/ml PMSS		1 ml
ME	Malic acid		100 mg
	NADP		10 mg
	MTT		10 mg
	EDTA		12 mg
	MgCl ₂		50 mg
	Tris/HCl 0.1 M, pH 8.6		25 ml
	+ PMS		

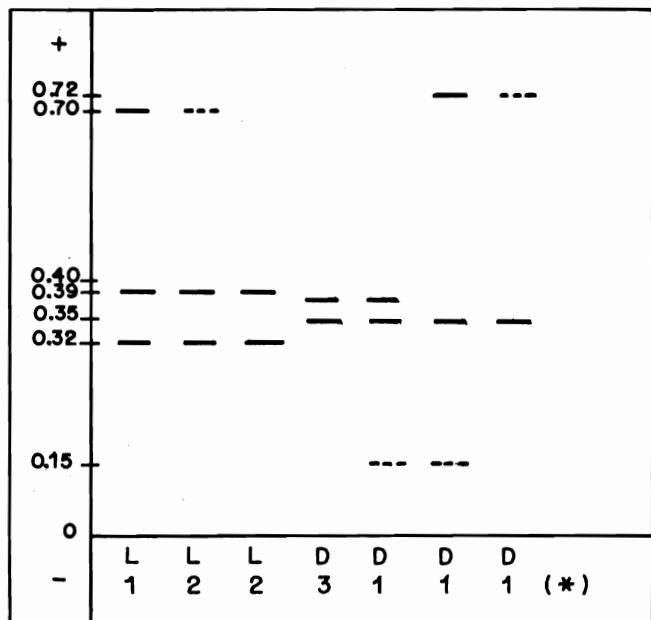
RESULTS AND DISCUSSION

SOD - All individuals analyzed but one presented SOD activity. This individual probably carried a null allele. Technical problems are unlikely since band absence was constant in several repetitions. Patterns are shown in Figures 1 and 2. Clearly different patterns were found for the two parental species and could be used as markers for these taxa. Maximum number of bands per individual was three in *L. leucocephala* (rm = 0.32, 0.40, 0.70) and also in *L. diversifolia*; four different bands were found in this species (rm = 0.12, 0.35, 0.39, 0.72), with no relation to ploidy level. In hybrids (Figure 2) up to four bands per individual were identified. This fourth band (rm = 0.50) probably resulted from a combination of polypeptide chains coded by the parental genes. "Hybrid" bands absent in progenitors are frequent among progenies (Wendel and Weeden, 1990). SOD patterns in the hybrid population presented *L. diversifolia* bands plus the new band, as shown in Figures 1 and 2. This is not in agreement with the data of Freitas *et al.* (1991a,b, 1995), who found predominance of *L. leucocephala* morphological characteristics.

ME - All individuals analyzed were monomorphic, presenting the same band (rm = 0.17). Lack of variability for this enzyme did not allow germplasm

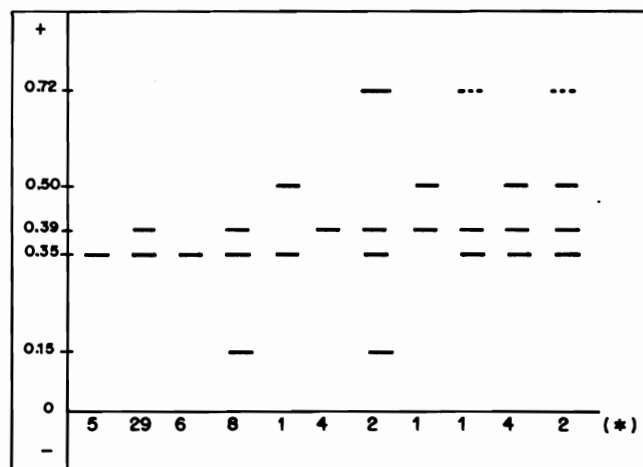
characterization or species and hybrids identification, as also reported by Schifino-Wittmann and Schlegel (1990).

EST - A total of 13 different anodic bands were detected, nine α (rm = 0.30, 0.35, 0.40, 0.45, 0.50, 0.55, 0.62, 0.75, 0.90) and four β (rm = 0.10, 0.20, 0.25, 0.28), ranging from two to six per individual (Figure 3). *L.*



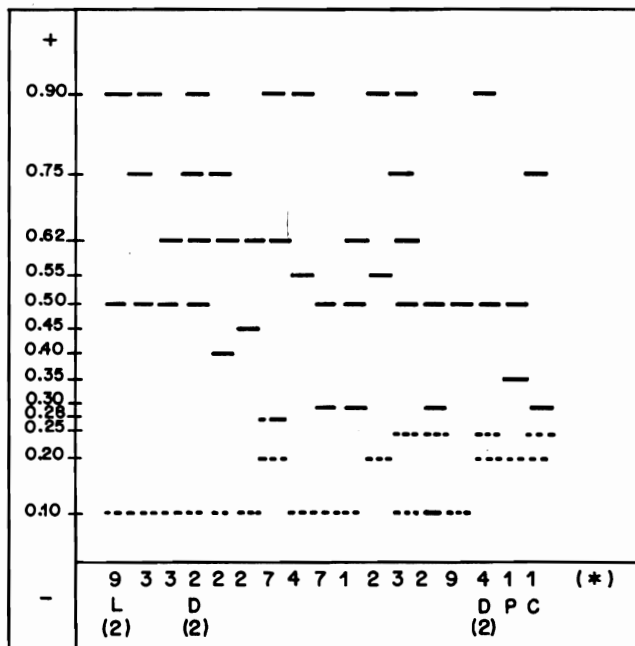
* Number of individuals
 — Strong bands
 ---- Weak bands

Figure 1 - SOD patterns for *Leucaena leucocephala* (L) and *L. diversifolia* (D).



* Number of individuals; —, strong bands; ----, weak bands.

Figure 2 - SOD patterns for hybrids between *Leucaena leucocephala* and *L. diversifolia*.



L - *L. leucocephala*
 D - *L. diversifolia*
 C - *L. leucocephala* cv. Cunningham
 P - *L. pulverulenta*
 * Number of individuals
 — α bands
 ---- β bands

Figure 3 - EST patterns for *Leucaena leucocephala* (L), *L. diversifolia* (D), their hybrids and *L. pulverulenta* (P).

leucocephala cv. Cunningham showed a unique and particular pattern (five bands), in contrast with the other *L. leucocephala* plants (three bands). *L. diversifolia* also showed two different patterns, with no relation to ploidy level. The only individual of *L. pulverulenta* examined had a specific band ($rm = 0.35$). Hybrids showed combined patterns between those of parental species, but also new bands (e.g. $rm = 0.28, 0.40, 0.45, 0.55$), band number ranging from three to six per individual. The band identified as a genetic marker of *L. diversifolia* in *L. leucocephala* × *L. diversifolia* ssp. *stenocarpa* hybrids (Schifino-Wittmann and Schlegel, 1990) was also found here but eventually appeared in some of the *L. leucocephala* individuals that we analyzed.

In general, the isozyme variability detected was low, when compared to the great morphological variation found by Freitas *et al.* (1991a,b, 1995), which could mean that, at least in the *Leucaena* material examined, these isozyme characters are much more conservative.

According to W. Sun (personal communication), isozyme patterns within *Leucaena leucocephala* ssp. *leucocephala* are very uniform and *L. leucocephala* and tetraploid *L. diversifolia* are much less polymorphic than other species. Harris *et al.* (1994) were able to separate the two *L. leucocephala* subspecies (*leucocephala* and *glabrata*) based on three isozyme systems and detected variation among natural populations.

The so often stressed narrow genetic bases found in cultivated or bred *Leucaena* (but not in natural populations), especially in *L. leucocephala* (Hughes, 1993), could partly explain the results presented here. The original *L. diversifolia* progenitors of our material were few and, so a "founder effect" would be expected.

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RESUMO

Padrões isoenzimáticos para esterase (EST); superoxidodismutase (SOC) e enzima málica (ME) foram analisados nas espécies e numa população de híbridos F₂, F₃ e F₄ resultantes de cruzamentos entre *Leucaena leucocephala* e *L. diversifolia* ssp. *diversifolia* (tetraplóide).

Não foi detectada variabilidade para ME, todos os indivíduos apresentando apenas uma banda ($rm = 0,17$). Para SOD, padrões característicos foram estabelecidos para *L. leucocephala* ($rm = 0,32, 0,40, 0,70$) e *L. diversifolia* ($rm = 0,12, 0,35, 0,39, 0,72$). Os indivíduos híbridos apresentaram padrões combinados, alguns mostrando uma nova banda ($rm = 0,50$) ausente nas espécies parentais. Para EST, foi encontrada grande variabilidade intraespecífica, assim como nos híbridos. Nove bandas α ($rm = 0,30, 0,35, 0,40, 0,45, 0,50, 0,55, 0,62, 0,75, 0,90$) e quatro bandas β ($rm = 0,10, 0,20, 0,25, 0,28$)

foram encontradas, alguns híbridos apresentando bandas ausentes nas espécies parentais. Padrões específicos para as espécies parentais não foram encontrados.

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