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Effects of buffer system pH and tissue storage on starch gel electrophoresis of allozymes in three tropical tree species

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Summary — The effects of 16 different electrophoresis buffer pHs, 4 tissue storage conditions and 5 storage times on starch gel electrophoresis of 18 enzymes were determined to design a genetic variation sampling strategy for an isozyme study of 3 tropical tree species, *Racosperma auriculiforme*, *R mangium*, and *Terminalia superba*. The pH of the buffer systems had a significant effect on the number of putative gene loci and alleles resolved, and the staining intensity of the 18 enzymes assayed. For *Racosperma* species, 2 buffer systems B_7 (Tris–citrate gel, pH 9.0: lithium hydroxide–borate electrode, pH 8.5) and H_7 (histidine–EDTA gel, pH 7.6: Tris–citrate electrode, pH 7.7) gave the highest average performance in resolving power. All buffer systems yielded poor results for *Terminalia*. Freezing of *Racosperma* embryos for up to 2 months did not seriously affect enzyme activity. However, freezing cotyledon tissue of *Terminalia* decreased enzyme activity over a 2-month period. In general, frozen tissues either with or without extraction buffer, were consistently better than frozen tissues with extraction buffer and DMSO. Three classes of enzymes were defined, based on their stability under the standardized storage conditions *in vivo*. Using the best buffer systems (B_7 and H_7) and tissue storage conditions (T_0 or T_1), 42, 43, and 32 zones of activity were resolved for *R auriculiforme*, *R mangium*, and *T superba*, respectively. Genetic inference of enzyme variants was made for 31 and 32 putative gene loci in *R auriculiforme* and *R mangium*, respectively. Mean number of putative alleles per locus was 3.0 for *R auriculiforme* and 2.4 for *R mangium*.

buffer system pH / starch gel electrophoresis / allozyme genetic inference / plant material storage / *Racosperma* / *Terminalia* / tropical tree

Résumé — Effets du pH du système de tampons et de la conservation des tissus en électrophorèse sur gel d'amidon d'allozymes chez 3 espèces d'arbres tropicaux. *En vue de planifier une stratégie d'échantillonnage de la variabilité génétique de 3 espèces d'arbres tropicaux,* Racos-

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perma auriculiforme, R mangium *et* Terminalia superba, *les effets de 16 différents pH de tampons d'électrophorèse (tableau I), de 4 conditions de conservation des tissus et de 5 durées de conservation ont été évalués pour l'électrophorèse sur gel d'amidon de 18 enzymes. La résolution du nombre de loci et d'allèles présumés possibles ainsi que l'intensité de coloration des 18 enzymes étaient influencées de manière sensible par le pH des systèmes de tampons. Pour les espèces de* Racosperma, *deux systèmes de tampons, B_7 (Tris-citrate, pH du gel 9.0: hydroxyde de lithium, borate- pH de l'électrode 8,5) et H_7 (histidine, EDTA, pH du gel 7,6: Tris-citrate pH de l'électrode 7,7) ont donné le meilleur pouvoir moyen de résolution (fig 1-11, tableau II). Tous les systèmes de tampons ont entraîné des résultats insatisfaisants chez* Terminalia. *La congélation des embryons de* Racosperma *pour plus de 2 mois n'a pas affecté sérieusement l'activité enzymatique. En revanche, la congélation des cotylédons de* Terminalia *au-delà de 2 mois a diminué l'activité enzymatique. En général, les tissus congélés avec ou sans tampon d'extraction, donnaient constamment de meilleurs résultats que les tissus congélés avec le tampon d'extraction supplémenté de DMSO (fig 12). Trois classes d'enzymes ont été définies, sur la base de leur stabilité sous les conditions* in vivo *standardisées (tableau III). En utilisant les meilleurs systèmes de tampons (B_7 et H_7) et conditions de conservation (T_0 ou T_1), 42, 43 et 32 zones d'activité étaient séparées respectivement pour* R auriculiforme, R mangium *et* T superba. *L'inférence génétique de 31 et 32 loci présumés a été conduite pour* R auriculiforme *et* R mangium, *respectivement (fig 13-17). Le nombre moyen d'allèles présumés par locus était de 3,0 pour* R auriculiforme *et de 2,4 pour* R mangium *(tableau IV).*

pH de tampons d'électrophorèse sur gel d'amidon / inférence génétique d'allozymes / conservation du matérial végétal* / Racosperma / Terminalia / *arbre tropical

INTRODUCTION

Isozyme analysis has been used over the past 2 decades to investigate the genetics of a large number of organisms, from fruit flies and wild herbs to humans (Nevo, 1978). One of the most widely used procedures for studying gene-based variation is through isozyme variation in starch gel electrophoresis. This technique has been especially powerful in the study of population genetics of forest tree species (Mitton, 1983; Hamrick and Loveless, 1989; El-Kassaby, 1991). Powell (1983), Hartl and Clark (1989), Lewontin (1991) and others pointed out that the validity of estimates of polymorphism based on electrophoresis is questionable: the amount of polymorphism may be underestimated because conventional electrophoresis fails to detect many amino acid substitutions. McLellan and Sherman (1991), using non-denaturating electrophoresis, reported that from 40–57% of proteins differing by single amino acids can be separated on a single gel. Their study implies that proteins with different amino acid sequences will have identical electrophoretic mobility in $\approx$ 50% of all comparisons using a single gel. However, the resolving power of electrophoresis could be enhanced by running a sequence of gels at different pH values (sequential electrophoresis), because proteins not separated at one pH may be separated at another. Sequential electrophoresis is presently one of the best methods for distinguishing among protein molecules (McLellan and Inouye, 1986). In the same way, isoelectric focusing (IEF), polyacrylamide gel electrophoresis (PAGE) and 2-dimensional (2-D) gel electrophoresis can be used to study the polymorphism of enzymes (McLellan *et al,* 1983; Görg *et al,* 1988a, 1988b), but the procedures may be difficult to apply to a large number of individuals required for population genetics.

Isozyme analysis requires material suitable for enzyme extraction. Seeds of forest trees, especially gymnosperms, have been extensively used for electrophoretic surveys of genetic variation and for the analysis of mating systems (Cheliak and Pitel, 1984; Adams and Birkes, 1991; El-Kassaby, 1991). The advantages of using seed material for isozyme analysis are, firstly, that storage conditions tend to be simpler than for other tissue types, secondly, that relatively little space is required to store large numbers of genotypes, and thirdly, that newly germinated embryos are relatively free of substances inhibiting enzyme activity (Loomis, 1974; Rhoades and Cates, 1976). However, for tropical species, seed collection is a major problem and it may prove difficult to obtain adequate samples (Gan *et al,* 1981; Liengsiri *et al,* 1990a; Wickneswari, 1991).

Tissue storage, optimum stage of germination, and subsequent storage of extracted enzymes need to be determined for each species (Vigneron, 1984; Pitel and Cheliak, 1986a, 1988; Pitel *et al,* 1989). Methods of protein extraction, electrode and gel buffer preparation, as well as enzyme staining recipes for temperate tree species are well characterized (Conkle *et al,* 1982; Cheliak and Pitel, 1984; Pitel and Cheliak, 1984, 1986a; Bousquet *et al,* 1987). More recently, Kephart (1990) has reviewed the technical aspects of plant enzyme electrophoresis. However, only a few of these procedures have been developed for tropical species (Vigneron, 1984; Hamrick and Loveless, 1986; Liengsiri *et al,* 1990a, 1990b; Wickneswari and Norwati, 1991). As many samples are prepared and analyzed simultaneously, pre-treatments that promote uniform germination of seed samples have also to be determined. With temperate species, once the enzymes have been successfully extracted and protected, they can be stored for extended times at –70 °C with little loss of activity (Cheliak and Pitel, 1984). Liengsiri *et al* (1990a) reported that, for certain tropical species such as *Pterocarpus macrocarpus* and *Dalbergia cochinchinensis*, storage of seed tissue in a refrigerator (4 °C) or a freezer (–20 °C) severely reduced enzyme activity. Cryogenic methods with liquid nitrogen and lyophilization have been used for storage (Hamrick and Loveless, 1986; Santi and Lemoine, 1990) but these facilities are not always available in developing countries. Thus, for tropical species, the challenge is to determine tissue storage, and enzyme extraction conditions which permit long-term storage and optimum resolution in the gels.

This paper reports the effects of 16 values of electrophoresis buffer pH, 4 tissue storage conditons, and 5 storage times on the capacity of starch gel electrophoresis to resolve enzymes from 3 tropical tree species, *Racosperma auriculiforme* (Cunn ex Benth) Pedley (formerly *Acacia auriculiformis*), *R mangium* (Willd) Pedley, comb nov (Formerly *A mangium*), and *Terminalia superba* Engler and Diels. The first 2 belonging to the family Leguminosae, are used as fast-growing trees for fuelwood plantations while the latter, a member of the family Combretaceae, is used for timber production in Zaïre. Genetic inference of enzyme variants is also presented for the first 2 species.

MATERIALS AND METHODS

Source of plant material

Bulked seed collections of 3 tropical tree species: *R auriculiforme* (exotic to Zaire), *R mangium* (exotic to Zaire), *T superba* (indigenous to Zaïre) were used in this study. The seeds of 13 populations of *A auriculiforme* and 13 populations of *R mangium* were provided by the Commonwealth Scientific and Industrial Research Organization (Australia), the Centre de Coopération Internationale en Recherche Agronomique

pour le Développement, Département Forêt (CIRAD-FORÊT) (Congo) and the Service National de Reboisement–Centre Forestier de Kinzono (Zaïre) and those of *Terminalia* were collected at Luki Biosphere Reserve (lat 5°37'S, long 13°6' E, alt 350 m) in Zaïre from 5 parent trees. More details on the origin of the *Racosperma* seeds are given elsewhere (Khasa *et al*, 1993a).

Seed germination

Seeds of *R mangium* were pretreated by immersing 3 vol seeds in 10 vol 100 °C water until cool (12–24 h). Seeds of *R auriculiforme* and *T superba* were chemically scarified with H_2SO_4 95–98% (v/v) for a period of 15 or 30 min, then rinsed under running tapwater for 15 min (Khasa, 1992, 1993). Pretreated seeds were germinated on Kimpak K-22 media (cellulose paper from Kimberly-Clark, WI, USA) in clear seed germination boxes (28 x 24 x 6 cm dimension, from Spencer-Lemaire Industries, Edmonton, Alberta, Canada) as described by Wang and Ackerman (1983). The Kimpaks were initially moistened to saturation point with distilled water. Germination was in Conviron G30 germinators (Controlled Environments, Winnipeg, Manitoba, Canada) with an 8 h–16 h photoperiod (day–night), 30 °C–20 °C temperature regime (day–night), and conditions of high humidity (85% RH). Light was supplied from fluorescent lamps at an intensity of ≈ 12 $\mu mE.m^{-2}.s^{-1}$.

Effect of electrophoresis buffer pH

Enzyme extraction

Newly germinated embryos of *Racosperma* were excised from the seed coat and were placed individually in 0.5-ml conical polystyrene sample cups (Elkay Products, Shewbury, MA). A small quantity (50 µl) of seed extraction buffer (30 mM Tris, 5 mM citric acid, 0.4 mM β-nicotinamide adenine dinucleotide (NAD), 0.2 mM β-nicotinamide adenine dinucleotide phosphate, sodium salt (NADP), 1 mM ascorbic acid, 1 mM ethylenediaminetetraacetate-disodium (EDTA), 0.1% (w/v) bovine serum albumin (BSA), pH adjusted to 7.0 with 1 M citric acid) was added to each cup.

From preliminary studies, cotyledon tissue of *Terminalia superba* was found to be better than radicle tissue for extracting enzymes. Therefore ≈ 100 mg of cotyledon tissue was used with 50 µl complex vegetative extraction buffer (0.05 M boric acid, 2% (v/v) tergitol, 2% (w/v) polyethylene–glycol (PEG 20 M), 7% (w/v) polyvinylpyrrolidone (PVP 40 M), 1% (w/v) PVP 360 M, 50 mM ascorbic acid, 0.4 mM NAD, 0.1% (w/v) BSA, 0.2 mM pyridoxal-5'-phosphate (P-5-P), 0.3 M sucrose, 12 mM cysteine–HCl, 1.3% (v/v) β-mercaptoethanol).

Electrophoresis

Prior to electrophoresis, both fresh and previously frozen embryos or cotyledons were homogenized with a power-driven Teflon rotating tissue grinder. Crude homogenate was absorbed onto 1 x 14 mm wicks cut from Whatman No 3 filter paper and loaded into 12.5% (w/v) starch gels prepared from hydrolyzed starch (Connaught Laboratories, Willowdale, Ontario, Canada). Each gel contained 20 samples of each of the populations of the 3 species and electrophoresis was repeated twice.

Two different running buffer systems (B or H) according to Cheliak and Pitel (1984) and Liengsiri *et al* (1990b) were tested with 16 pH conditions ranging from pH 5.6–9.3 (table I). The electrophoresis was carried out to reveal the activity of 18 enzymes: acid phosphatase (ACP, EC 3.1.3.2), aconitase (ACO, EC 4.2.1.3), aldolase (ALD, EC 4.1.2.13), alkaline phosphatase (ALP, 3.1.3.1), aspartate aminotransferase (AAT, EC 2.6.1.1), diaphorase (DIA, EC 1.8.1.4), esterase-colorimetric (EST-c, EC 3.1.1.1), glucose-6-phosphate dehydrogenase (G6P-DH, EC 1.1.1.49), isocitrate dehydrogenase (IDH, EC 1.1.1.42), leucine aminopeptidase (LAP, EC 3.4.11.1), malate dehydrogenase (MDH, EC 1.1.1.37), malic enzyme (ME, EC 1.1.1.40), nicotinamide adenine dinucleotide dehydrogenase (NADH DH, EC 1.6.99.3), phosphoenolpyruvate carboxylase (PC, EC 4.1.1.31), 6-phospho-gluconate dehydrogenase (6-PGDH, EC 1.1.1.44), phosphoglucose isomerase (PGI, EC 5.3.1.9), phosphoglucomutase (PGM, EC 5.4.2.2), shikimic acid dehydrogenase (SDH, EC 1.1.1.25). These enzymes were stained following Cheliak and Pitel (1984) and Liengsiri *et al* (1990a) with minor modifications.

Table I. Buffer systems and electrophoretic conditions tested.

Buffer system [a]	*Gel buffer pH*	*Electrode buffer pH*	*Electrophoretic conditions* [b]
H_1	5.6	5.8	
H_2	6.0	5.8	
H_3	6.3	6.5	
H_4	6.7	6.5	I = 50 mA/gel
H_5	7.0	7.1	T = 4–5 h
H_6	7.3	7.1	
H_7	7.6	7.7	
H_8	8.0	7.7	
B_1	7.3	7.8	
B_2	7.6	7.8	
B_3	8.0	8.1	
B_4	8.3	8.1	I = 30 mA/gel
B_5	8.5	8.1	T = 4–5 h
B_6	8.8	8.5	
B_7	9.0	8.5	
B_8	9.3	8.5	

[a] B gel buffer: 0.03 M Tris, 0.005 M citric acid, 1% (v/v) B electrode buffer; pH adjusted with 1 M Tris; B gel buffer is diluted in a 1:9 ratio before use (*ie* 1 vol B gel buffer: 9 vol distilled water); B electrode buffer: 0.06 M lithium hydroxide, 0.3 M boric acid; pH adjusted with 1 N NaOH except for B_1 with 1 M citric acid; H gel buffer: 0.05 M histidine-HCl, 1.4 mM EDTA; pH adjusted with 1 M Tris; H gel buffer is diluted in a 1:4 ratio before use (*ie* vol H gel buffer: 4 vol distilled water); H electrode buffer: 0.125 M Tris, pH adjusted with 1 M citric acid. [b] I = Current intensity applied to the HBI 500 V and Ephortec™ 1 000 V power supplies (Haake Buchler Instruments, NJ, USA), respectively for B and H gels. *T* = duration of electrophoresis.

The resolving power and the staining intensity were evaluated for each enzyme and pH condition by using a 6-step score (0 = bad resolution, 1 = poor, 2 = average, 3 = good, 4 = very good, 5 = excellent) and by estimating the migration distance of the common allozyme (standard) within a zone of activity compared to the total distance that the buffer front migrated (R_f). For each pH buffer system, the scores were averaged for all the enzyme zones assuming 5 as a maximum score and expressed as a percentage of the maximum score in order to identify the best buffer system.

Effect of tissue storage conditions and freezing periods

In this experiment, 4 tissue storage conditions and 5 storage times were examined. The storage conditions were: T_1 (frozen tissue without extraction buffer), T_2 (frozen tissue immersed in 50 µl of extraction buffer), T_3 (frozen tissue immersed in 20 µl of dimethyl sulfoxide (DMSO) acting as a cryoprotective agent (see Kephart, 1990) + 30 µl extraction buffer), T_4 (frozen tissue immersed in 30 µL DMSO + 20 µl extraction buffer). T_0 (fresh tissue) was considered as the standard. For T_1–T_4, the 5 storage times tested were: 1 wk, 1, 2, 3 and 6 months. Before grinding the samples, 50 µl of sample extraction buffer was added to the treatments T_0 and T_1 and frozen tissues were allowed to thaw. Extraction buffers and methods used in this experiment were those described above. For each combination of species, tissue storage conditions, and storage times, twenty samples were then run following protocols described in Experiment 1 by using B_7 and H_7 buffer systems, which proved to be the most reliable (see below). Enzyme activity was also assessed visually using a 6-step score as above, where 0 means no enzyme activity and 5 is the standard corresponding to the enzyme activity in fresh tissue. For each combination of tissue, storage condition and storage time, the scores were also averaged across the enzyme zones and expressed in percentage relative to the standard (T_0) to define the average remaining percentage of enzyme activity (ARPEA), which was used to identify the best tissue storage condition and storage time.

Genetic inference of enzyme variants in Racosperma

Using the best buffer systems (B_7 and H_7) and tissue storage conditions (T_0 or T_1) presented herein (see below), genetic inference of enzyme variants for *Racosperma* species was performed by comparison with results previously reported in these species (Moran *et al,* 1989a, b) and by the examination of the active subunit composition of each enzyme. At least 60 seeds from each of 13 different populations for each *Racosperma* species were analysed for the inference of allozymes. Putative allelic identity was con-

firmed across populations within species by running different populations on the same gel. When more than one zone of activity was detected for a particular enzyme, the most-anodally-migrating zone of activity (nominally a putative locus) was designated as 1 and any other numbered according to decreasing mobility. Within each putative gene locus, the most anodal allozyme (nomically a putative allele) was assigned the number 1, 2 was the next most anodal and so on. R_{fa} is the mobility of the various allozymes. For each species, the mean number of putative alleles per locus (A_s), including the null alleles, was calculated following the formula $A_s = 1/m\ \Sigma a_i$ where m is the number of putative loci scored, and a_i is the number of putative alleles observed at locus i. Because of the small sample size used and the poor resolution obtained in *Terminalia superba*, genetic inference of enzyme variants was not conducted in this species.

RESULTS

Effect of electrophoresis buffer pH

The 18 enzyme systems used in this study displayed 42, 43, and 32 zones of activity for *R auriculiforme*, *R mangium*, and *T superba*, respectively (see below for description). The effect of buffer pH on some of these zones is shown in figures 1–10. In general, clear and consistent zones of activity were observed for both *Racosperma* species while poorly resolved zones were typical for *T superba*.

The buffer systems resulting in highest levels of enzyme activity and resolving power across the different enzyme systems assayed for the 3 species are shown in table II. Certain enzymes such as ACO, ALP, MDH, and SDH proved to have broad pH range tolerance, particularly for *T superba*. On the basis of the averaged scores in percentage, B_7 and H_7 were the best buffer systems among the different B and H buffer systems assayed for both *Racosperma* species (fig 11). For *T superba*, B_7, B_8 and H_7 were the best buffer systems but displayed poor resolution and weak staining intensities for most of the enzymes tested.

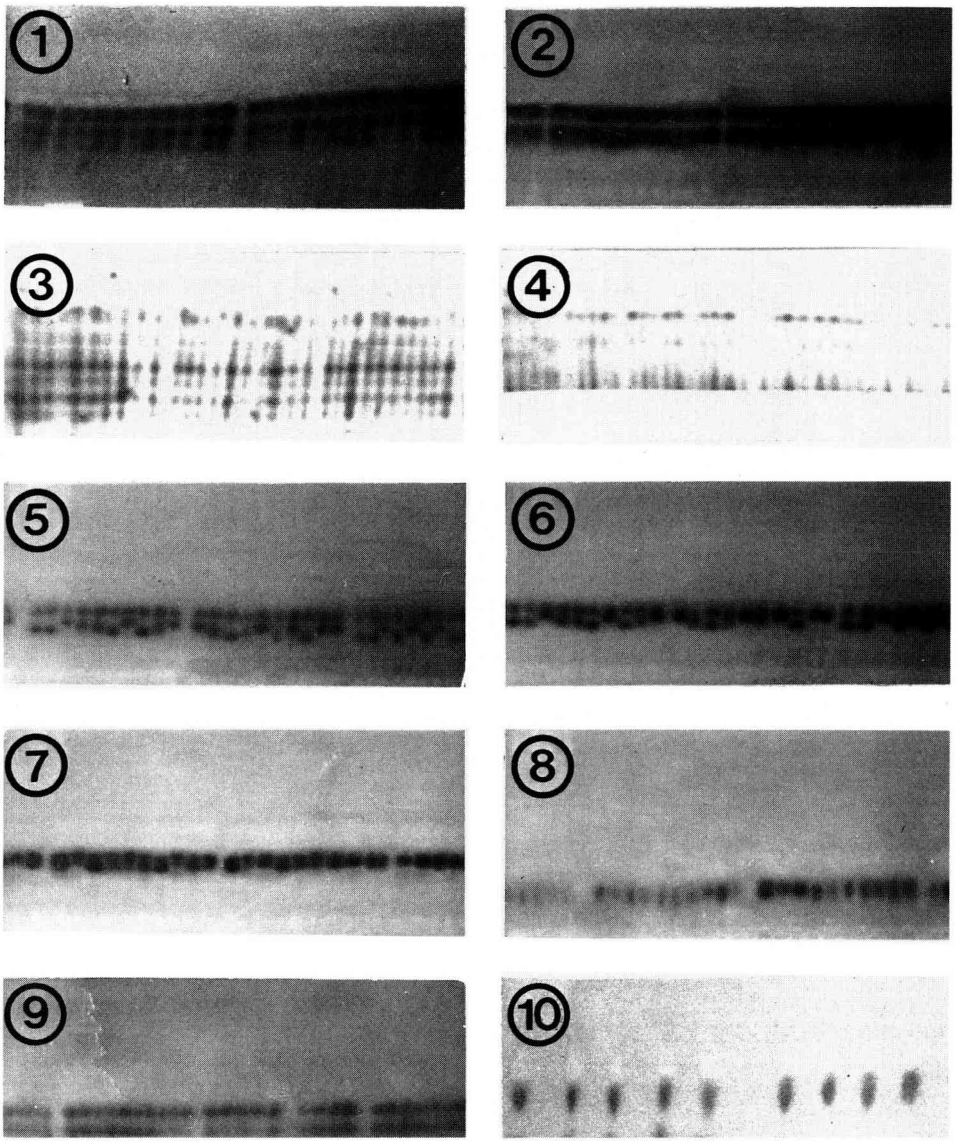
Acid phosphatase (ACP)

When analysed with the B_5 buffer system, 3 zones of activity were observed for each of the 3 species. The R_f's were 0.32, 0.16, and –0.03 for both *Racosperma* species. The third zone (Acp#3) was stained on the cathodal strip. For *T superba*, the R_f's were 0.32, 0.22, and 0.12. Using the B_7 buffer system, all 3 zones migrated anodally in *Racosperma*.

Table II. Buffer systems resulting in highest levels of resolving power and enzyme activity for 18 enzyme systems [a].

Enzyme system	*Buffer system* [b]		
	R auriculiforme	R mangium	T superba
ACP	B_5	B_5	B_5
AAT	B_7	B_7	B_8
DIA	B_7	B_7	B_7/B_8
EST-C	B_7	B_7	B_7
LAP	B_7/B_8	B_7/B_8	B_8
NADHDH	B_7	B_7	–
PC	B_7	B_5	B_8
PGI	B_7/B_8	B_7/B_8	B_8
ACO	H_6/H_7	H_7/H_8	$H_4/H_5/H_6/H_7$
ALD	H_8	H_8	H_8
ALP	H_6/H_7	H_5/H_7	H_6/H_7
G_6P-DH	H_6/H_7	H_6/H_7	H_6/H_7
IDH	H_7	H_7	H_3/H_7
MDH	H_1	H_1	$H_2/H_3/H_4$
ME	H_6/H_7	H_6	H_3/H_4
6-PGDH	H_7	H_7	H_2
PGM	H_7	H_7	H_7/H_8
SDH	H_4/H_6	H_2	$H_2/H_6/H_8$

[a] Raw scores avaible from the authors upon request; [b] see table I for definition of buffer systems.



Figs 1–10. 1. PGM enzyme activity of *Racosperma* using H_7 buffer system. Note the presence of 3 zones of activity. **2.** PGM enzyme activity of *Racosperma* using H_3 buffer system. Note the presence of only 2 zones of activity. **3.** EST-c enzyme activity of *Racosperma* using B_7 buffer system. Note the presence of 8 zones of activity. **4.** EST-c enzyme activity of *Racosperma* using B_3, B_4 and B_5 buffer systems. Note the presence of only 3 poorly resolved zones of activity. **5.** 6-PGDH enzyme activity of *Racosperma* using H_7 buffer system. Note the presence of 2 well resolved zones of activity. **6.** 6-PGDH enzyme activity of *Racosperma* using H_3 buffer system. Note the presence of 2 zones of activity closer than in figure 5. **7.** G6P-DH enzyme activity of *Racosperma* using H_7 buffer system. Note the presence of 1 well resolved zone of activity. **8.** G6P-DH enzyme activity of *Racosperma* using H_5 buffer system. Note the presence of 1 zone of activity showing a blur. **9.** PGI enzyme activity of *Racosperma* using B_7 buffer system. Note the presence of 2 well resolved zones of activity. **10.** PGI enzyme activity of *Racosperma* using B_3 buffer system. Note the presence of only 1 poorly resolved zone of activity showing a tail, the second zone is too close to the origin of the gel.

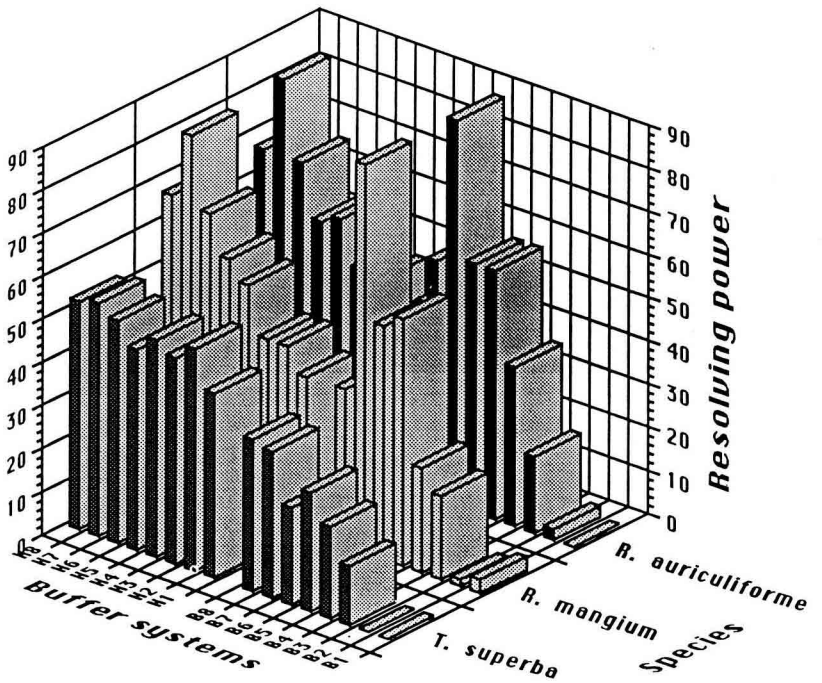


Fig 11. Average resolving power of 18 enzymes for 3 tropical tree species and 16 buffer systems.

Aconitase (ACO)

Using the H_7 buffer system, 2 zones of activity having R_f's of 0.50 and 0.38 were detected for both *Racosperma* species. The more anodal zone was achromatic whereas the second developed a bluish background. Only one blue background zone with R_f of 0.64 was present for *T superba*, when analysed with H_4 buffer system.

Aldolase (ALD)

Using the H_8 buffer system, 2 zones stained for both *Racosperma* species with R_f's of 0.30 and 0.12. The more anodal zone probably represents a single locus with a total of 3 single-banded phenotypes while the variants appeared as single-banded phenotypes in the more cathodal zone (Ald#2). For *T superba*, one clear and consistent band with R_f of 0.22 could usually be observed.

Alkaline phosphatase (ALP)

A singe zone of activity was observed with R_f's of 0.18 and 0.10 for *Racosperma* species and *T superba*, respectively, when analysed with H_6 buffer system. A 1.5-mm slice is preferred because thinner gel slices showed weak staining of bands.

Aspartate aminotransferase (AAT)

Using the B_7 buffer system, 3 zones of activity were detected. The R_f's were 0.35, 0.27, 0.09 for *R auriculiforme*, 0.27, 0.23, 0.09 for *R mangium* and 0.36, 0.22, 0.18 for *T superba*. With the B_5 buffer system, the most cathodal zone (Aat#3) for *Racosperma* species was close to the origin of the gel and was indistinct and unscorable. This suggests a zwitterion, which has its isoelectric point close to the pH condition used.

Diaphorase (DIA)

With the B_7 buffer system, 3 zones of activity were evident. However only 2 zones could be consistently scored. The R_f's of the first 2 zones were 0.32 and 0.24 for both *Racosperma* species. For *T superba*, the 2 zones were indistinct and impossible to score.

Esterase-colorimetric (EST-c)

With the B_7 buffer system, 7 and 8 zones of activity were observed for *R auriculiforme* and *R mangium* respectively, with R_f's of 0.60, 0.50, 0.41, 0.29, 0.21, 0.14, 0.07 and 0.60, 0.50, 0.45, 0.40, 0.34, 0.29, 0.21, 0.14. When B_3 and B_4 buffer systems were used, the 3 least anodal zones (Est#6, Est#7 and Est#8) stained on the cathodal strip and the resolution was bad. *T superba* showed 4 zones of activity for EST-c but they were poorly resolved.

Glucose-6-phosphate dehydrogenase (G6P-DH)

When analysed with the H_7 buffer system, a single zone of activity was evident in the 3 species. Staining intensity and resolving power were poor for *T superba*. The R_f's of the observed zone were 0.36 and 0.40 for *R auriculiforme* and *R mangium* respectively, and 0.31 for *T superba*.

Isocitrate dehydrogenase (IDH)

Using the H_7 buffer system, one zone of activity was observed with R_f's of 0.40 and 0.38 for *Racosperma* species and *T superba*, respectively.

Leucine aminopeptidase (LAP)

Two zones of activity were detected when analysed with the B_7 or B_8 buffer system. The R_f's were 0.39 and 0.32 for both *Racosperma* species. When analysed with any other buffer system, the 2 zones were close and indistinguishable. Two poorly resolved zones were also observed for *T superba*.

Malate dehydrogenase (MDH)

Using the H_7 buffer system, 3 zones of activity (Mdh#1, Mdh#2, Mdh#3) could usually be observed, with R_f's of 0.35, 0.29, 0.06 for the *Racosperma* species, and 4 zones with R_f's of 0.42, 0.41, 0.15, –0.06 for *T superba*. For the *Racosperma* species, the most anodal (Mdh#1) stained intensely, the next most anodal (Mdh#2) was often obscured by the excessively heavy stain at Mdh#1 so that slight mobility differences at Mdh#2 may often be difficult to detect. A putative interzone was apparently present in population of *R auriculiforme* between Mdh#2 and Mdh#3 (Khasa *et al*, 1993a). The third zone (Mdh#3) displayed faint bands in *Racosperma* species. For *T superba*, the first 2 zones were comigrating. While Mdh#1, Mdh#2, and Mdh#3 stained on the anodal gel strip, the fourth zone was stained on the cathodal strip when the H_1 buffer system was used. Using the H_8 buffer system, Mdh#4 was very close to the origin of the gel but migrated anodally for *T superba*.

Malic enzyme (ME)

Two zones of activity with R_f's of 0.39 and 0.12 were observed for *Racosperma* species and *T superba* when analysed with the H_7 buffer system.

Nicotinamide adenine dinucleotide dehydrogenase (NADHDH)

Three zones of activity were observed for the *Racosperma* species, but the most anodal zone stained inconsistently and therefore could not be scored. The most cathodal zone (Nadhdh#3) was faint. With the B_7 buffer system, the R_f's of Nadhdh#2 and Nadhdh#3 were 0.26, 0.21 and 0.23, 0.19 for *R auriculiforme* and *R mangium*, respectively. No enzyme activity was detected for *T superba*.

Phosphoenol pyruvate carboxylase (PC)

When analysed with the B_5 buffer system, 1 zone of activity with R_f of 0.17 was observed for the 3 species. As staining is strong but washes off the gel quickly, this enzyme must be scored at the optimal time.

Phosphoglucose isomerase (PGI)

With the B_7 buffer system, 2 zones of activity (Pgi#1 and Pgi#2) were observed. The R_f's were 0.30 and 0.18 for both *Racosperma* species. When the B_3 buffer was used, the second zone Pgi#2 was too close to the origin to be scored. Only 1 zone of PGI activity was detected for *T superba* with an R_f of 0.30.

Phosphoglucomutase (PGM)

When analysed with the H_7 buffer system, 3 zones of activity were observed for the *Racosperma* species. Under low pH conditions, only 2 zones could be scored. The R_f's for *R auriculiforme* and *R mangium* were 0.52, 0.38, 0.33 and 0.46, 0.38, 0.33 respectively. For *T superba*, 2 zones with R_f's of 0.48 and 0.27 were detected.

6-phosphogluconate dehydrogenase (6-PGDH)

When analysed with the H_7 buffer system, 2 zones with R_f's of 0.40 and 0.33 were found for the 3 species. At high pH conditions, only the second zone was detected for *T superba*.

Shikimic acid dehydrogenase (SDH)

With the H_2 buffer system, a single zone of activity with an R_f of 0.41 was found for *Racosperma* species. Two zones stained strongly with R_f's of 0.35 and 0.23 for *T superba*.

Effect of tissue storage conditions and storage times

Based on their sensitivity to cold storage after different tissue storage times and following 4 storage conditions, we have defined 3 classes of enzyme (table III): 1) high stability enzymes (HSE) which include AAT, EST, 6-PGDH, PGI, and PGM for which between 67–100% of the enzyme activity remained for the best treatment after 6 months of freezing; 2) medium stability enzyme (MSE) including ACP, ALP, G6-PDH, LAP, and MDH (recovery between 33–66%); 3) low stability enzyme (LSE) including ACO, ALD, DIA, IDH, PC, ME, NADHDH, and SDH (recovery < 33%). The average recovery for the 18 enzymes assayed (fig 12) indicated that embryos of *Racosperma* may be stored in a freezer for

Table III. Average remaining percentage of enzyme activity ARPEA for 3 levels of enzyme stability, 5 tissue freezing periods and 4 storage conditions (T_1–T_4) [a].

Enzyme	*Freezing period* [b]	*Species*											
		R auriculiforme				R mangium				T superba			
		T_1	T_2	T_3	T_4	T_1	T_2	T_3	T_4	T_1	T_2	T_3	T_4
HSE [c]													
	1 W	100 (0) [f]	100 (0)	98 (2)	90 (9)	100 (0)	100 (0)	87 (9)	87 (9)	74 (30)	69 (28)	51 (31)	46 (31)
	1 M	94 (8)	99 (3)	94 (8)	85 (8)	95 (6)	99 (5)	85 (9)	83 (7)	59 (27)	60 (28)	37 (32)	27 (23)
	2 M	89 (10)	85 (7)	87 (10)	74 (9)	85 (10)	82 (6)	77 (7)	77 (10)	13 (22)	14 (23)	11 (23)	18 (22)
	3 M	81 (4)	83 (4)	78 (11)	52 (18)	73 (8)	79 (3)	78 (6)	65 (18)	0	0	0	0
	6 M	74 (6)	78 (8)	72 (10)	55 (23)	71 (8)	69 (8)	67 (16)	57 (31)	ND [g]	ND	ND	ND
MSE [d]													
	1 W	100 (0)	93 (9)	78 (18)	66 (17)	99 (2)	96 (8)	82 (18)	78 (15)	78 (20)	82 (18)	56 (52)	56 (52)
	1 M	86 (13)	77 (12)	74 (22)	56 (18)	82 (8)	84 (7)	72 (8)	62 (15)	68 (18)	75 (19)	35 (39)	39 (40)
	2 M	70 (19)	66 (7)	53 (18)	52 (18)	72 (10)	68 (10)	60 (6)	52 (9)	29 (20)	23 (24)	19 (26)	22 (23)
	3 M	56 (15)	49 (15)	36 (26)	34 (24)	59 (15)	55 (9)	46 (26)	39 (23)	7 (15)	9 (19)	9 (19)	8.7 (19.4)
	6 M	45 (14)	40 (19)	33 (26)	30 (26)	50 (12)	51 (11)	46 (26)	30 (29)	ND	ND	ND	ND
LSE [e]													
	1 W	95 (7)	86 (17)	64 (22)	51 (22)	93 (9)	84 (16)	65 (19)	60 (17)	72 (31)	71 (30)	50 (43)	42 (35)
	1 M	57 (21)	55 (21)	42 (20)	32 (17)	64 (20)	63 (21)	50 (18)	44 (10)	31 (30)	38 (34)	17 (20)	16 (23)
	2 M	41 (33)	36 (32)	25 (22)	20 (12)	46 (24)	45 (30)	36 (14)	20 (19)	15 (25)	11 (19)	12 (20)	13 (23)
	3 M	14 (16)	7 (10)	10 (15)	3 (6)	18 (29)	24 (26)	21 (14)	17 (19)	8 (21)	5 (14)	6 (17)	7 (18)
	6 M	4 (7)	4 (8)	3 (5)	2 (4)	8 (17)	11 (23)	8 (10)	6 (11)	ND	ND	ND	ND

[a] Storage conditions: T_0 (fresh tissue), T_1 (frozen tissue without extraction buffer), T_2 (frozen tissue immersed in 50 µL of extraction buffer), T_3 (frozen tissue immersed in 20 µL of DMSO) + 30 µL extraction buffer), T_4 (frozen tissue immersed in 30 µL DMSO + 20 µl extraction buffer). [b] Freezing period: 1 week, 1, 2, 3, 6 months. [c] HSE: high stability enzymes including AAT, EST, 6-PGDH, PGI and PGM. [d] MSE: medium stability enzymes including ACP, ALP, G_6-PDH, LAP, and MDH. [e] LSE: low stability enzymes including ACO, ALD, DIA, IDH, PC, ME, NADHDH and SDH. [f] Parenthetical numbers are standard deviations. [g] ND: not determined.

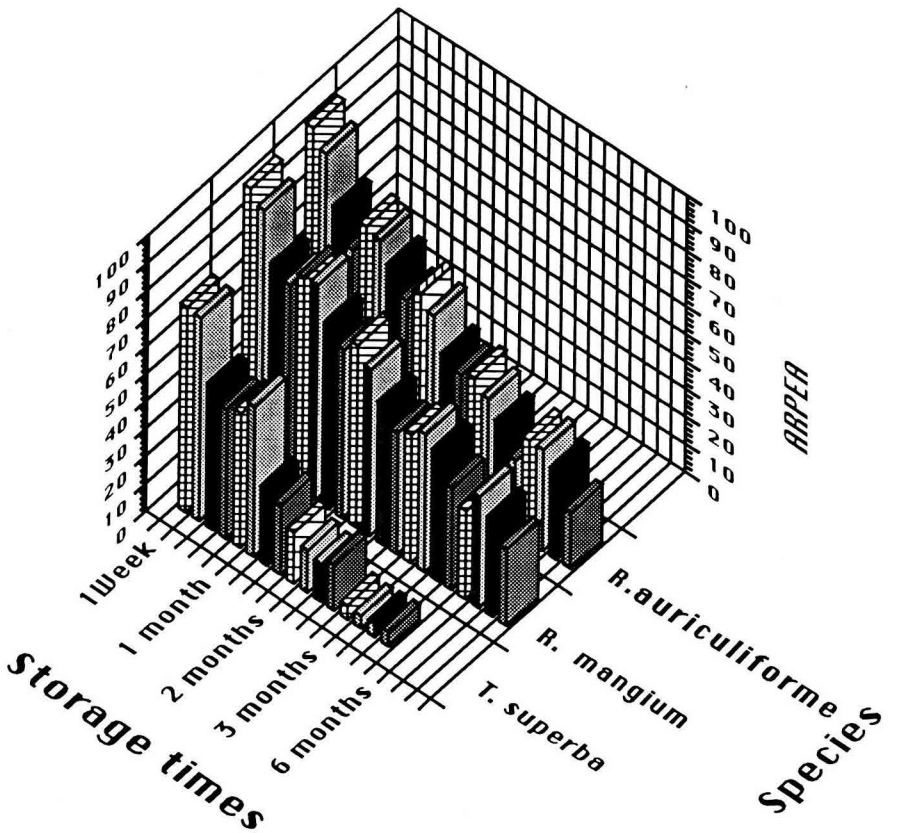


Fig 12. Average remaining percentage of enzyme activity (ARPEA) for 5 tissue storage times and 4 storage conditions for 3 tropical tree species and 2 buffer systems. ▨ : T1; ▨ : T2; ■ : T3; ▨ : T4.

≈ 2 months and still show an average recovery > 60% whereas an average recovery of ≈ 50% was observed after 1 month of cotyledon tissue freezing for *T superba*. After 2 months of freezing for *T superba*, the average recovery was < 50% and the results were highly variable from one enzyme to the other as indicated by the large standard deviations (results available from the authors upon request). The storage conditions T_1 and T_2 resulted in the highest enzyme activity on average with apparently no significant difference whereas addition of DMSO (T_3 and T_4) decreases enzyme activity markedly. Preliminary studies indicated that weaker banding patterns were also obtained when glycerol was used as a cryoprotectant.

Genetic inference of enzyme variants

Putative gene loci and allozyme variants in *Racosperma* are presented in table IV. Thirty-one and 32 putative gene loci were inferred for *R auriculiforme* and *R mangium*, respectively. Of these loci, 28 and 25 were polymorphic for *R auriculiforme* and *R mangium*, respectively. The average numbers of putative alleles per locus were 3.0 and 2.4 for *R auriculiforme* and *R mangium*, respectively, and the respective numbers of putative alleles per polymorphic locus were 3.4 and 3.1. Zymogram phenotypes for monomorphic or polymorphic putative isozyme loci in *Racosperma* are illustrated in figures 13–17. In the present study, inheritance of enzyme variants was not inferred in *Terminalia* but has been proposed for some loci by Vigneron (1984).

Table IV. List of putative loci and allozymes observed in *R auficuliforme* and *R mangium*.

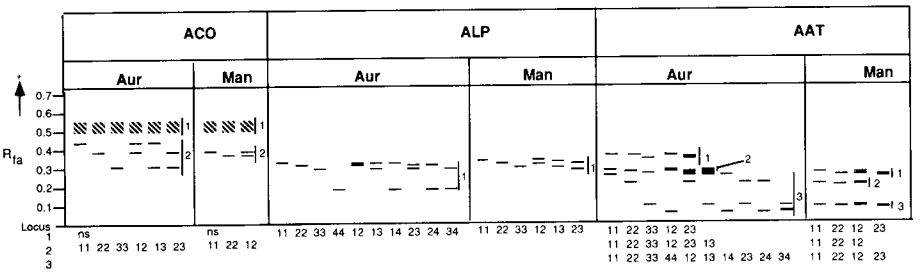
Enzyme [a]	*Buffer system used* [b]	*Locus*	*No of alleles detected at each locus*	
			R auriculiforme	R mangium
ACO	H_7	*Aco-2*	3	2
ALP	H_7	*Alp*	4	3
AAT	B_7	*Aat-1*	3	3
		Aat-2	3	2
		Aat-3	4	3
EST-c	B_7	*Est-1*	4	3
		Est-2	4	3
		Est-3	3	4
		Est-4	3	3
		Est-5	3	3
		Est-6	3	2
		Est-7	1	1
		Est-8	–	1
G_6P-DH	H_7	*G6pdh*	3	3
IDH	H_7	*Idh*	3	1
LAP	B_7	*Lap-1*	4	3
		Lap-2	4	3
MDH	H_7	*Mdh-1*	2	1
		Mdh-3	2	3
ME	H_7	*Me-1*	3	3
		Me-2	4	3
NADHDH	B_7	*Nadhdh-2*	4	3
		Nadhdh-3	2	1
PC	B_7	*Pc*	1	1
PGI	B_7	*Pgi-1*	3	3
		Pgi-2	3	3
PGM	H_7	*Pgm-1*	3	3
		Pgm-2	3	3
		Pgm-3	4	3
6-PGDH	H_7	*6Pgdh-1*	1	1
		6Pgdh-2	3	2
SDH	H_7	*Sdh*	4	2

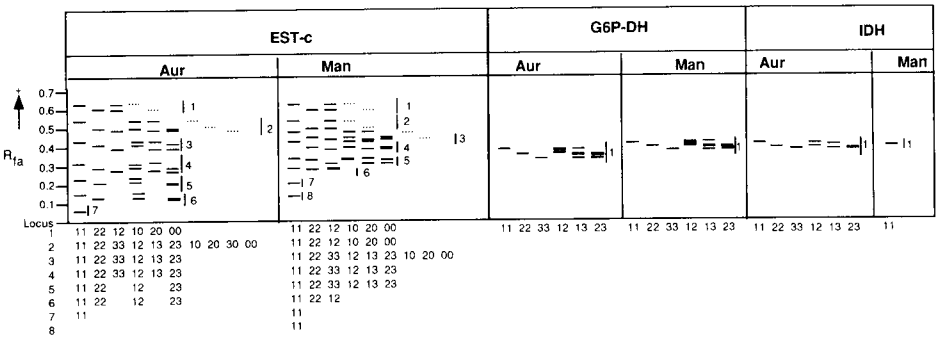
[a] See *Materials and Methods* for enzyme nomenclature; [b] see table I for definitions. –: does not exist.

DISCUSSION

Starch gel electrophoresis has been frequently used in a successful manner for surveys of isozyme variation. However, this technique is susceptible to quantitative inaccuracy and irreproducibility (Gordon *et al*, 1988; Walters *et al*, 1989; Kephart, 1990) because of inconsistent staining, uninterpretable inheritance patterns, or other problems. Many factors may be responsible for these problems (Kephart, 1990). Some of these factors include the nature of the tissue and type of storage conditions, protocols for enzyme extraction, starch gel preparation and electrophoretic conditions, gel and electrode buffer characteristics, staining and genetic interpretation of enzyme phenotypes. The production of unreliable or reliable results depends much more on the skills of the experimenter.

In our first experiment, we have investigated the effect of buffer pH (B and H gel and electrode buffers) on the resolving power and staining intensities of electromorphs. Follwing a conservative approach by altering the pH of the buffer systems in increments of ≈ 0.4 pH units (Kephart, 1990), it was possible to identify the best


ACO
ALP
AAT
Aur
Man
Aur
Man
Aur
Man
+
Rfa
0.7
0.6
0.5
0.4
0.3
0.2
0.1
Locus
1
2
3
ns
11 22 33 12 13 23
ns
11 22 12
11 22 33 44 12 13 14 23 24 34
11 22 33 12 13 23
11 22 33 12 23
11 22 33 12 23 13
11 22 33 44 12 13 14 23 24 34
11 22 12 23
11 22 12
11 22 12 23


EST-c
G6P-DH
IDH
Aur
Man
Aur
Man
Aur
Man
+
Rfa
0.7
0.6
0.5
0.4
0.3
0.2
0.1
Locus
1
2
3
4
5
6
7
8
11 22 12 10 20 00
11 22 33 12 13 23 10 20 30 00
11 22 33 12 13 23
11 22 33 12 13 23
11 22 12 23
11 22 12 23
11
11 22 12 10 20 00
11 22 12 10 20 00
11 22 33 12 13 23 10 20 00
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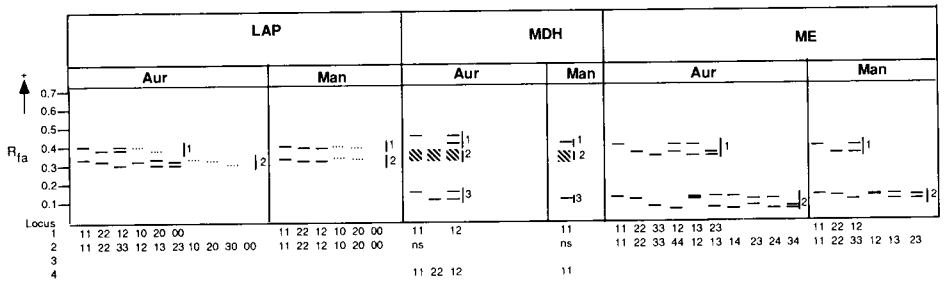
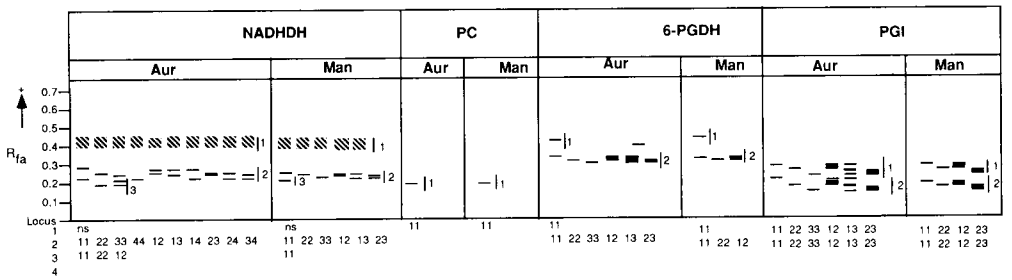
11 22 33 12 13 23
11 22 33 12 13 23
11 22 33 12 13 23
11


LAP
MDH
ME
Aur
Man
Aur
Man
Aur
Man
+
Rfa
0.7
0.6
0.5
0.4
0.3
0.2
0.1
Locus
1
2
3
4
11 22 12 10 20 00
11 22 33 12 13 23 10 20 30 00
11 22 12 10 20 00
11 22 12 10 20 00
11
ns
11 22 12
12
ns
11
11 22 33 12 13 23
11 22 33 44 12 13 14 23 24 34
11 22 12
11 22 33 12 13 23


NADHDH
PC
6-PGDH
PGI
Aur
Man
Aur
Man
Aur
Man
Aur
Man
+
Rfa
0.7
0.6
0.5
0.4
0.3
0.2
0.1
Locus
1
2
3
4
ns
11 22 33 44 12 13 14 23 24 34
11 22 12
ns
11 22 33 12 13 23
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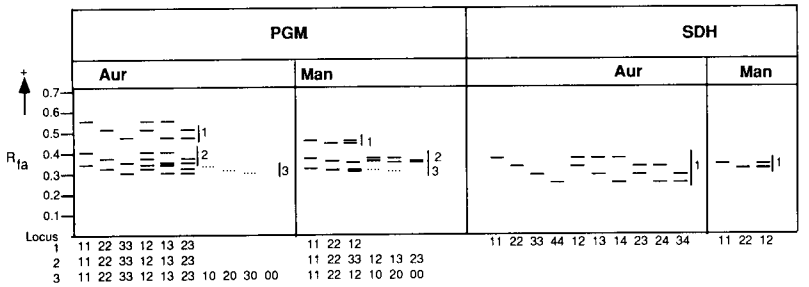
11 22 33 12 13 23
11 22 33 12 13 23
11 22 12 23
11 22 12 23


PGM
SDH
Aur
Man
Aur
Man
+
Rfa
0.7
0.6
0.5
0.4
0.3
0.2
0.1
Locus
1
2
3
11 22 33 12 13 23
11 22 33 12 13 23
11 22 33 12 13 23 10 20 30 00
11 22 12
11 22 33 12 13 23
11 22 12 10 20 00
11 22 33 44 12 13 14 23 24 34
11 22 12

pH conditions of buffers for each of 18 enzyme systems (table II). Using 12 gel-electrode buffer systems, 22 out of 40 enzyme systems assayed in *Vitis* species were successfully resolved (Walters *et al,* 1989). However, a compromise must be struck between resolving power in terms of the number of zones scored, and keeping the whole process economical to avoid unnecessary expenditure of chemicals and time. Using only H and B running buffer systems in 3 tropical tree species (*Pterocarpus macrocarpus, Dalbergia cohcinchinensis*, and *Pinus kesiya*), a satisfactory number of zones of enzyme activity was obtained by staining 15 enzyme systems (Liengsiri *et al,* 1990b). Likely, from our results of average scores (fig 11), we recommend the use of B_7 and H_7 buffer systems for *Racosperma* species to stain satisfactorily the 18 enzymes used in this study.

According to results presented in table IV, the number of putative loci and alleles resolved was greater than that previously reported by Moran *et al* (1989a, b) in *Racosperma*. Using Tris–citrate and morpholine-citrate buffers and 18 enzymes, Moran *et al* (1989b) scored 30 loci in *R mangium*, as compared to 32 putative loci from 18 enzymes in this study. From 12 enzyme systems assayed in *R auriculiforme*, Moran *et al* (1989a) scored 19 loci, as compared to 31 putative loci from 18 enzymes in this study. The mean number of putative alleles per locus (A_s) was 3.0 and 2.4 respectively for *R auriculiforme* and *R mangium* in this study, as compared to published estimates of 2.5 and 1.4, respectively (Moran *et al,* 1989a, b). Different sampling and analytical procedures such as buffer systems and pH conditions likely account for most of these differences. In addition, more polymorphic loci have been observed in this study, especially for *R mangium* which has been described as genetically depauperate (Moran *et al,* 1989b).

The resolution of extracts of *T superba* was poor when compared to *Racosperma*, even with B_7 and H_7 buffer systems. But the number of zones resolved in this study (32) was substantially greater than that reported by Vigneron (1984), who scored 4 zones for EST-c, 3 zones for AAT, 2 zones for LAP, 2 zones for PGM, 1 zone for PGI, 2 zones for ACP, and 2 zones for MDH. A browning effect was also observed in *Terminalia* homogenates when the radicle was used instead of cotyledon tissues, suggesting a loss of enzyme activity resulting from phenoloxidase products (see Kephart, 1990). It is obvious that buffer systems developed for one species may have to be modified depending on the tissue and enzyme of interest for another species. In that case, the divide and conquer approach (Kephart, 1990), where a wide variety of buffer systems under different pH conditions and enzyme combinations must be applied with prior experimentation on different extraction buffers and different volume to various plant tissue ratios.

Proteins, which are zwitterions, carry positive and negative charges. Their net charge, and thus their migration depends on the pH of the buffer system. For example, using the B_3 and B_5 buffer systems respectively, even with longer run times, Pgi#2 and Aat#3 zones for *Racosperma* species were close to the origin of the gel

Figs 13–17. Diagrams of banding patterns for enzyme systems in *R auriculiforme* and *R mangium.* At the bottom of the figure, the inferred genotypes and allelic designations are given for each putative locus resolved satisfactorily; ns means not scored. The vertical axis shows the R_{fa} of the various putative allozymes. Bands indicated by dashed lines are putative heterozygotes with null allele while a blank for the same locus is the putative null allele. Numbers at the vertical lines of the right of the zymogram indicate the zone of activity (nominally a putative locus).

and did not migrate because at their isoelectric points, these isozymes are electrically neutral and do not migrate. For some pH conditions (B_3, B_4, B_5 and H_3), 3 zones of EST-c (Est#6, Est#7, Est#8), 1 zone of ACP (Acp#3), and a few bands of ALP migrated cathodally for the *Racosperma* species, as did Mdh#4 for *T superba.* At high pH conditions, these electromorphs migrated anodally. Therefore the pH affects the charge and separation of enzymes allowing the possibility of increasing the number of zones of enzyme activity. It is relevant to note that the R_f values and numbers of bands may also change if different electrode and gel buffers and/or pH conditions are used. This point is well illustrated in this study for enzymes ALP, MDH, PC and SDH (see R_f values of enzyme description in results and figures 13–17). On the other hand, most allozyme surveys use gels with pH values close to the isoelectric point of the proteins, so that a given difference in charge of the molecules produces a greater difference in mobility than if a pH far from the isoelectric point was used (McLellan, 1984).

The way to achieve the desired pH is another concern for some enzymes. For example, we did not obtain good resolution for EST-c even with the B_7 and B_8 buffer systems when NaOH (0.1 N) was used to adjust the pH, as suggested by Liengsiri *et al* (1990a). This could be explained by the fact that NaOH increases the ionic strength ($Ic = 1/2 \Sigma z_i^2 c_i$, where z_i is the valency of the ion and *ci* its concentration) in the buffer. Buffers of high ionic strength result in greater heat production (Andrews, 1981; see Kephart, 1990). Heating was also observed when the differential pH between electrode and gel buffers was large. Gelfi and Righetti (1984) also reported a relationship between the pH gradient, buffering power, and ionic strength. Therefore, we recommend that the pH of B gel buffers be adjusted by using 1 M Tris because this organic compound produces weaker ionic strength even when it increases molarity. Hence, buffers used to prepare gels are more sensitive than the electrode buffer where acidic or basic titrant may be used to adjust pH. For enzymes of broad pH range (tolerance), the composition and molarity of buffer solutions are critical in improving resolution.

Our results showed that the length of time that plant tissues could be refrigerated or frozen and still retain enzyme activity was variable between species, tissues, and enzymes. For high stability enzymes, it was possible to detect at least 60% of remaining enzyme activity for the best tissue storage condition after a 6 month freezing period of *Racosperma* embryos. Other researchers reported a great loss of activity and indistinct banding pattern for several enzyme systems from other plant species after short periods of storage (see Kephart, 1990; Liengsiri *et al,* 1990a). On the other hand, after 2 months, freezing cotyledon tissue of *T superba* yielded unsatisfactory enzyme activity. Heterogeneity was also observed from one enzyme to the other, some being more stable than others regarding tissue storage in frozen conditions. *In vitro* experimentation would be necessary to confirm the 3 classes of enzyme stability defined here *in vivo.*

The storage conditions T_1 and T_2 were the best, indicating that the possible stabilization effect of DMSO during long periods of freezing is not evident for these species. Apparently, its addition only served to dilute the extract, thus decreasing banding intensity. Smaller quantities of cryoprotectant would be necessary to test this hypothesis. On the other hand, a net positive effect of DMSO to stabilize extracts during long periods of ultra-cold storage was reported in other species, when tissues were homogenized prior to storage (Kephart, 1990). It is likely that the potential stabilization effect of DMSO is more dependent on

extraction procedure and technique of storage than on species: perhaps with whole tissues, DMSO more readily diffuses across cell membranes than other extraction buffer components, or selectively transports certain buffer components, resulting in adverse internal concentrations detrimental on enzyme activity. Thus, DMSO-treated tissues might show decreased activity primarily for this reason and in addition to any species specific or dilution effects. In contrast, with mechanically homogenized extracts treated with DMSO, cells are disrupted, and the protective agents of the extraction buffer might be able to reach and better protect the enzymes, likely resulting in a net positive effect of DMSO relative to tissues homogenized in buffer only. Further investigations are needed to clarify these questions. As T_1 was slightly superior to T_2 for *Racosperma*, it also seems to freeze better the *Racosperma* embryos without the extraction buffer. Probably, in presence of extraction buffer, the cell walls may rupture more quickly than its in absence, then exposing enzymes to denaturing secondary metabolites.

Furthermore, for all operations, except incubation, it is essential to keep the temperature at or below 4 °C. Even if an array of operating temperatures was not tested in this study, it has generally been demonstrated that when the temperature is above 40 °C, the activity of most enzymes decreases severely (Pitel and Cheliak, 1986a). Finally, the pretreatments to ensure uniform germination are of great importance as many samples are analysed simultaneously and as differential germination of seeds could bias results of allozyme variation in the population (Crawford, 1990). In fact, scarifying seeds with H_2SO_4 instead of hot water gave better enzyme activity especially for *R auriculiforme* while hot water was better for *R mangium.* Nicking the seed coat of *Racosperma* species has also yielded uniform germination (Moran *et al,* 1989a, b; Wickneswari and Norwati, 1991).

The loci and allozyme variants inferred herein are considered putative as they are not fully supported by segregation studies. However, consistent scoring was possible across the populations investigated with the proposed genetic models. Some of these genetic models have been used successfully to study the mating system using single-tree progeny genotype arrays (Khasa *et al,* 1993b). Analysis of progeny arrays from controlled crosses or half-sib families is necessary to confirm the genetic models, especially for loci where null alleles were detected. This is the case for Est#1, Est#2, Est#3, Lap#1, Lap#2, and Pgm#3. Among the loci scored, Moran *et al* (1989a, b) did not report null alleles. However, null alleles were reported in *Larix laricina* and other conifers (Cheliak and Pitel, 1985; Iewandowski and Mejnartowicz, 1990), and in *Terminalia superba* (Vigneron, 1984). The presence of null alleles may be related to seed aging, either from prestorage treatment, or due to long storage times in the seed bank (Cheliak and Pitel, 1985), or due to artifacts resulting in using systems with synthetic substrates (Pitel *et al* 1987).

Regarding enzyme subunit composition and compartmentalization, little information exists on tropical species (Vigneron, 1984; Wickneswari, 1991). No such information exists in *Racosperma* studies by Moran *et al* (1989a, b). As suggested by Bousquet *et al* (1987) and Crawford (1990), the use of pollen as the source of enzymes and the examination of the progeny of seeds from controlled crosses or half-sib families, should be undertaken to study the active subunit compositions of some enzymes such as AAT, IDH, MDH, ME, which did not clearly agree with the published literature (Crawford, 1990; Kephart, 1990).

Modifying effects of enzyme subunit structure during extraction and/or electrophoretic procedures may occur, producing atypical heterozygotes. Such atypical heterozygous isozyme patterns may result from both asymmetry of position or asymmetry of staining intensity (up to total loss) of one homopolymeric or the heteropolymeric form (Richardson *et al,* 1986).

In view of the important variation in enzyme activity and resolution of banding patterns encountered by using different tissue preparations, different electrophoresis buffer pH's as well as the significant variation between species and enzymes, it seems necessary, for every new species being studied, to assess the effects of extraction buffers, buffer systems, and conditions of material storage and handling on the activity of a number of enzyme systems, ideally in a factorial design. Furthermore, a genetic base that is broad enough to be representative of allozyme variants likely to be encountered for the species of interest should be used. In addition, within any good buffer system, this study showed that different pH conditions of the buffer system may be required to obtain optimal enzyme resolution and staining, all other sources of variation being held constant. If accurate analytical methods are developed at an early stage, the number of loci and alleles will be maximized and the genetic inference of allozyme variants will be more consistent and less subject to unexpected adjustments in the course of or after the experimental phase of the study.

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