CHARACTERIZATION OF ISOZYMES OF THREE TROPICAL TREE SPECIES - EFFECT OF EXTRACTION AND RUNNING BUFFERS ON STAINING INTENSITY AND RESOLUTION

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LIENGSIRI, C., PIEWLUANG, C. & BOYLE, T.J.B. 1990. Characterization of isozymes of three tropical tree species - effect of extraction and running buffers on staining intensity and resolution. The staining intensity and resolution of 27 enzyme systems using a combination of eight extraction buffers and four gel-electrode buffer systems was assessed for various tissue types in *Pterocarpus macrocarpus, Dalbergia cochinchinensis* and *Pinus kesiya.* Embryos of all three species stained well for most enzyme systems. Young leaf extracts of *P. macrocarpus* and *D. cochinchinensis* produced stains for many enzyme systems, but dormant buds failed to produce enzyme activity. Needle tissues of *P. kesiya* were not assayed, but megagametophytes were easily stained. Based on preliminary trials, two additional extraction buffers were devised and tested to combine the best characteristics of several others. Recommendations are given for buffer systems to be used and enzymes to be assayed for all three species.

Key words: Tropical trees - isozymes - electrophoresis - extraction - staining - resolution

Introduction

The forest resources of tropical southeast Asia have been heavily exploited, especially since the end of the Second World War. In tropical Asia, 1.8 million *ha* of closed forest were lost per year in the period 1976 to 1985 (United Nations 1986), and it is only recently that active management of these resources has been undertaken. Effective management requires a thorough understanding of many aspects of the biology, ecology, and genetics of the species involved. Such knowledge is particularly difficult to obtain in the tropical zone because of the immense species diversity.

Genetic studies can be used to identify superior populations (provenances), and play a role in the subsequent selection and breeding of the most desirable individuals within these populations. Genetic conservation efforts, which are of critical importance in the tropics, will also benefit from a survey of the amount and distribution of genetic variation within a species. The technique of isoenzyme (isozyme) analysis provides a rapid and relatively economical method to obtain such information. Many types of tissue may be sampled for isozyme analysis, and the final choice of tissue depends on its availability and biochemical activity.

Seeds provide some of the easiest tissues for analysis because storage tends to be simpler than for other tissue types and newly germinated embryos are relatively free of the "secondary plant products" that can inhibit enzyme activity (Loomis 1974). For these reasons, despite problems associated with the restriction of sampling to only seed-producing trees, the use of seed for isozyme analysis is very common. When tissue that is relatively free from damaging agents (such as phenols, quinones, and tannins) is used, the electrophoretic process is greatly simplified. Torres *et al.* (1978), studying isozymes in avocado (*Persea americana*) mesocarp, simply squashed samples onto filter paper wicks. Similarly, Torres (1974) homogenized cotyledons of mature sunflower (*Helianthus annuus*) with a simple Tris-HCl buffer.

For tissues containing higher concentrations of secondary products, measures must be taken to prevent damage by removing phenolic compounds. Such techniques have been reviewed by Loomis and Battaile (1966), Anderson (1968), Loomis (1974), Haissig and Schipper (1978), and Loomis *et al.* (1979) among others. They include control of pH to prevent ionic interactions, the addition of various polymers to absorb phenols and prevent reactions with enzymes, and the addition of compounds to prevent quinone production (Loomis & Battaile 1966, Anderson 1968). In this last category various thiols, such as cysteine and mercaptoethanol and similar compounds like ascorbic acid, have been found to be most effective (Anderson 1968). Also, bovine serum albumin has been found to bind phenols.

Leaf tissue is a very convenient source of enzymes because it is usually plentiful except in deciduous species during the period of dormancy. However, leaves also tend to contain high concentrations of secondary plant products and are also subject to rapid deterioration. Gan *et al.* (1981) experienced some difficulty in obtaining satisfactory resolution of isozymes in leaves of *Xerospermum intermedium* because of high concentrations of tannins, which required the addition of several protective agents to the extraction buffer in order to improve resolution. To overcome problems of degradation in using leaf tissue of many species of tropical trees, Hamrick and Loveless (1986) employed vacuum drying on the same day as collection, followed by storage at -65°*C* until the samples were assayed. Using this technique, no loss of enzyme activity was detected after storage for several months.

Occasionally, other tissue types may also be used, such as dissected buds and young bark tissue of several conifer species (Pitel & Cheliak 1984). Pollen is another tissue type that is relatively free from secondary plant products, and has been used in studies of imcompatibility in maize (Makinen & Brewbaker 1967, Makinen & MacDonald 1968). However, pollen obviously suffers from many of the same drawbacks as seed in terms of availability, and collection, and storage of pollen is quite difficult for many tropical species.

In this study, we characterised the isozymes of three tropical tree species using starch gel electrophoresis, and examined the effect of extraction and running buffers on staining intensity and resolution.

Materials and methods

Eight extraction buffer solutions (Table 1) and up to four running buffer combinations (Table 2) were used to characterize isozymes in mature vegetative tissue and embryonic tissue of *Pterocarpus macrocarpus* and *Dalbergia cochinchinensis* and in megagametophytic and embryonic tissue of *Pinus kesiya*. The intensity and resolution of the stains were assessed visually as ranging from no staining, or weak and indistinct, to strong and distinct.

Table 1. Extraction buffers used in the study

Number	Formulation				Reference
1	Tris	0.03*M*	Ascorbic acid	0.001*M*	Yeh & O'Malley
	Na citrate		EDTA	0.01*M*	(1980) (modified)
	NAD	0.4*mM*	Bovine albumin	0.1%	
	NADP	0.2*mM*			
			pH adjusted to 7.0 with 0.1*M* Tris		
2	Boric acid	0.05*M*	NAD	0.4*mM*	Craker *et al.*
	Tergitol	2%	Bovine albumin	0.1%	(1969) (modified)
	PEG 20M	2%	P-5-P	0.2*mM*	
	PVP 40M	7%	Sucrose	0.3*M*	
	PVP 360M	1%	Cysteine-HCl	12*mM*	
	Ascorbic acid	50*mM*	β-mercaptoethanol	1.3%	
			pH adjusted to 7.1 with 1*M* Tris		
3	Tris-Hcl pH 7.5	0.1*M*	10% $MgCl_2$	2%	Feret (1971)
	Ascorbic acid	0.01*M*	10% $CaCl_2$	2%	(modified)
	Cysteine-HCl	5.4*mM*	Sucrose	0.5*M*	
	Tween 80	1%	β-mercaptoethanol	1%	
			pH adjusted to 7.5 with 1*M* Tris		
4	PVP 40M	7%	Bovine albumin	0.1%	Cheliak (1983)
	PVP 360M	1%	NAD	0.4*mM*	
	Sucrose	0.3*M*	NADP	0.3*mM*	
	EDTA	0.5*mM*	P-5-P	0.2*mM*	
	Dithiothreitol	1*mM*	β-mercaptoethanol	0.7%	
	Ascorbid acid	1*mM*			
			pH adjusted to 6.7 with 1*M* Tris		
5	Tris-HCl pH 8.0	0.1*M*	Ascorbic acid	6*mM*	Feret (1971)
	Sucrose	0.5*M*	Tween 80	1%	
	Cysteine-HCl	6*mM*			
			pH adjusted to 8.0 with 1*M* Tris		
6	Tris-HCl pH 7.5	0.1*M*	NAD	0.4*mM*	Feret (1971)
	Sucrose	0.5*M*	Bovine albumin	0.1%	
	Cysteine-HCl	6*mM*	Dithiothreitol	1*mM*	
	Ascorbic acid	6*mM*	EDTA	0.5*mM*	
	Tween 80	1%	Tergitol 15-S-9	1%	
	PVP 40M	7%	β-mercaptoethanol	0.7%	
	PVP 360M	1%			
			pH adjusted to 7.5 with 1*M* Tris		
7	Borate pH 9.8	0.05*M*	PEG 20M	1%	Craker *et al.*
	Tergitol 15-S-9	1%	β-mercaptoethanol	0.3%	(1969)
			pH adjusted to 9.8 with 1*N* NaOH		
8	Phosphate pH 6.8	0.1*M*	Tween 80	1%	Riov & Brown
	PVP 40M	2%	PEG	2%	(1976) (modified)
			pH adjusted to 6.8 with 1*M* phosphate (NaH_2PO_4)		
9	Tris-HCl pH 8.0	0.1*M*	$MgCl_2$	0.2%	
	Ascorbic acid	0.01*M*	$CaCl_2$	0.2%	
	Cysteine-HCl	5.4*mM*	PEG20M	1%	
	Sucrose	0.5*M*	Tergitol	1%	
	Tween 80	1%	β-mercaptoethanol	0.3%	
			pH adjusted to 8.0 with 1*M* Tris		
10	Borate pH 8.0	0.05*M*	$MgCl_2$	0.2%	
	Ascorbic acid	0.01*M*	$CaCl_2$	0.2%	
	Cysteine-HCl	5.4*mM*	PEG20M	1%	
	Sucrose	0.5*M*	Tergitol	1%	
	Tween 80	1%	β-mercaptoethanol	0.3%	
			pH adjusted to 8.0 with 1*N* NaOH		

Abbreviations: NAD - Nicotinamide adenine dinucleotide; NADP - Nicotinamide adenine dinucleotide phosphate; EDTA - Ethylene diamine tetra-acetic acid; PEG - Polyethylene glycol; PVP - Polyvinyl pyrrolidine; P-5-P- Pyridoxal-5-phosphate

Notes: All percentages are volume-by-volume, with the exceptions of bovine albumin, PEG, PVP, $MgCl_2$, and $CaCl_2$. b-mercaptoethanol added after pH adjustment.

Table 2. Running buffer systems

Name	Electrode buffer		Gel buffer		Reference
H	Tris	0.125*M*	Histidine-HCl	0.05*M*	Pitel & Cheliak (1984)
			EDTA	1.4*mM*	
	pH adjusted to 7.0 with 1*M* citric acid		pH adjusted to 7.0 with 1*M* Tris		
B	Lithium hydroxide	0.06*M*	Tris	0.03*M*	Ridgeway *et al.* (1970)
	Boric acid	0.3*M*	Citric acid	0.005*M*	
			Electrode buffer B	1%	
	pH adjusted to 8.1 with 1*N* NaOH		pH adjusted to 8.5 with 1*N* NaOH		
P	Boric acid	0.31*M*	Tris	0.08*M*	Schaal & Anderson (1974)
	NaOH	0.063*M*			
	pH adjusted to 8.1 with NaOH or Boric acid		pH adjusted to 8.65 with 1*M* Citric acid		
TC	Tris	0.13*M*	Electrode buffer TC		Siciliano & Shaw (1976)
	Citric acid	0.043*M*	diluted in 1:14 ratio		
	pH adjusted to 7.0 with 10*N* NaOH				

Notes: H gel buffer is diluted in a 1:4 ratio before use; B gel buffer is diluted in a 1:10 ratio before use; pH of P gel buffer must be checked and readjusted if necessary immediately before use.

Mature vegetative tissue was prepared by grinding approximately 2 *cm* of freshly collected, immature leaf samples in about 0.02 *ml* of the extraction buffer, using a rotating teflon grinding head, or by grinding, at room temperature, one vegetative bud with 0.5 *ml* of the buffer, and a small quantity of sterile sand using a mortar and pestle. Embryonic angiosperm tissue was prepared by imbibing seed for two or three days, then grinding the emerging embryonic root in 0.02 *ml* of the extraction buffer, again using a rotating teflon grinding head. For *P. kesiya*, seed can be stored at 4°C for long periods without significan deterioration. In preparation for isozyme analysis, seed was imbibed for between four and six days, and the emerging embryo was separated from the megagametophyte when the embryonic radicle was approximately 5 *mm* emerged from the seed coat.

In all cases, the resulting homogenate was absorbed onto filter paper (Whatman No. 3) wicks, approximately 14 × 1 *mm* in dimension. These are then introduced into a 12.5% starch (Connaught Laboratory, Ontario, Canada) gel by making a vertical cut along the long axis of the gel, about 1 *cm* from one edge, placing the wicks vertically along the resulting edge, and then pushing the two slabs together. The procedure for electrophoresis was similar to that described by Liengsiri *et al.* (1990). Once the enzyme had migrated a sufficient distance through the gel, as indicated by the progress of a coloured dye (about 5 *cm*), the gels were sliced into 1 *mm* thick slices, which were then stained for the enzymes shown in Table 3.

Table 3. Enzyme systems assayed

Name	E.C. Code	Abbreviation
Adenylate kinase	2.7.4.3	AK
Alcohol dehydrogenase	1.1.1.1	ADH
Aldolase	4.1.2.13	ALD
Aspartate aminotransferase	2.6.1.1	AAT
Diaphorase	1.6.4.3	DIA
Esterase	3.1.1.1	EST
Fumarase	4.2.1.2	FUM
Glucose dehydrogenase	1.1.1.47	GLUDH
Glucose-6-phosphate dehydrogenase	1.1.1.49	G6PDH
Glutamate dehydrogenase	1.4.1.3	GDH
Glyceraldehyde-3-phosphate dehydrogenase	1.2.1.12	G3PDH
Glycerate-2-dehydrogenase	1.1.1.29	G2DH
Hexokinase	2.7.1.1	HK
Isocitrate dehydrogenase	1.1.1.42	IDH
Leucine aminopeptidase	3.4.11.1	LAP
Malate dehydrogenase	1.1.1.37	MDH
Malic enzyme	1.1.1.40	ME
Menadione reductase	1.6.99.2	MR
Nicotinamide adenine dinucleotide dehydrogenase	1.6.99.3	NADHDH
Nicotinamide adenine dinucleotide phosphate dehydrogenase	1.6.99.1	NADPHDH
Peptidase	3.4.13.11	PEP
Phosphoenolpyruvate carboxylase	4.1.1.31	PC
Phosphoglucomutase	2.7.5.1	PGM
6-Phosphogluconic dehydrogenase	1.1.1.44	6PGD
Phosphoglucose isomerase	5.3.1.9	PGI
Shikimic acid dehydrogenase	1.1.1.25	SDH
Succinate dehydrogenase	1.3.99.1	SUDH

After a series of preliminary trials, two additional extraction buffers (numbers 9 and 10, Table 1) were developed in an attempt to improve on the overall staining intensity and resolution of as many enzymes as possible.

Results and discussion

For all three species, embryonic tissue stained well for the majority of enzyme systems, using many combinations of extraction and running buffer systems (Tables 4, 5 & 6). Young leaf extracts of *Pterocarpus macrocarpus* and *Dalbergia cochinchinensis* also yielded good results in many cases but, for both species, enzyme activity rapidly deteriorated with increasing age of the leaves. This was associated with distinctive browning or reddening of the tissue homogenate, especially for some extraction buffers (*e.g.* numbers 1, 5, and 8). Such discolouration of the homogenate is typically the result of oxidation or polymerization of enzyme functional groups by quinones, which are powerful oxidizing agents (Anderson 1968, Loomis 1974). The prevention of quinone production can be achieved by the addition of protective agents such as cysteine, mercaptoethanol, ascorbic acid, or bovine serum albumin (Anderson 1968). Therefore, the addition of, or an increase in concentration of such agents in those extraction buffers that failed to prevent browning might improve their efficacy. The use of tissue dissected from dormant axillary buds of both species

Table 4. *Pterocarpus macrocarpus* staining results for embryonic tissue (above) and leaf tissue (below) (Symbols: - weak staining; + good staining, fair resolution; * good staining and resolution; blank = no test)

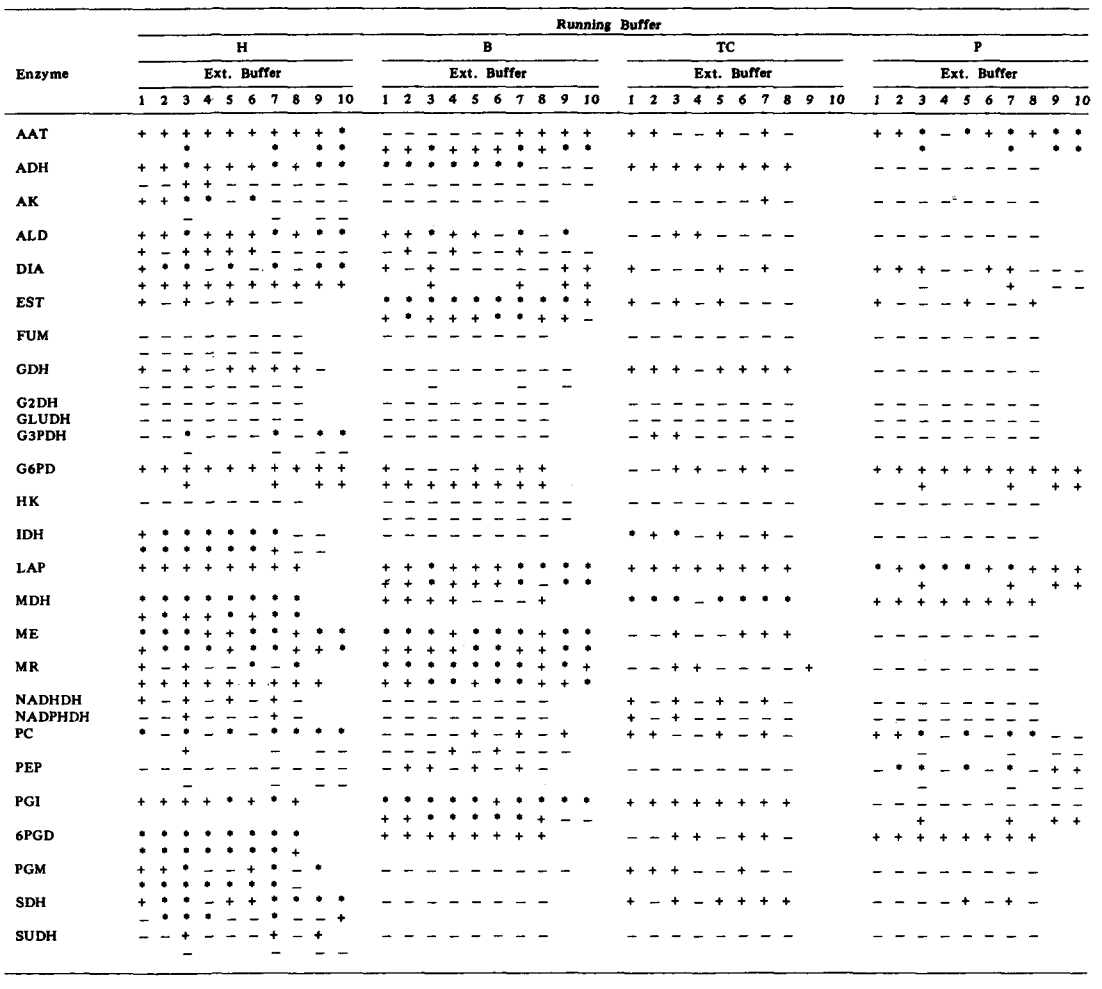

Table 5. *Dalbergia cochinchinensis* staining results for embryonic tissue (above) and leaf tissue (below); symbols as in Table 4

Enzyme	Running Buffer: H — Ext. Buffer 1	2	3	4	5	6	7	8	9	10	B — Ext. Buffer 1	2	3	4	5	6	7	8	9	10	TC — Ext. Buffer 1	2	3	4	5	6	7	8	9	10	P — Ext. Buffer 1	2	3	4	5	6	7	8	9	10
AAT	•	•	•	•	•	•	•	•	•	•	+	+	+	+	+	+	+	+	+	+	•	•	•	•	•	•	•	•			+	+	•	+	+	+	•	+	•	•
			–				–		–	+	–	–	–	–	–	–	–	–	+	+												–							+	–
ADH	•	•	•	•	•	•	•	•	•	•	•	–	+	•	•	+	–	–	+	–	•	•	•	•	•	•	•	•			–	–	–	–	–	–	–	–		
	–	–	–	–	–	–	–	–	+	•	–	–	–	–	–	–	–	–	–	–																				
AK	•	–	•	–	•	•	•	•	+	+	–	–	–	–	–	–	–	–			•	–	+	–	+	+	+	+			–	–	–	–	–	–	–	–		
	–	–	–	–	–	–	–	–	•	•																													
ALD	–	–	•	–	+	+	•	+	•	•	+	–	+	+	+	+	+	+	+		+	–	+	+	+	+	+	+			–	–	+	+	–	–	–	–		
	+	+	•	+	+	+	•	+	+	•																													
DIA	+	+	•	+	+	+	+	+	+	•	•	•	•	•	•	•	•	•	•	•	+	+	+	+	+	+	+	+			+	–	–	–	–	–	–	–	–	–
	–	+	+	–	+	–	+	+	+	+			–				–		+	+													–				–	–		
EST	+	+	+	+	+	+	+	+			+	+	•	•	•	+	•	•	•	•	+	+	+	+	+	+	+	+			+	+	+	+	+	+	+	+		
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FUM	–	–	–	–	–	–	–	–			–	–	–	–	–	–	–	–			–	–	–	–	–	–	–	–			–	–	–	–	–	–	–	–		
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GDH	–	–	–	–	–	–	–	–	–		–	–	–	–	–	–	–	–	–		–	–	–	–	–	–	–	–			–	–	–	–	–	–	–	–		
	–	–	–	–	–	–	–	–	–		–	–	–	–	–	–	–	–	–																				
G2DH	–	–	–	–	–	–	–	–	–		–	–	–	–	–	–	–	–			–	–	–	–	–	–	–	–			–	–	–	–	–	–	–	–		
GLUDH	–	–	–	–	–	–	–	–	–		–	–	–	–	–	–	–	–			–	–	–	–	–	–	–	–			–	–	–	–	–	–	–	–		
G3PDH	–	+	•	•	•	•	•	–	•	•	–	–	+	–	–	–	+	–			–	–	–	–	–	–	–	–			–	–	–	–	–	–	–	–		
			•				•		•	•																													
G6PD	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	+	–			+	+	+	+	+	+	+	+			+	+	+	+	+	+	+	+	+	+
			+				+		+	+	+	+	+	+	+	+	+	+																						
HK	+	–	+	+	+	–	+	–	–		–	–	–	–	–	–	–	–	–		–	–	–	–	–	–	–	–			–	–	–	–	–	–	–	–		
			–				–		–	–	–	–	–	–	–	–	–	–	–																					
IDH	•	•	•	•	•	•	•	•	•		+	–	–	–	–	–	–	–			•	•	•	•	•	•	•	•			–	–	–	–	–	–	–	–		
	+	+	•	•	•	+	•	+	•																														
LAP	–	–	–	–	–	–	–	–			–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–			–	+	•	+	–	+	+	–	–	–
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MDH	•	•	•	•	•	•	•	•			+	+	+	+	+	+	+	+			+	+	+	+	+	+	+	+			+	+	+	+	+	+	+	+		
	+	+	+	+	+	+	+																																
ME	•	•	•	•	•	•	•	•	•	•	–	•	•	•	•	•	•	–	+	+	–	+	+	+	+	+	+	–			–	–	–	–	–	–	–	–		
	–	–	+	–	–	–	+	–	•	•	+	•	•	•	•	•	•	–	•	•																				
MR[1]	+	•	•	•	•	•	•	+	+		+	+	•	•	•	•	•	•	•	•	+	–	–	+	+	+	+	–			•	•	•	•	•	•	•	•		
	–	–	–	–	–	–	–	–	+		+	+	+	+	+	+	+	–	•	•																				
NADHDH	–	–	–	–	–	–	–	–	–		–	–	–	–	–	–	–	–			–	–	–	–	–	–	–	–			–	–	–	–	–	–	–	–		
NADPHDH	–	–	–	–	–	–	–	–	–		–	–	–	–	–	–	–	–			–	–	–	–	–	–	–	–			–	–	–	–	–	–	–	–		
PC	–	–	•	–	•	–	•	–	–	–	+	+	–	•	+	–	+	–	–		–	–	–	–	–	–	–	–			•	+	+	+	+	–	+	–	–	–
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PEP	–	–	–	–	–	–	–	–	–	–	+	+	+	+	+	+	+	+			–	–	–	–	–	–	–	–			+	+	+	–	+	–	+	–	+	+
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PGI	+	+	+	+	+	+	+	+			•	•	•	•	•	•	•	•	•		+	+	+	+	+	+	+	+			•	•	•	•	•	•	•	•		
											+	+	•	+	+	+	•	–	•																			+	+
6PGD	•	•	•	•	•	•	•	•			+	+	+	+	+	+	+	+			+	–	+	+	+	–	+	+			–	–	–	–	–	–	–	–		
	+	•	+	+	•	+	•	+																																
PGM	•	•	•	+	+	+	•	+	•		–	–	•	•	•	–	–	–	–		+	–	+	+	+	+	+	+			•	•	•	+	–	+	+	–		
	–	+	•	+	+	+	•	+	•				–																											
SDH	+	+	+	–	–	+	+	–	+	+	–	–	+	+	–	–	–	–			–	–	–	–	–	–	–	–			–	–	–	–	–	–	–	–		
	+	•	•	•	+	•	•	+	•	•																														
SUDH	–	–	–	–	–	–	–	–	+	+	–	–	–	–	•	–	–	–			–	–	–	–	–	–	–	–			–	–	–	–	–	–	–	–		
			–				–		–	–																														

[1] For MR, Cathodal strip stained on B only, with good results for buffers 3, 5, 6, 7 (9 and 10 not tested).

Table 6. *Pinus kesiya* embryo (above) and gametophyte (below) staining results; symbols as in Table 4; only B and H systems used

	Running Buffer																			
	H										B									
	Extraction Buffer										Extraction Buffer									
Enzyme	1	2	3	4	5	6	7	8	9	10	1	2	3	4	5	6	7	8	9	10
AAT		•	+				–		•	•			+				+		•	•
	•		+				+		•		•		+				+		•	
ADH		–	–				–		–	–		–	–				–		–	–
	+	–	–				–		–		–		–				–		+	
AK			–				–		–											
			–				–		–											
ALD		–	–				–		+	+		+	–				–		+	+
	+		–				–		•		+		–				–		•	
DIA		+	+				–		+	+		–	+				–		•	•
	•		•				–		•		•		+				–		+	
EST		–							–	–		–	–				–		–	–
	–										–		–				–		•	
FUM		–							+	•										
	+																			
GDH		–	–				–		–	–		–	–				–		–	–
	–		–				–		–		–		–				–		–	
G2DH		–							–	–		–							–	–
	–										–									
GLUDH		–							–	–		–							–	–
	–										–									
G3PDH		+	–				–		–	+										
	–		–				–		–											
G6PD		–							•	•		–							+	+
	•										+									
HK		–	–				–		–	+		–	–				–		–	–
	+		–				–		–		–		–				–		–	
IDH		•	•				–		•	•										
	•		+				–		•											
LAP												+	–				–		+	•
	•										•		–				–		–	
MDH		•							•	•										
	•																			
ME		–	–				–		•	+		–	–				–			+
	•		–				–		–		–		–				–		•	
MR		•	+				–		•	•		–	+				–		•	+
	•		+				–		•		+		+				–		•	
NADHDH		–							–	–		–							–	–
	–										–									
PC		–	–				–		–	–		–	–				–		–	–
	–		–				–		–		–		–				–		–	
PEP		–							–	–		+							+	+
	–										+									
PGI													•				+		•	•
											•		•				–		•	
6PGD		+							•	+										
	•																			
PGM		•	•				•		•	•										
	•		•				•		•				–				–		–	
SDH		+	–				–		•	•			–				–		–	
	•		–				–		–											
SUDH			–				–		–											
			–				–		–											

resulted in almost no enzyme activity, and the homogenate often turned brown, indicating probable oxidation of the enzymes by quinones.

Extraction buffers 9 and 10 (Table 1) were developed to improve overall staining quality and to increase the number of enzymes scored for allozyme variation. Buffer number 9 tended to be ineffective in preventing browning of the homogenate. Buffer number 10, however, yielded results that were as good as, or better than, any other extraction buffer in almost every case.

For *Pterocarpus* embryos, good staining and resolution were obtained for one or more extraction buffers on the H running system for 15 enzyme systems (AAT, ADH, AK, ALD, DIA, G3PDH, IDH, MDH, ME, MR, PC, PGI, 6PGD, PGM, and SDH) (Table 4). For leaves of *Pterocarpus*, not all enzyme systems were assayed, but seven yielded good results (Table 4). Similarly, for *Dalbergia* embryos, 13 enzyme systems stained well on the H running buffer system - the same ones as for *Pterocarpus*, with the exceptions of PGI and SDH (Table 5). Nine enzymes stained strongly for *Dalbergia* leaves. On the B running buffer system, fewer enzymes stained strongly, but those which had not stained on the H system included EST and LAP for *Pterocarpus* embryos and leaves, and EST and PGM for *Dalbergia*. The TC and P running buffer systems were similar to the B system in terms of both the number and range of enzymes successfully stained. However, peptidase stained very strongly on the P system for *Pterocarpus*, where it had failed on the other three systems. No differences were detected in banding patterns for leaves at different stages of development.

Needle tissue of *P. kesiya* was not available and could not be assayed, but good results were obtained for both embryos and megagametophytes (Table 6). No assays were attempted with either the TC or the P systems. On the H system, megagametophytes stained well for 12 enzymes (AAT, ALD, DIA, G6PD, IDH, LAP, MDH, ME, MR, 6PGD, PGM, and SDH), with an additional two enzymes (EST and PGI) on the B system. Similar results were obtained for embryos. As for *Pterocarpus* and *Dalbergia*, extraction buffer number 10, where tested, was as good as, or better than other extraction buffers. The inheritance and linkage of many of these enzyme loci have been reported for *P. kesiya* by Boyle *et al.* (1990).

In population genetics studies, it is desirable to use as many genetic characters as possible, and this is one of the major advantages of isoenzyme analysis. However, the chemicals used in buffer systems and staining recipes are expensive, so a balance must be struck between increasing the number of enzyme loci scored and keeping the process economical. The number of enzymes that can be stained from a single gel depends on the thickness of the gel and the number of slices into which it can be sliced. Thicker gels are more susceptible to heating effects that can destroy enzymes during electrophoresis, and gels that are approximately 1 *cm* thick, which can be sliced into nine or ten 1 *mm* thick slices, are generally used. Running gels on additional buffer systems obviously increases the number of possible gel slices and enzymes to be stained, but at a cost of considerable extra work.

Based on results obtained in this study, recommendations can be made for running buffer systems and enzymes to be stained in order to optimize the use

of time and money (Table 7). For both *Pterocarpus* and *Dalbergia* embryos, nine enzyme systems are suggested on the H buffer system and six on the B system. Depending on the species, these 15 enzymes code for between 25 and 30 loci, which is a satisfactory number for most applications. Although most loci migrate towards the anode during electrophoresis, and therefore stain on the anodal gel strip, for EST, MR, and DIA in *Dalbergia* additional loci are stained on the cathodal strip. For leaves of *Pterocarpus*, only a total of ten enzymes are recommended for staining.

Table 7. Recommendations for enzyme systems to be used in population genetics studies

Enzyme	*Pterocarpus*		*Dalbergia*		*Pinus kesiya*	
	H	B	H	B	H	B
PGM (embryonic)*	✓		✓		✓	
(leaf)*	✓		✓			
IDH	✓		✓		✓	
	✓		✓ (2)			
MDH	✓		✓		✓	
	✓		✓			
6PGD	✓		✓		✓	
	✓		✓			
G6PDH					✓	
MR		✓ (2)		✓ (2)	✓	
		✓ (2)		✓ (2)		
FUM					✓	
SDH	✓ (2)					
			✓		✓ (1 1/2)	
ME		✓ (2)	✓			
		✓ (2)	✓		✓ (2)	
AAT		✓ (1 1/2)		✓ (1 1/2)		✓
		✓ (1 1/2)		✓ (1 1/2)		
PGI		✓		✓		✓
		✓		✓		
LAP		✓		✓ (3)		
		✓		✓ (3)		✓ (2)
DIA	✓			✓		
				✓		✓ (1 1/2)
EST		✓		✓		
		✓		✓		
ADH	✓		✓			
			✓ (2)			
ALD	✓ (2)	✓				
G3PDH	✓ (2)		✓			
			✓			

* For *Pterocarpus* and *Dalbergia* recommendations for embryonic tissue are given above and for leaf tissue below. For *Pinus kesiya*, recommendations for embryonic and megagametophytic tissue are identical. Figures in parentheses indicate the factor by which the amount of substrate given in Liengsiri *et al.* (1990) should be increased for best results.

For *P. kesiya*, 15 enzyme systems can also be stained using a combination of the H and B systems for both megagametophytes and embryos (Table 7). Due to differences in migration distance and similarities of the staining recipes, PGM and IDH can be stained on the same gel slice. For all the enzymes listed in Tables 4 to 7, the staining recipes used were those given in Liengsiri *et al.* (1990) but,

in Table 7, the numbers in parentheses next to an enzyme indicate that the amount of substrate should be increased by that factor.

Conclusions

By using a combination of extraction and running buffer systems, a large number of enzymes can be stained for both embryonic and leaf tissue of *P. macrocarpus* and *D. cochinchinensis* and for embryonic and megagametophytic tissue of *P. kesiya*. Problems were encountered with loss of enzyme activity in leaf extracts, particularly of *Pterocarpus*, probably due to the action of quinones. The development of a new extraction buffer, however, solved most of these problems. Extraction buffer number 10 produced good results for most enzymes tested for *Pterocarpus* and *Dalbergia* embryos and leaves. For *P. kesiya*, buffer number 1 can be used for megagametophytes, as it is cheaper. For embryos, either buffer number 10 or a 1:1 mixture of 10 and 1 is best.

In all three species, a satisfactory number of enzyme loci can be assayed by staining 15 enzyme systems, using H and B running buffer systems. This essentially halves the daily workload, compared with using all four systems.

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