USE OF ISOZYME ANALYSIS IN A PROPOSED *ACACIA MANGIUM* × *ACACIA AURICULIFORMIS* HYBRID SEED PRODUCTION ORCHARD

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WICKNESWARI, R. 1989. Use of isozyme analysis in a proposed *Acacia mangium* × *Acacia auriculiformis* hybrid seed production orchard. Isozyme analysis of *Acacia mangium* and *Acacia auriculiformis* from different provenances showed that *A. mangium* has a 22 genotype at *Gdh-1* whereas *A. auriculiformis* has an 11 genotype. *A. mangium* × *A. auriculiformis* hybrids were identified as broad fuzzy banded 12 genotype at this locus, indicating the probable hexameric molecular structure of glutamate dehydrogenase in acacias. The gene for *Gdh-1* was expressed in callus tissues, seed leaves, juvenile bipinnate leaves and mature phyllodes. This isozyme marker would prove to be useful for early identification of hybrid seedlings from open-pollinating hybridising orchards of the two species.

Key words: *Acacia mangium*-*Acacia auriculiformis*- hybrid-isozyme-locus-allele

Introduction

Acacia mangium Willd and *Acacia auriculiformis* A. Cunn. ex Benth. are two tropical acacias natural to Australia, Papua New Guinea and Indonesia. There is considerable interest in growing these species for timber production in southeast Asia. They are also excellent for fuelwood and pulp and paper production (Turnbull 1986). In addition, these acacia species, especially *A. mangium* are widely used in rehabilitation of degraded lands (Turnbull 1986, Sim 1987)

A. mangium has a lower genetic diversity (H = 0.017, Moran *et al.* 1989a) than *A. auriculiformis* (H = 0.146, Moran *et al.* 1989b). Furthermore, *A. mangium* is genetically depauperate compared to wind-pollinated conifers (H = 0.207, Hamrick *et al.* 1981), animal-pollinated eucalypts (H = 0.186, Moran & Hopper 1987) and tropical rain forest trees (H = 0.111, Hamrick & Loveless 1986). Hybridisation with near relatives would thus be a practicable breeding option for *A. mangium.*

In fact, *A. mangium* and *A. auriculiformis* planted closely do form hybrids. Spontaneous hybrids of them have been reported from plantation grown trees in Sabah (Sim 1987) and Taiwan (Kiang *et al.* 1989). Artificial hybrids through

controlled pollination have also been produced between these two species (R. Wickneswari *et al.* unpublished). In natural populations too such hybrids have been reported, for example in Papua New Guinea (Skelton 1987, Gunn *et al.* 1989). These hybrids may have great potential for plantation forestry by combining desirable properties of the parent species (Bowen 1981, Rufelds & Lapongan 1986). The hybrids tend to grow vigorously, have better form than *A. auriculiformis*, and have lighter branching than *A. mangium*.

In order to develop a systematic program for breeding and testing the artificial hybrid, it is essential to be able to reliably identify the hybrids. This would expedite the hybrid breeding program. Hybrids could be produced in seedling or biclonal hybridising orchards of selected trees of the two species. For reliable identification, isozyme analysis (R. Wickneswari *et al.* unpublished) and seedling morphology studies (Rufelds 1988) have been initiated. This study reports the use of isozyme markers in hybrid identification.

Material and methods

Plant materials

Callus tissues and shoots with bipinnate leaves were obtained from one-month-old micropropagated cultures. Open-pollinated seeds from a spontaneous F_1 *A. mangium* × *A. auriculiformis* hybrid (identified using morphological characters) in Ulu Kukut, Sabah were germinated aseptically. Stem nodal segments from five such seedlings were used as ex-plants for the *in vitro* cultures.

Seed leaves were excised from one to two-week-old seedlings germinated in sand beds. One hundred and fifty three seedlings from an *A. mangium* × *A. auriculiformis* hybrid described above were assayed. One seed leaf per seedling was used.

Fresh and freeze-dried phyllodes from 14 seven-y-old *A. mangium* trees from seven provenances in Queensland, Australia, 27 four-y-old *A. auriculiformis* trees from four provenances in Papua New Guinea and 34 six-month-old seedlings from control-pollinated *Acacia* seeds from a crossing programme (R. Wickneswari *et al.* unpublished) were assayed. The interspecific crosses consisted of both *A. mangium* and *A. auriculiformis* maternal parents. In this study the progenies from the different crosses were bulked.

Tissue extraction

Seed leaves were crushed with a glass rod in one drop of modified cold leaf buffer of Cheliak and Pitel (1984) in microtitre wells. The leaf buffer consisted of 25 *mg* ascorbic acid, 85 *mg* ethylenediaminetetraacetic acid (disodium salt, dihydrate), 190 *mg* sodium disulphite, 400 *mg* Borax, 1000 *mg* egg albumin, 50

dihydrate), 190 *mg* sodium disulphite, 400 *mg* Borax, 1000 *mg* egg albumin, 50 *mg* dithiothreitol, 450 *mg* sodium diethyldithiocarbamate, 10 *mg* nicotinamide adenine dinucleotide phosphate, 20 *mg* nicotinamide adenine dinucleotide, 1 *mg* pyridoxal-5-phosphate and 50 *ml* polyvinyl pyrrolidone (PVP) - sucrose buffer pH 6.8. The PVP-sucrose buffer was prepared using 100 *g* sucrose, 140 *g* PVP-40 (polyvinyl pyrrolidone, molecular weight 40,000) and 20 *g* PVP-360 (polyvinyl pyrrolidone, molecular weight 360,000) per 1000 *ml* 0.1 M phosphate buffer pH 6.8.

Callus tissues, micropropagated shoots with bipinnate leaves and phyllodes were ground in liquid nitrogen to a fine frozen powder in a mortar and pestle. The fine powdered tissues were then transferred to a glass filter funnel lined with high strength filtering cloth and mixed well with an equal volume of cold leaf buffer. A few drops of tissue extract were squeezed out of the filtering cloth into Eppendorf tubes.

Table 1. Enzyme stains

Enzyme	Enzyme abbreviation	Enzyme commission number	Staining method	Staining chemicals	Reference
Glutamate dehydrogenase	GDH	E.C. 1.4.1.2	Staining tray	20 *ml* 0.1M tris pH 7.5 80 μl 10 mM $CaCl_2$ 1.2 *ml* NAD, 10 *mg* ml^{-1} 0.8 *ml* MTT, 10 *mg* ml^{-1} 0.8 *ml* PMS, 2 *mg* ml^{-1} 400 *mg* sodium glutamate	Hartmann *et al.* 1973 (modified)
Peroxidase	PER	E.C. 1.11.1.7	Staining tray	17.5 *ml* 95% ethanol 7.0 *ml* 0.2M acetate buffer pH 4.6 25 *mg* θ - dianisidine 0.5 *ml* 3% H_20_2	Brewbaker *et al.* 1968
Phosphoglucomutase	PGM	E.C. 2.7.5.1	Agar overlay	10 *ml* 0.1M tris pH 7.5 and 100 *mg* agar (boil and cool to 60°C) 10 *ml* 0.1M tris pH 7.5 0.6 *ml* NADP, 5 *mg* ml^{-1} 0.4 *ml* MTT, 10 *mg* ml^{-1} 0.4 *ml* PMS, 2 *mg* ml^{-1} 0.2 *ml* 1M MgCl. $6H_2O$ 30 *mg* glucose-1-phosphate 20 I.U. glucose-6-phosphate dehydrogenase	Tanksley 1979
Shikimate dehydrogenase	SDH	E.C. 1.1.1.25	Agar overlay	10 *ml* 0.1M tris pH 7.5 and 100 *mg* agar (boil and cool to 60°C) 10 *ml* 0.1M tris pH 7.5 0.6 *ml* NADP, 5 *mg* ml^{-1} 0.4 *ml* MTT, 10 *mg* ml^{-1} 0.4 *ml* PMS, 2 *mg* ml^{-1} 20 *mg* shikimic acid	Tanksley & Rick 1980

Electrophoresis

Tissue extracts were absorbed onto chromatography paper wicks and loaded

acid (monohydrate)). Gels were run for 5 to 6 *h* at 70 *mA* constant current using borate tank buffer pH 8.6 (0.3 M boric acid and 0.1 M sodium hydroxide).

Gels were cut into five slices (the top slice was discarded) and stained for glutamate dehydrogenase (*GDH*), phosphoglucomutase (*PGM*), shikimate dehydrogenase (*SDH*) and peroxidase (*PER*). Enzyme staining recipes are given in Table 1.

The genetics of the loci were inferred from single-tree progeny arrays and by comparison of enzyme systems in *A. mangium* (Moran *et al.* 1989a), *A. auriculiformis* (Moran *et al.* 1989b) and reciprocal crosses between *A. mangium* and *A. auriculiformis* (R. Wickneswari *et al.* unpublished) . Allelic identity within and between species was based on similar electrophoretic mobilities.

Results

Eight loci were scored from the four enzyme systems assayed. All eight loci were present in both *A. mangium* and *A. auriculiformis*, but not all were present in the different plant tissues. Table 2 shows the isozyme gene loci that were expressed in the different plant tissues.

Table 2. Expression of isozyme gene loci in different plant tissues of *Acacia mangium* and *Acacia auriculiformis*

Isozyme gene loci *	Plant tissues			
	Callus tissues	Seed leaves	Juvenile bipinnate leaves	Mature phyllodes
Gdh-1	+	+	+	+
Per-1	+	+	+	+
Per-2	+	+	+	+
Per-3	-	-	+	+
Per-4	-	-	+	+
Pgm-1	+	+	+	+
Pgm-2	+	-	+	+
Sdh-1	+	+	+	+

* The isozyme gene loci are denoted by three letters followed by a number , where 1 is the most anodal, 2 is the next most anodal *et cetera*
\+ isozyme gene locus is expressed
\- isozyme gene locus is not expressed

Bands for all loci migrated anodally except for *Per-4* which migrated cathodally. *Per-3* was invariant in both *A. mangium* and *A. auriculiformis*. *Per-4* was only expressed in *A. auriculiformis*. These two loci were thus not scored.

Table 3 summarises the isozyme genotypes scored for *A. mangium* and *A. auriculiformis* callus tissues, seed leaves, juvenile bipinnate leaves and mature

phyllodes. *Pgm-1*, *Pgm-2* and *Sdh-1* were polymorphic in both *A. mangium* and *A. auriculiformis* (Figures 1 & 2). *Per-1* and *Per-2* were polymorphic in *A. auriculiformis* but monomorphic in *A. mangium* (Figure 3). *Gdh-1* was monomorphic at different allelic positions in *A. mangium* and *A. auriculiformis*. All *A. mangium* were homozygous for allele 2 at *Gdh-1* whereas all *A. auriculiformis* were homozygous for allele 1 at *Gdh-1* (Figure 4). Hybrids of the two species were detected by a 12 genotype at *Gdh-1* (Figure 4). The 12 genotype at *Gdh-1* was observed as a broad fuzzy band indicating the probable hexameric molecular structure of glutamate dehydrogenase in acacia species. This banding pattern with multiple bands merging together is common in plants and various models have been proposed to explain the single heterozygote bands (Pryor 1974).

Table 3. Isozyme genotypes of *Acacia mangium* and *Acacia auriculiformis* and possible isozyme genotypes of their hybrids

Isozyme gene loci	Isozyme genotypes*		
	A. mangium	*A. auriculiformis*	*A. mangium* × *A. auriculiformis*
Gdh-1	22	11	12
Per-1	11	11; 12; 22	11; 12
Per-2	22	11; 12; 22	12; 22
Pgm-1	11; 12; 22;	11; 12; 22	11; 12; 22
Pgm-2	11; 12; 22;	11; 12; 22	11; 12; 22
Sdh-1	11; 12; 22;	11; 12; 22	11; 12; 22

* Isozyme genotypes are denoted by allelic combinations at any given locus; alleles are represented by numbers, where 1 is the most anodal, 2 is the next most anodal *et cetera*

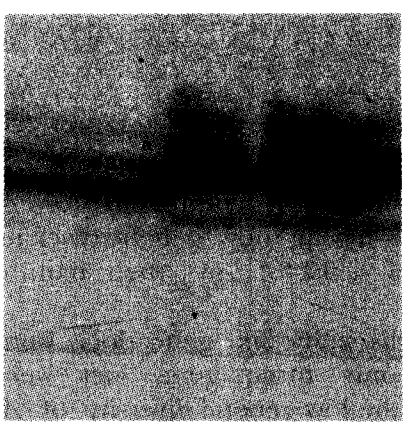

Figure 1. Zymogram of phosphoglucomutase of *Acacia* phyllodes showing 22 homozygote (a) and 12 heterozygote (b) at *Pgm-1* and 11 (c) and 22 (d) homozygotes at *Pgm-2*

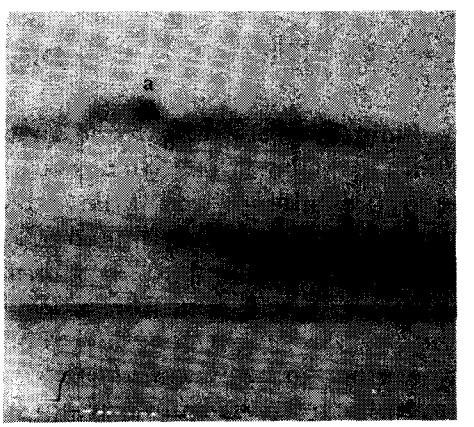

Figure 2. Zymogram of shikimate dehydrogenase of *Acacia* phyllodes showing 11 (a) and 22 (b) homozygotes at *Sdh-1*

One out of the five micropropagated cultures was an identifiable hybrid, the rest were *A. mangium* genotypes. Fifty-seven of the 153 open pollinated seedlings assayed using seed leaves were identifiable hybrids, the remainder had *A. mangium* (56 seedlings) or *A. auriculiformis* genotypes (33 seedlings). The 57 hybrid seedlings were potted and allowed to grow in the nursery. Seven seedlings produced very faint bands that could not be scored. Of the control-pollinated interspecific *Acacia* seedlings assayed using phyllodes, 17 were identifiable hybrids, 11 had *A. mangium* genotypes and six had *A. auriculiformis* genotypes. Selfs and hybrids were produced in these interspecific crosses because pollinations were carried out using unemasculated flowers.

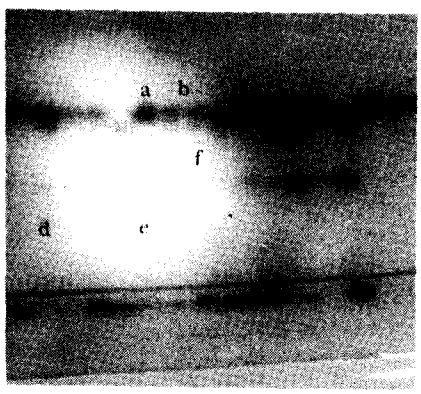

Figure 3. Zymogram of peroxidase of *Acacia* seed leaves showing 11 (a) and 22 (c) homozygotes and 12 (b) heterozygote at *Per-1* and 12 (d) heterozygote and 22 (e) and 11 (f) homozygotes at *Per-2*

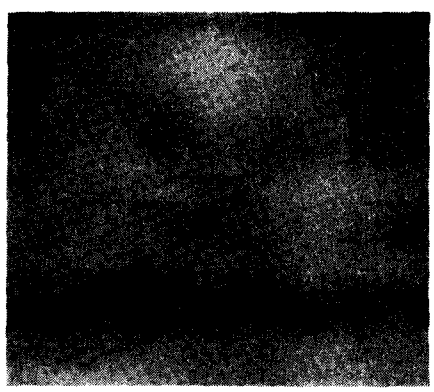

Figure 4. Zymogram of glutamate dehydrogenase of *Acacia* seed leaves showing 22 (a) and 11 (c) homozygotes and 12 (b) heterozygote at *Gdh-1*

Discussion

Preliminary work on isozyme analysis of *A. mangium* and *A. auriculiformis* planted in Malaysia (R. Wicneswari unpublished) showed that generally *A. mangium* exhibited very little enzyme polymorphism compared to *A. auriculiformis*. This is attributed to *A. mangium* having low genetic diversity (H = 0.017) compared to *A. auriculiformis* (H = 0.146) in their natural populations (Moran *et al.* 1989a, 1989b).

Out of the eight gene loci reported in this study, six gene loci were polymorphic in *A. auriculiformis* whereas only three gene loci were polymorphic in *A. mangium*, further indicating the low genetic diversity of *A. mangium*.

All *A. mangium* assayed in this study had a 22 genotype at *Gdh-1*. This has

also been reported by Moran *et al.* (1989a). They looked at eleven natural populations of *A. mangium* in Australia, Papua New Guinea and Indonesia and found all individuals to be of the 22 genotype at this locus except for one out of 80 progenies from a population in Boite, Papua New Guinea which had a 23 genotype (J. C. Bell personal communication). This was probably an outcross with *Acacia crassicarpa* which occurs in this provenance. In the case of *A. auriculiformis*, all individuals had 11 genotypes at *Gdh-1*, concurring with the findings of Moran *et al.* (1989b). Kiang *et al.* used peroxidase to identify hybrids from open-pollinated seeds of *A. mangium* trees grown side by side with *A. auriculiformis* in a plantation. In this study, however, peroxidase was polymorphic in *A. auriculiformis*. Hence this marker is less reliable than glutamate dehydrogenase for the identification of hybrids from open-pollinating hybridising orchards of the two species.

The gene for *Gdh-1* was expressed in callus tissues and in juvenile as well as mature leaves of *A. mangium* and *A. auriculiformis*, implying that hybrids can be determined and verified at any stage in the growth of the species. The fact that *Gdh-1* is expressed in seed leaves, allows hybrid determination to be carried out as early as one to two weeks after seeds are sown in the nursery. Growth of seedlings with one seed leaf excised was not impaired.

Hence, in a hybrid breeding program of the two species, *Gdh-1* can be used as a genetic marker to screen for the hybrids in open-pollinating hybridising orchards. A reliable seedling morphology guide, if developed together with the isozyme screening would cut costs and time in a hybrid breeding program for this species. Then, leaf samples of seedlings need not be sent to a laboratory. for an isozyme analysis. This study is currently being investigated.

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