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Use of isoenzymes to differentiate growth categories of *Pericopsis mooniana* trees

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Abstract

Leaf isoenzymes of *Pericopsis mooniana* from twenty-one trees at forest plantation were evaluated for their use in identification of elite trees among heterogeneous population. Trees were grouped morphologically, before leaf extracts were separated by one-dimensional polyacrylamide gel electrophoresis. Isoenzyme analysis were carried out for peroxidase, esterase, alcohol dehydrogenase, formate dehydrogenase, acid phosphatase, aspartate aminotransferase, isocitrate dehydrogenase, malate dehydrogenase, leucine aminopeptidase, phosphoglucoisomerase, phosphogluconate dehydrogenase, phosphoglucomutase, and shikimate dehydrogenase. From the thirteen enzymes studied only four gave distinct banding patterns. Level of significance of appearing particular band for each enzyme of a given category was investigated using χ^2-test, followed by cluster analysis for categorization. The isozyme type A of formate dehydrogenase showed promising results that could be used for differentiating trees of categories investigated.

Aditional key words: formate dehydrogenase, 1-D PAGE.

Introduction

P. mooniana processes high quality timber. It fixes N_2 thus providing an excellent species for reforestation particularly in eroded soil. Therefore, demarcation of its elite

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Abbreviations: AAT - aspartate aminotransferase; ACP - acid phosphatase; ADH - alcohol dehydrogenase; EST - esterase; FDH - formate dehydrogenase; IDH - isocitrate dehydrogenase; LAP - leucine amino peptidase; MDH - malate dehydrogenase; PER - peroxidase; PGD phosphogluconate dehydrogenase; PGI - phosphoglucoisomerase; PGM - phosphoglucomutase; PVP - polyvinyl - pyrrolidone; SKD - shikimate dehydrogenase; Tris - tris (hydroxymethyl) aminomethane.

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individuals from a heterogeneous population for further propagation is an important issue to preserve this endangered species.

The DNA markers provide direct information of genetic code inherited as needed for exact identification of plants. However, the DNA techniques are expensive; hence they cannot be utilized for routine identification. The gel electrophoresis of isoenzymes offers an alternative tool as a biochemical marker (Schaal *et al.* 1991) providing essential data that can be utilized for identification purposes. Further, these markers are simply inherited, comparatively inexpensive, and largely unaffected to external environmental factors.

The aim of this research was to determine isoenzyme maps of *P. mooniana* in order to identify elite trees from a heterogeneous population. These maps are not available to date for *P. mooniana* species. In achieving the objective we hypothesized that the isoenzyme patterns are closely associated with morphological characters such as girth, height and apical dominance (reduced lateral branching). Therefore, particular emphasis was made to assess the contribution from these parameters in the development of isoenzyme-based identification scheme.

Materials and methods

Adult trees of *Pericopsis mooniana*, age of about 48 years, from a stand at Badagamuwa Forest Plantation, Sri Lanka (average annual rainfall 1000 mm; low humic gley soil, pH 5.8 - 6.0) were chosen for the study. In order to differentiate the best performing trees (BP) from the least performing trees (LP), the following morphological characteristics were assessed: *1*) girth at breast height 150 - 180 cm for BP and 60 - 80 cm for LP, *2*) height to the first branch 750 to 1050 cm for BP and less than 300 cm for LP, and *3*) number of lateral branches less than 7 for BP and more than 12 for LP. Data obtained were treated statistically by mid-ranking method to calculate frequency distribution patterns ($P < 0.05$) in order to select trees for both categories. This assessment was made for the data collected over a period of three years.

Young leaf (1 - 2 weeks old) samples were extracted for gel electrophoresis following slightly modified procedures of Hames (1981) and Soltis *et al.* (1983). Approximately a 15 g of tissue was macerated at 4 °C using mortar and pestle with about 0.5 cm^3 of extraction buffer [composition: 45 mM Tris-citrate, pH 8.3, 12 mM KCl, 21 mM $MgCl_2$, 1.0 mM ethylene diamine tetraacetic acid (EDTA)-disodium salt, 1.0 mM PVP-40, 0.5 % Triton, 0.3 mM dithiothreitol (DTT)] for about 10 min. Crude protein supernatant was separated by micro-centrifugation at 14 500 *g* (4 °C) for 10 min (ALC 4214, *Analis*, Namur, Belgium). This was utilized for further separation of proteins by 10 % one-dimensional polyacryamide gel electrophoresis (1-D PAGE; 1500 *Advantec*, Tokyo, Japan). Electrophoretic runs were performed at 40 mA for 4 h. The 1-D PAGE was repeated three to four times in all samples for each enzyme tested. Thirteen enzymes were stained to determine isoenzyme patterns. Staining procedure was done according to Wendel and Weeden (1989). The following buffers were used for enzyme separation:

Enzyme	Buffer system	pH
Acid phosphatase (ACP) E.C. 3.1.3.2	Tris-citrate	7.0
Alcohol dehydrogenase (ADH) E.C. 1.1.1.1	sodium-borate/Tris-citrate	8.0/8.6
Aspartate aminotransferase (AAT) E.C. 2.6.1.1	sodium-borate/Tris-citrate	8.0/8.6
Esterase (EST) E.C. 3.1.1.1	Li-borate/Tris-citrate	8.3/8.3
Formate dehydrogenase (FDH) E.C. 1.2.1.2	Tris-borate-EDTA	8.6
Isocitrate dehydrogenase (IDH) E.C. 1.1.1.41	histidine-citrate	5.7
Leucine aminopeptidase (LAP) E.C. 3.4.11.1	Li-borate/Tris-citrate	8.3/8.3
Malate dehydrogenase (MDH) E.C. 1.1.1.37	histidine-citrate	5.7
Peroxidase (PER) E.C. 1.11.1.7	Li-borate/Tris-citrate	8.3/8.3
Phosphoglucomutase (PGM) E.C. 5.4.2.2	histidine-citrate	5.7
Phosphogluconate dehydrogenase (PGD) E.C. 1.1.1.44	histidine-citrate	7.0
Phosphoglucoisomerase (PGI) E.C. 5.3.1.9	sodium-borate/Tris-citrate	8.0/8.6
Shikimate dehydrogenase (SKD) E.C. 1.1.1.25	Tris-citrate	7.0

Zymograms were scanned by a densitometer and associated *Image Master* (*version 1.0*) program (*Pharmacia*, Uppsala, Sweden). This process ensures the accuracy in the measurements of relative migration (R_f), thickness and intensity thus assuring gel uniformity. Hierarchic clustering of trees of both groups was analyzed using the unweighted pair-group method with arithmetic averaging (UPGMA) based on their similarity values using the *Gel Compar* software program.

Results and discussion

From 13 selected enzymes, only four enzymes PER, EST, ADH and FDH gave isoenzyme-banding patterns. The AAT, IDH, MDH, ACP and PGD did not produce any significant bands. The LAP, PGI, SKD and PGM showed some smearing behaviour alone the lanes; however, no bands were detected. The reasons might be that: *1*) these isoenzymes are not strongly expressed in the tissues examined, or *2*) they may be denaturared, inactivated, or unstable during the extraction or electrophoresis.

Peroxidase (PER): Three isoenzymes of PER and five variable bands were observed (Fig. 1). Previously, PER has been used for identification of many plant species (*e.g.* Kuhns and Frezts 1978, Roya *et al.* 1997, Gracia *et al.*, 1982, Brewbaker *et al.* 1985). The reported numbers of isoenzymes were between 2 to 13.

From the three isoenzymes observed for PER, only type A was common in both categories (Table 1). The occurrence frequency of type A is high in both categories (BP = 88.3 % and LP = 100 %). Therefore, these results cannot be used to differentiate BPs and LPs; hence they cannot be employed in the present context.

Alcohol dehydrogenase (ADH) and esterase (EST): ADH gave three isoenzymes as well as three variable bands. Our data on ADH isoenzymes are in agreement with the forest tree species *Pterocarpus macrocarpus* (Liengsiri *et al.* 1994). However, in

Olea europea cultivars, only one zone of activity with either one banded or a two banded pattern has been observed for ADH (Trujillo and Rallo 1995). EST yielded maximum number of isoenzymes (six) and five variable bands (Fig. 1). However, this banding pattern does not agree with previous data reported for *Fortunella* (Rahman and Nito 1994) and *Hordeum vulgare* (Kahler and Allard 1970).

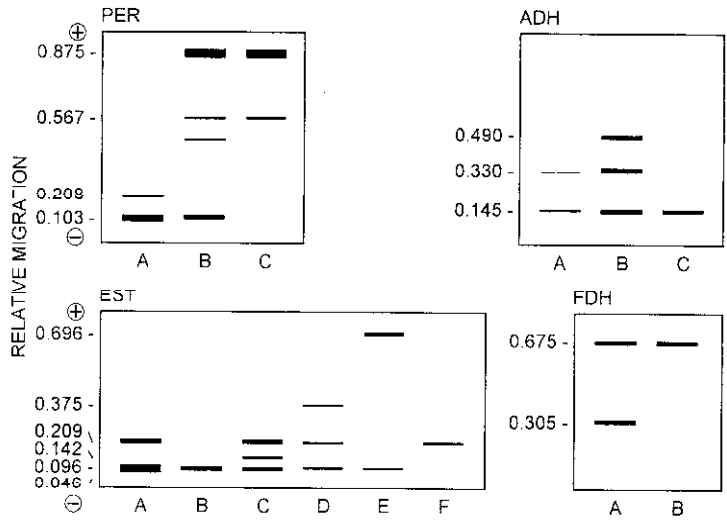

Fig. 1. The relative migration distance (R_f) of the distinct isoenzyme-banding pattern in *P. mooniana* of both categories (BP - best performing trees, LP - least performing trees). The following classification was used (enzyme name/isoenzyme type): PER/ABC, ADH/ABC, EST/ABCDEF, and FDH/AB.

The relative occurrence of isoenzymes observed in ADH and EST is highly variable (Table 1). Further, the isoenzyme type F is not present to a measurable extent in BP category. However, this isoenzyme type is present in LP category, thus giving some significant values ($P < 0.01$; BP = NB and LP = 48.48 %). We decided not to use this isoenzyme type for differentiation of two categories of *P. mooniana* due to negative results obtained in BP category.

Formate dehydrogenase (FDH): In contrast to the enzymes examined so far, the FDH behaved differently resulting two isoenzymes (A and B) with two variable bands. So far, FDH has not widely been used for identification of plant species. Few available data showed only one isoenzyme for FDH (Fairnelli *et al.*, 1983; Wendel and Parks, 1982). An exact explanation for this observation is not possible presently. However, following general remarks can be made to account this apparent discrepancy: variability of banding patterns in different tissues (Feret and Bergman 1976, Wise and Morrison 1971, Yurenkova *et al.* 1995), plant age or season (Thurman *et al.* 1965, Scandalios 1969, Pierce and Brewbacker 1973, Schwartz *et al.* 1994).

FDH gave two isoenzymes designated as A and B. The occurrence frequency of type A in BP category is significantly higher than in LP ($P < 0.01$; BP = 89.80 % and

LP = 6.06 %). Therefore, these data were processed further using multivariate statistical method to determine the similarity characteristics between two categories.

Table 1. Frequency [%] of isoenzymes observed in best performing (BP) and least performing (LP) trees of *P. mooniana*. The frequency values given in 3rd and 4th columns were calculated as: $f = (\Sigma X_A^{enz}/\Sigma BP_T) \times 100$, and $X_A^{enz} = \Sigma n_A^{enz}/\Sigma n_T^{enz}$, where X_A^{enz} = mean relative frequency of type A occurrence per enzyme, n_A^{enz} = type A occurrence frequency, BP_T = total number of replicates, n_T^{enz} = total number of trees in a category. The enzymes that yielded distinct bands were used for the calculation. Isoenzyme types (A, B, C, ...) correspond to those in Fig. 1, NB - no bands. χ^2 categorical comparison was used to determine variable bands that showed distinct variability with respect to BP and LP category of *P. mooniana* (* - $P < 0.05$, ** - $P < 0.01$).

Enzyme	Isoenzyme	BP	LP
Peroxidase	A	88.20	100.00
	B	3.40	NB
	C	8.30	NB
Esterase	A	NB	10.60
	B	23.40	3.03*
	C	13.30	3.03
	D	NB	4.56
	E	NB	27.27
	F	NB	48.48**
	NB	63.30	3.03
Alcohol dehydrogenase	A	28.34	19.69
	B	19.99	51.52
	C	51.65	28.79
Formate dehydrogenase	A	89.89	6.06**
	B	6.40	NB
	NB	3.71	93.94**

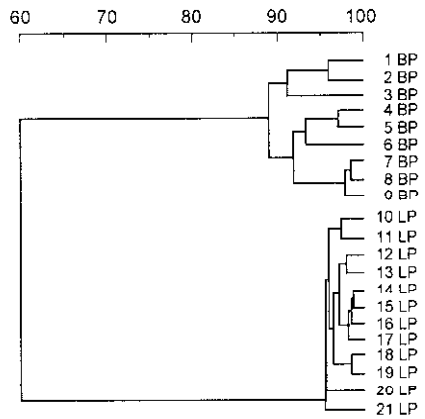

Fig. 2. Dendogram showing UPGMA clustering of FDH in *P. mooniana*. BP - best performing trees, LP - least performing trees. Scale denotes similarity values [%].

The dendogram (Fig. 2) obtained for isozyme type A of FDH confirmed the similarity between banding patterns. The BP category of *P. mooniana* is related closely, and it gave 89 % of similarity. The LP category also showed highly similar results (similarity value = 95 %). However, when the data were analyzed collectively using BP and LP categories, only a 4 % similarity value was obtained. This finding supports the conclusion that we obtained from frequency distribution (Table 1). The isoenzyme type A of FDH is recommended for identification and, hence a biochemical marker. Therefore, isozyme polymorphism in a population of *P. mooniana* is proved as a feasible method for differentiation of growth categories.

References

Brewbaker, J.L., Nagai, C., Liu, E.H.: Genetic polymorphism of 13 maize peroxidases. - Heredity **76**: 159-167, 1985.

Fairnelli, M.P., Fry, D.W., Richardson, K.E.: Isolation, purification and partial characterization of formate dehydrogenase from soybean seed. - Plant Physiol. **73**: 858-859, 1983.

Feret, P.P., Bergman, F.: Gel electrophoresis of proteins and enzymes. - In: Miksche, J.P. (ed.): Modern Methods in Forest Genetics. Pp. 1-49. Springer-Verlag, New York 1976.

Gracia, P., De La Vega, P., Benito, C.: The inheritance of rye seed peroxidases. - Theor. appl. Genet. **61**: 341-351, 1982.

Hames, B.D.: An introduction to polyacrylamide gel electrophoresis. - In: Hames, B.D., Rickwood, D. (ed.): Gel Electrophoresis of Proteins, a Practical Approach. Pp. 1-91. IRL Press, Oxford 1981.

Kahler, A.L., Allard, R.W.: Genetics of isozyme variant in barley. I. Esterases. - Crop Sci. **10**: 444-448, 1970.

Kuhns, L.J., Fretz, T.A.: Distinguishing rose cultivars by polyacrylamide gel electrophoresis. II. Isoenzyme variation among cultivars. - Amer. Soc. hort. Sci. **103**: 509-516, 1978.

Jiengsiri, C., Yeh, F.C., Boyle, T.J.B.: Isozyme analysis of a tropical forest tree *Pterocarpus macrocarpus* Kurz. in Thailand. - Forest Ecol. Manage. **74**: 13-22, 1994.

Pierce, L.C., Brewbaker, J.L.: Applications of isozyme analysis in horticultural science. - HortScience **8**: 17-22, 1973.

Rahman, M.M., Nito, N.: Phylogenetic relationship in the kumquat (*Fortunella*) as revealed by isozyme analysis. - Scientia Hort. **57**: 17-28, 1994.

Royo, J.B., Cabello, F., Miranda, S., Gogocena, Y., Gonzalez, J., Moreno, S., Itoiz, R., Ortiz, J.M.: The use of isoenzymes in characterization of grapevines (*Vitis vinifera* L.) influence of the environment and time of sampling. - Scientia Hort. **69**: 145-155, 1997.

Scandalios, J.G.: Genetic control of multiple molecular forms of enzymes in plants. A review. - Biochem. Genet. **3**: 37-79, 1969.

Schaal, B.A., Leverich, W.L., Rogstad, S.H.: A comparison of methods for assessing genetic variation in plant conservation biology. - In: Falk, D.A., Holsinger, K.E. (ed.): Genetics and Conservation of Rare Plants. Pp. 123-134. Oxford University Press, Oxford 1991.

Schwarts, H.M., Biedron, S.I., Von Holdtand, M.M., Rehm, S.: A study of some plant esterases. - Phytochemistry **3**: 189-200, 1964.

Soltis, D.E., Haufler, C.H., Darrow, D.C., Gastony, G.J.: Starch gel electrophoresis of fern: a compilation of grinding buffers, gel and electrode buffers and staining schedules - Amer. Fern J. **73**: 9-27, 1983.

Thurman, D.A., Palin, C., Laycock, M.V.: Isozymic nature of L-glutamic dehydrogenase of higher plants. - Nature **207**: 193-194, 1965.

Trujillo, I., Rallo, L., Arus, P.: Identifying olive cultivars by isozyme analysis. - Amer. Soc. hort.

Sci. **120**: 318-324. 1995.

Wendel, J.F., Parks, C.R.: Genetic control of isozyme variation in *Camellia japonica* L. - Heredity **73**: 197-204, 1982.

Wendel, J.F., Weeden, N.F.: Visualization and interpretation of plant isozymes. - In: Soltis, D.E., Soltis, P.S. (ed.): Isozymes in Plant Biology. Pp. 5-45. Dioscorides Press, Portland 1989.

Wise, B., Morrison, M.: Location of isozyme forms of peroxidase in the cotton plant. - Phytochemistry **10**: 2355-2359, 1971.

Yurenkova, S.I., Khotyleva, L.V., Tsebrikov, Y.V.: Tissue-specific expression of esterase isoenzymes in *Linum usitatissimum* L. - Biol. Plant. **37**: 375-379, 1995.