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Identification of genotypes using leaf isozymes: A study to assess biochemical gene markers in walnut

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Abstract

Leaf isozyme patterns have been observed in *Juglans nigra* and eight cultivars of *Juglans regia* for their identification in the field. The results showed differences in specific relative mobility values for different isozymes in different cultivars of walnut. Of the six enzyme systems that were localised on native gels, only esterase and malate dehydrogenase showed wide diversity in R_m values for different isozymes. A total of 16 loci and 24 alleles were observed for five enzyme systems, out of which 10 loci were polymorphic. *J. nigra* was found to be more heterozygous and polymorphic than *J. regia.* PPO I, PRO I and EST III were found as reliable markers for distinguishing the two species of walnut. The values obtained for similarity co-efficient were used to make the dendrogram. Among the cultivars of *J. regia*, the most diverse relationship was found between 'Tutle' and 'Blackmore' whereas 'Blackmore' and 'Payne' were nearest.

Key words: *Juglans* spp., identification, marker, isozyme, dendrogram, allozymes, walnut.

Introduction

Walnut (*Juglans* spp.) is an important nut tree, which belongs to family *Juglandaceae*. The genus *Juglans* contains about 20 species, all producing edible nuts. Among these the English or Persian walnut (*Juglans regia* L.) is the most widely cultivated species (McGranahan and Leslie, 1990). The utility of walnut tree is beyond comparison but the fruit and the timber has been put to maximum use specially in dietry and industrial application.

The focus in the identification studies has now been shifted towards the chemical based approaches. This has been greatly due to the lack of reliable morphological gene markers in plants. The genetic studies in case of fruit and nut trees are thus, greatly hindered (Arulsekar and Parfitt, 1986). The biochemical and DNA markers have been a powerful approach. Gel electrophoresis of enzymes has played a key role in studying genetic variation of plants, therefore, the use of isozymes especially in plant genetics and breeding has been extensive. DNA marker application, however, is more demanding in terms of infrastructure and expertise to get reproducible results (Cardena *et al.,* 1998). Therefore, for some routine applications of marker-assisted selection as in the case of cultivar differentiation and progeny legitimacy, analysis of leaf proteins is easier to perform and analyse.

Fruiting in walnut starts several years after the plantation and therefore, an early assessment of the planting material becomes a necessity for farmers in its cultivation. The identification at an early stage is also important for breeders in improvement programme. In India, almost all walnut plantation being of seedling origin exhibit tremendous genetic variation. Therefore, the systematic evaluation of walnut germplasm is essential for selecting superior genotypes to build gene repository (Sharma and Kumar, 1994). Although, a study to decifer the genetic structure of walnut genotype in India was undertaken (Vyas *et al.,* 2002), there is no report on the use of isozyme gene markers for identification.

The present study was therefore undertaken to analyse *J. nigra* L. and 8 cultivars of *J. regia,* using leaf isozymes for identification, selection, conservation and improvement

Material and methods

Juglans nigra and eight different cultivars of *J. regia* were used in the present study. The cultivars of *J. regia* included 'ACO 38853', 'Blackmore', 'Gobind', 'Hartley', 'KX Giant', 'Lake English', 'Payne' and 'Tutle'. The plant material was available in the germplasm collection being maintained at the University farm of Horticulture and Forestry, Solan.

Green leaves were sampled on ice in the early hours of the day. The extractable total crude protein was taken out by crushing the leaf samples in homogenisation buffer. 100 mg of leaf tissue was crushed in 1 ml of homogenisation buffer, which consisted of tris citrate buffer (pH 8.0) containing 5 mM EDTA and 2 mM DTT. To check the oxidation by phenols, insoluble polyvinyl pyrolidone at the rate of 0.75 g g^{-1} of leaf tissue was also added to the crushing buffer. Well homogenised paste was centrifuged in the refrigerated centrifuge and the supernatant was taken for further processing. The supernatant was kept at -80°C and used as crude enzyme extract. 100 mg of the soluble crude protein was loaded for each cultivar in all the enzyme systems.

Horizontal polyacrylamide gel electrophoresis was employed to resolve the bands of esterases (EST), acid phosphatases (ACP), alkaline phosphatases (ALP), malate dehydoregenases (MDH), peroxidases (PRO) and polyphenol oxidases (PPO) using lithium-borate buffer at pH 8.3. Gels were prepared according to the method of Arulsekar and Parfitt (1986). The staining of the gels for different

enzymes was done according to the methods given by Vallejos (1983). Similarity coefficient values which implicated the direct extention of isozyme pattern were calculated according to Hayhome and Pfeiester (1983). Dendrogram was developed with unweighted pair group method analysis (UPGMA) in accordance with Sokal and Sneath (1963) using similarity co-efficients.

Results and discussion

All the enzymes except ALP were resolved well on the native gels. No clear separation of isozyme bands could be obtained for ALP and hence, it was not included in the identification studies. Among the enzymes studied the EST, MDH, and ACP were particularly helpful in identification of different cultivars. Sixteen loci and 24 alleles were observed for five enzymes with the number of loci coding for individual enzymes ranging from monomorphic to polymorphic with 4 alleles.

Each isozyme band was marked with a particular relative mobility (Rm) value based on its movement in relation to the tracking dye. Each different zone of enzyme activity was considered to be coded by different locus as described by Soltis and Rieseberg (1986). When more than one locus was observed for an enzyme, loci were numbered sequentially with the most anodally migrating locus designated as I. For each gene locus, the putative allozyme specifying the fastest form was called as a, and the progressively slower forms were called b, c, d, etc.

Four different loci were observed for esterases (Fig. 1), all of which were polymorphic. EST Ib and EST IIa were present in all cultivars of *Juglans regia*. The cultivars 'Tutle' and 'KX Giant' did not show EST Ia, whereas EST IIb was absent in 'Gobind' and 'Blackmore'. EST IIIa and c were present in cultivars 'Tutle', 'Lake English', 'KX Giant' and 'Hartley', whereas EST IIIb was present in cultivars 'Payne', 'Blackmore' and 'ACO'. EST III was absent in cultivar 'Gobind'. Fourth zone of activity was visible only in *J. nigra* and 'Gobind'. This zone has two isozymes EST IVa & b. However, EST IVb was not observed in 'Gobind'. Arulsekar *et al.* (1986) and Germain *et al.* (1993) found only three zones of activity in this enzyme with former reporting relatively fewer bands while the latter, a large number of them. Differences in the number of bands might appear due to the differences in the gel concentrations used in the present study and the earlier studies. Also, Aly *et al.* (1992) while working with the esterase isozyme of walnut somatic embryos showed that they did not resolve clearly to determine the parentage. In the present study, however, the third locus showed a dark and consistent band with three differernt alleles a, b, and c with Rm values of 0.295, 0.271 and 0.239, respectively.

Relatively small numbers of bands were observed for ACP, which had two polymorphic loci out of a total of three (Fig. 2). The most cathodal band (Rm 0.194) was monomorphic and the two faster loci were polymorphic. Earlier, Battistini and Sansavini (1991) had also found a faster zone with two alleles to be polymorphic and slower a monomorphic zone which was single allele controlled. Its discreet presence made it a choice for an isozyme marker. The distinct zone ACP III with a single band was present in all the species and cultivars. ACP III & ACP Ib was present in all the samples tested. ACP Ia was present in cultivars 'Tutle', Lake English', 'Hartley' and in *J. nigra.* Loci ACP II was absent in *J. nigra*, while allele ACP IIa was absent in 'Tutle', 'Lake English' and 'Hartley' and present in other cultivars. The allele ACP IIb was, however, present only in cultivars 'Payne' and 'Blackmore'.

The highest number of bands was observed in MDH as it showed four different loci (Fig. 3), two of which, (MDH II and MDH IV) were polymorphic. The identifiable enzymatic banding of MDH was distinct in respect that the MDH I was present in all the genotypes. The absence of a particular allele or loci was identifiable of certain genotypes as MDH II, III and IV of *J. nigra*; MDH II of 'Gobind' and 'Lake English'; MDH III of 'Lake English' and 'Hartley'; MDH IVa of 'Lake English' and MDH IVb of 'Payne', 'Hartley', 'Gobind' and 'Blackmore'. Aleta *et al.* (1993) had also observed four different zones in MDH in eight progenies of walnut but earlier Aleta *et al.* (1990) had reported complex interpretation of this enzyme in 29 walnut cultivars based on 4 different phenotypes with 6, 7 and 8 bands. However, Solar *et al.* (1993) observed only three zones of activity with MDH I to be monomorphic with single band.

Peroxidase is one of the most worked out and widely investigated enzyme system, and is highly variable in higher plants. Three different zones of activity for peroxidase from walnut leaf extracts were found in the present investigation. The least anodal zone showed a single dark and thick band (PRO III), which was present in all species and cultivars (Fig. 4). The most anodal loci PRO I was heterozygous and present only in *J. nigra*, whereas PRO IIa and IIb were present only in 'Gobind' and PRO IIb and PRO IIc were identifiable in 'KX Giant only. Earlier, Solar *et al.* (1994) had reported poor separation of these bands in the leaves of walnut cultivars.

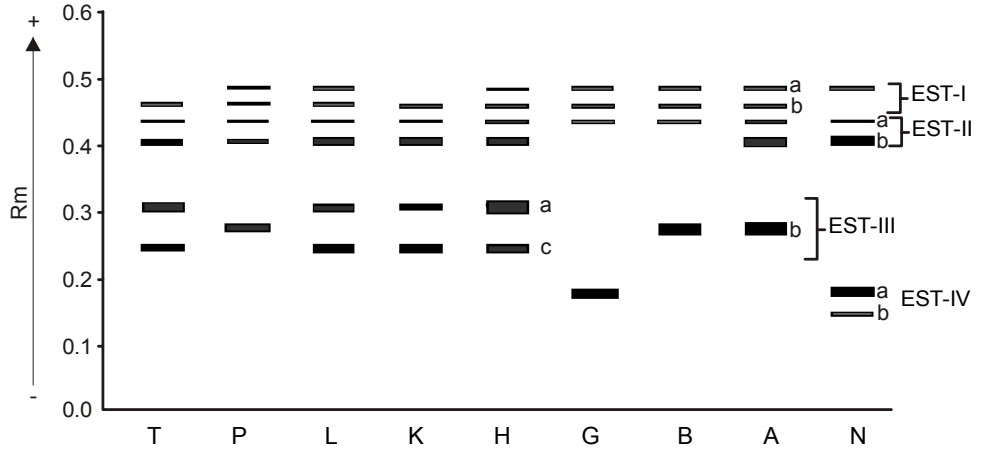

Fig. 1. Zymogram pattern of esterases in *J. nigra* (N) and cultivars of *J. regia* viz., 'Tutle'(T), 'Payne' (P), 'Lake English' (L), 'K x Giant' (K), 'Hartley' (H), 'Gobind' (G), 'Blackmore' (B) &'ACO' (A).

The fifth enzyme system PPO showed two loci, both of which were monomorphic (Fig. 5). In the present study it was found to be the most clear and distinct enzyme when it comes to the differentiation of the two walnut species. However, it could not be used to differentiate the inter-cultivar differences for the absence of heterozygosity between various cultivars of *Juglans regia.*

Since, the true picture of the diversity among the cultivars can only be assessed by their behaviour on the native gels, therefore, the similarity co-efficients based on the co-efficient of Jaccard were assessed. These represented the direct application of enzyme phenotype and the true picture of phylogeny. The values of similarity co-efficients (Table 1) were used to construct the phylogenetic tree of the studied genotypes (Fig. 6). *J. nigra* represented a different branch in dendrogram with maximum value of only 0.474 for similarity co-efficient. It also showed the relatedness between different cultivars, which was greatest between 'Blackmore' - 'Payne' with a value of 0.938 and the least between 'Lake English' - 'Blackmore' and 'Lake English'- 'Gobind' with a value of 0.450.

The present investigations into the leaf isozyme systems of walnut

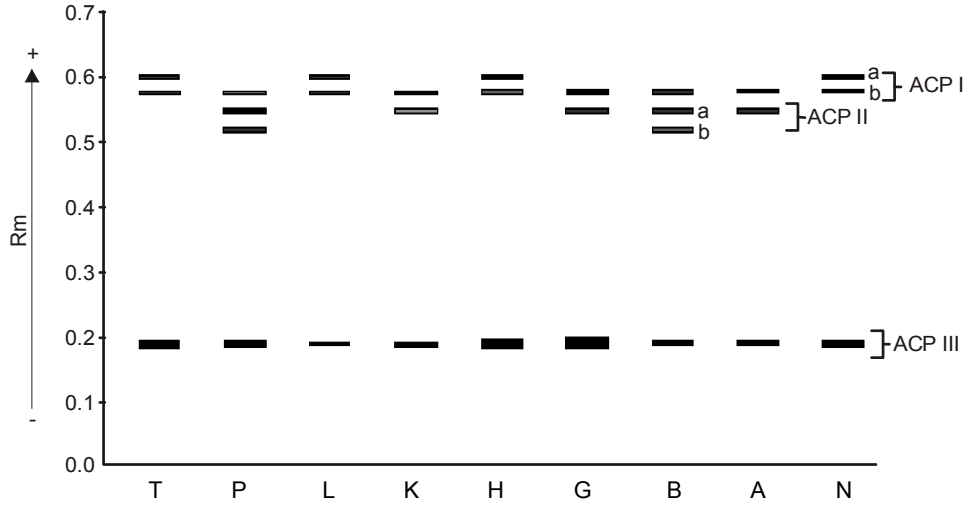

Fig. 2. Zymogram pattern of acid phosphatases in *J. nigra* (N) and cultivars of *J. regia* viz., 'Tutle'(T), 'Payne' (P), 'Lake English' (L), 'K x Giant' (K), 'Hartley' (H), 'Gobind' (G), 'Blackmore' (B) &'ACO' (A).

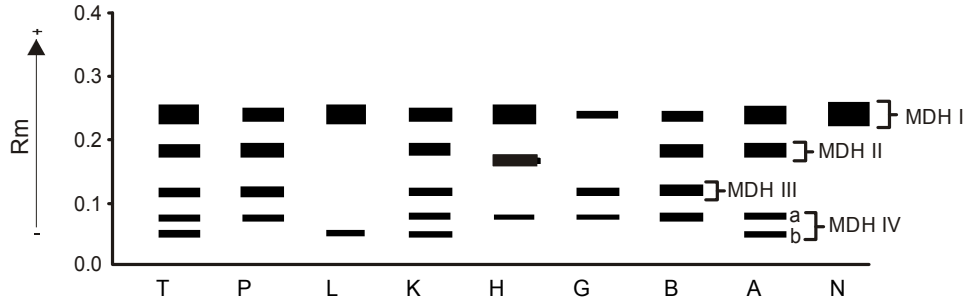

Fig. 3. Zymogram pattern of malate dehydrogenases in *J. nigra* (N) and cultivars of *J. regia* viz., 'Tutle'(T), 'Payne' (P), 'Lake English' (L), 'K x Giant' (K), 'Hartley' (H), 'Gobind' (G), 'Blackmore' (B) &'ACO' (A).

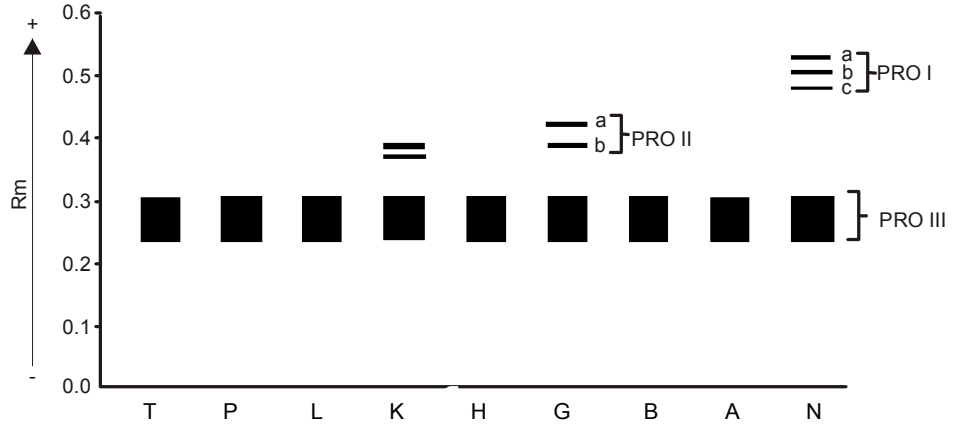

Fig. 4. Zymogram pattern of peroxidases in *J. nigra* (N) and cultivars of *J. regia* viz., 'Tutle'(T), 'Payne' (P), 'Lake English' (L), 'K x Giant' (K), 'Hartley' (H), 'Gobind' (G), 'Blackmore' (B) &'ACO' (A).

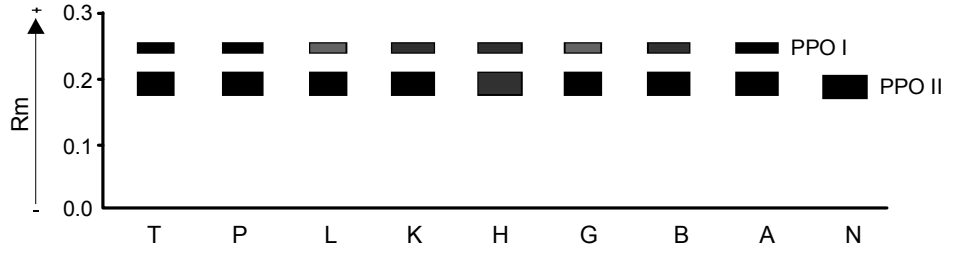

Fig. 5. Zymogram pattern of polyphenol oxidases in *J. nigra* (N) and cultivars of *J. regia* viz., 'Tutle'(T), 'Payne' (P), 'Lake English' (L), 'K x Giant' (K), 'Hartley' (H), 'Gobind' (G), 'Blackmore' (B) &'ACO' (A).

Table 1. Similarity coefficient matrix based on isozyme pattern. It denotes the matches/non matches between phenotype patterns of isozymes

	J. nigra	*J. regia*							
		ACO	Blackmore	Gobind	Hartley	KxGiant	Lake English	Payne	Tutle
J. nigra	-	-	-	-	-	-	-	-	-
J. regia									
ACO	0.381	-	-	-	-	-	-	-	-
Blackmore	0.318	0.765	-	-	-	-	-	-	-
Gobind	0.381	0.579	0.667	-	-	-	-	-	-
Hartley	0.450	0.579	0.500	0.500	-	-	-	-	-
KX Giant	0.280	0.650	0.571	0.571	0.571	-	-	-	-
Lake English	0.474	0.611	0.450	0.450	0.813	0.600	-	-	-
Payne	0.364	0.824	0.938	0.632	0.550	0.619	0.500	-	-
Tutle	0.364	0.632	0.550	0.476	0.722	0.789	0.765	0.600	-

reveal that esterase and malate dehydrogenase enzyme systems can be used for identification of different genotypes in walnut. The presence or absence of PPO I, PRO I and EST III was found remarkable for identification of the two species of walnut namely *J. regia* and *J. nigra*. The differences found in various isozymes for the enzyme systems studied can therefore be used to identify the variations obtained within a species and between cultivars. The dendrogram also showed a close relationship between the different cultivars of *J. regia*, whereas *J. nigra* was distinctly different. Two distinct groups of cultivars in *J. regia* were observed.

To conclude, the results obtained in this study can be useful in current walnut breeding programs, allowing the identification of new cultivars as well as the assessment of genetic similarity among genotypes which will help in selecting the best parents to obtain new genetic combinations. The simple allelic interpretation used in the present study is useful for generalization of the technique for routine purposes. However, this interpretation may not be very useful for deducing the genetic structure of the walnut genotype.

These will however be of immense importance for the walnut growers and foresters to assess the planting material essentially at an early stage.

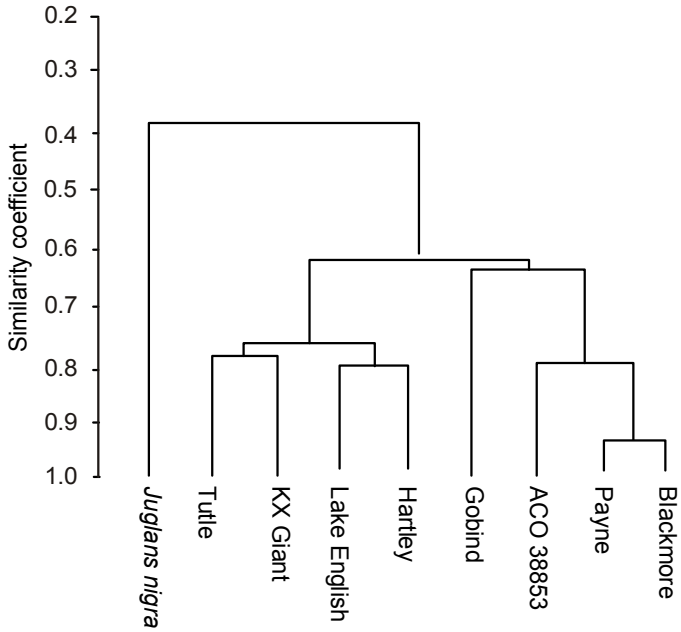

Fig. 6. Dendrogram of *J. nigra* and *J. regia* cultivars based on similarity coefficients

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