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Isozyme Variation and Genetic Relationships among Three *Plumbago* Species

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Keywords

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Abstract

The purpose of the study is to understand the genetic affinities and variation among three *Plumbago* species by means of isoenzyme viz., esterase, peroxidase, poly phenol oxidase and Restriction Fragment Length Polymorphism using Eco RI, Hind III and Bam HI. Each and every species expressed their similarities and variation by the presence and absence of their banding profiles in the gel system. A total of 32 bands were observed in the isozyme system of *Plumbago* species. The RFLP profile of the *Plumbago* species showed seven bands in three active zones / regions. The cladogram shown that two clusters, of which cluster 2 includes only one species viz., *P. auriculata* showed 100% of divergence with other two species. Cluster 1 (C_1) showed two nodal (N) branches (C_1N^1 and C_2N^2). C_1N^1 was *P. zeylanica* and C_1N^2 was *P. rosea.*

1. Introduction

India is a major centre of origin and diversity of crop and medicinal plants. It holds an extraordinary significance among the top gene-rich countries of the world relating to its abundantly rich land race diversity in medicinal, agricultural and horticultural crops and their wild relatives. India possesses about 20,000 species of higher plants and one third of it being endemic and 500 species are categorized to have medicinal value. The South Western Ghats is one of the major repositories of endemic and medicinal plants. It harbors around 4,000 species of higher plants of which 450 species belonging to 150 natural orders are endangered. The tools of modern biotechnology are being increasingly applied for plant diversity characterization and undoubtedly they have a major role in assisting plant conservation programmes. However, their value is dependent upon ensuring that biotechnological methods are targeted effectively and utilized as complementary and enabling technologies. Most importantly, they must be applied in the appropriate context. There are four main areas of biotechnology, which can assist plant conservation programmes Molecular markers technology, Molecular diagnostics, Tissue culture *(in vitro* technologies) and Cryopreservation. Genetic information provided by morphological characters is often limited and expression of quantitative trait is subjected to strong environmental influence. In 1960s the biochemical method based on seed, leaf protein and enzymes electrophoresis were introduced, which proved particularly useful in analysis of genetic diversity as they reveal differences between seed storage proteins or enzymes encoded by different alleles at one (allozymes) or more gene loci (isozymes). Use of biochemical methods eliminates the environmental influence. The ability to identify genetic variation is indispensable to effective management and use of genetic resources. Electrophoretic separation methods are increasingly playing an important role in genetic diversity analysis and conservation of plant genetic resources. These methods are being used as complementary strategies to traditional approaches for assessment of genetic diversity. The analysis can be performed at any growth stage using any plant part and it requires only small amounts of materials. Following the advances in molecular biology in the last decades variety of different methods have been developed for analysis of genetic diversity. These methods differ with respect to technical requirements, level of polymorphism detected, reproducibility and cost [1]. In recent years, limitations of morphological and biochemical markers has been overcome by biochemical and molecular markers. Among the different molecular markers, Isozymes are relatively cheaper and simple to use in variety of applications in plant research. Isozymes have been used extensively in plants for both taxonomic and population analyses [2 - 9]. Isozymes have the advantage that material is processed by an efficient and inexpensive technique without requiring prior knowledge of the genome. Isozymes have the advantage of being easy to isolate, requiring very small amount of isozymes without the need of blotting and radioactive detection and are moderately reproducible. In this

research, an attempt has been made to fingerprint and study their genetic relativity of *Plumbago* species viz., *Plumbago zeylanica, Plumbago rosea* and *Plumbago auriculata* using isozyme (biochemical markers), which could be very helpful for characterization germplasm management and genetical improvements. In addition, an attempt has been made to study diversity in isozyme profiles for understanding the species inter-relationship and variation among *Plumbago*, to provide further indications about their genetic relations.

2. Materials and Methods

The species of *Plumbago* viz., *Plumbago zeylanica* (Lane 1)*, Plumbago rosea* (Lane 2) and *Plumbago auriculata* (Lane 3 were collected from the wild and established in the botanic garden of Muthayammal College of Arts and Sciences, Rasipuram, Namakkal, Tamil Nadu, India. For Isoenzyme analysis, 500 mg of freshly harvested young leaves were taken and homogenized with 3.5 ml of ice-cold homogenizing buffer in a pre-chilled pestle and mortar. For peroxidase, the young shoots were homogenized with 0.1M phosphate buffer (pH 7.0) and centrifuged at 12,000 rpm for 10min. For esterase, the young leaves were collected and ground with pre-chilled isolation buffer (0.1M phosphate buffer pH 9.2) and centrifuged at 12,000 rpm for 10 min. For acid and alkaline phosphatase the young leaves were harvested and homogenized in a mortar and pestle with citrate buffer and centrifuged at 20,000 rpm for 10 min. The supernatant was subjected to electrophoresis as described by Sadasivam & Manickam [10]) on PAGE. For the detection of isozymes on the gels, the staining solution, were prepared as per Sadasivam & Manickam [10]. After the electrophoresis, the gels were incubated in the staining solution for few minutes under dark condition till the clear bands appeared. The gels were fixed with 7% acetic acid solution for 30 min, washed with distilled water and photographed using the gel documentation system, Genei Bangalore, India. DNA was isolated by using CTAB protocol developed by Doyle and Doyle [11] with minor alterations described by Tel-Zur *et al.,* [12]. Isolated genomic DNA (4µg) was digested for 1 h with 8 U of Hind III, Bam HI and Eco RI under 37°C. The digested DNA fragments were separated on 1% agarose at 5V/cm.

3. Results

Esterase

Multiple zones of activity were obtained for this enzyme (Iso-esterase) system EST 2 to 10. Zone two limited with only one band EST 2^1 with 0.103 MW-Rf values. It was shared by *P. rosea* and *P. auriculata*. Similar to zone two, zone three also showed only one band (EST 3^1), it's (0.206897) showed unique presence only in *P. auriculata*. EST 4^1 (0.327586) was restricted to *P. rosea* and EST 4^2 (0.327586) was showed its unique presence in *P. auriculata*. EST 5^1 (0.465517) was shared by *P. rosea* and *P. auriculata*. EST 6^1 (0.534483) was restricted with *P. zeylanica*. EST 7^1 (0.603448) was shared by all the selected three species. EST 7^2 (0.655172) was present only in *P. rosea*. EST 8^1 (0.706897), EST 9^1 (0.810345), EST 9^2 (0.896552) and EST 10^1 (0.965517) were expressed only in *P. auriculata* (Fig. 1 A: Table – 1).

Peroxidase

Six zones of activity (PRX 1 – 6) were observed in this enzyme system. The first band PRX 1^1 (0.054545) was restricted to *P. auriculata*. Second band MW-RF 0.090909 (PRX 1^2) was shared by two selected species *P. zeylanica* and *P. rosea*. PRX 2^1 (0.181818) and PRX 3^1 (0.290909) were present only in *P. zeylanica*. PRX 4^1 (0.381818) was showed it's jointly presence in *P. zeylanica* and *P. auricultata*. PRX 5^1 (0.418182) was expressed in *P. zeylanica* and *P. rosea*. PRX 6^1 (0.563636) was restricted to *P. rosea* (Table 1: Fig. 1 B).

Poly Phenol Oxidase

Four zones of activity were observed for this enzyme system PPO 2, 5, 6 and 7. PPO 2^1 (0.178082) and PPO 7^1 (0.657534) were present only in *P. auricultata*. PP0 5^1 (0.424658) was restricted to *P. zeylanica*, PPO 5^2 (0.474) was present only in *P. rosea* and PPO 6^1 (0.560) was unique to *P. zeylanica*. PPO 6^1 (0.520548) was shared by *P. zeylanica* and *P. auricultata* (Table -1: Fig. 1 C).

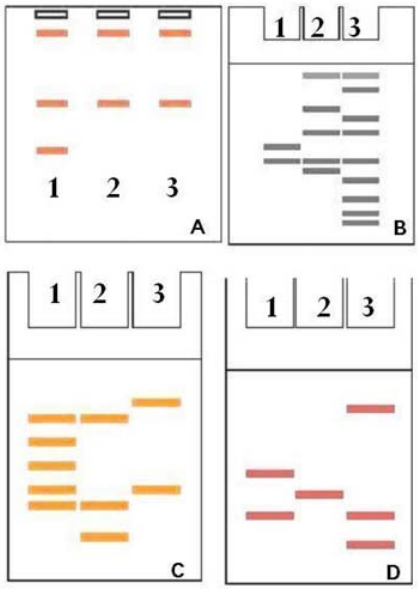
Table 1: Molecular Profile of *Plumbago* species

MW-Rf	Positions	*P. zeylanica*	*P. rosea*	*P. auriculata*
		Esterase		
0.103448	EST 2^1	-	+	+
0.206897	EST 3^1	-	-	+
0.327586	EST 4^1	-	+	-
0.396552	EST 4^2	-	-	+
0.465517	EST 5^1	-	+	+
0.534483	EST 6^1	+	-	-
0.603448	EST 7^1	+	+	+
0.655172	EST 7^2	-	+	-
0.706897	EST 8^1	-	-	+
0.810345	EST 9^1	-	-	+
0.896552	EST 9^2	-	-	+
0.965517	EST 10^1	-	-	+
		Peroxidase		
0.054545	PRX 1^1	-	-	+
0.090909	PRX 1^2	+	+	-
0.181818	PRX 2^1	+	-	-
0.290909	PRX 3^1	+	-	-
0.381818	PRX 4^1	+	-	+
0.418182	PRX 5^1	+	+	-
0.563636	PRX 6^1	-	+	-
		Poly Phenol Oxidase		
0.178082	PPO 2^1	-	-	+
0.424658	PPO 5^1	+	-	-
0.479452	PPO 5^2	-	+	-
0.520548	PPO 6^1	+	-	+
0.657534	PPO 7^1	-	-	+
		DNA Restriction by HIND III, Bam HI and Eco R I		
0.119266		+	+	+
0.321101		+	+	+
0.412844		+	-	-

DNA Restriction with Restriction Enzymes

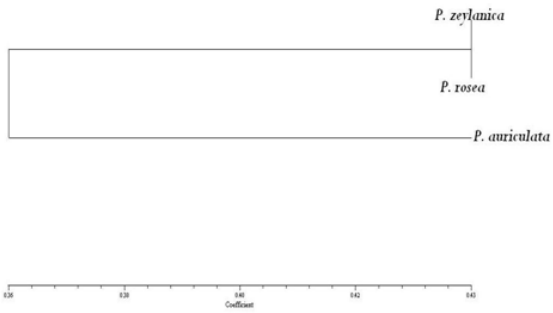
Three zones of activity were observed in the genomic DNA restriction**.** *P. zeylanica, P. rosea* and *P. auricultata* showed two common bands with 0.119266 and 0.321101 MW-Rf values. *P. zeylanica* alone showed unique band with 0.412844 MW- Rf values (Table -1: Fig. 1 D). Cluster analysis was performed using isozyme data and it produced stable and consistent patterns (Figure 1). The cladogram shown that two clusters, of which cluster 2 includes only one species viz., P. auriculata showed 100% of divergence with other two species. Cluster 1 (C_1) showed two nodal (N) branches (C_1N^1 and C_2N^2). C_1N^1 was *P. zeylanica* and C_1N^2 was *P. rosea* (Fig. 2).

Fig. 1: Isozyme and RFLP profile of *Plumbago* species



A- RFLP profile of *Plumbago* species
B - Iso-esterase Profile of *Plumbago* species
C- Isoperoxidase Profile of *Plumbago* species and
D - Poly Phenol Oxidase Profile of *Plumbago* species
(1- *Plumbago zeylanica;* 2 – *Plumbago rosea* and 3 – *Plumbago auriculata*)

Fig. 2. Cladogram of the selected *Plumbago* species based on Isozyme and RFLP profile



4. Discussion

Genetic characterization of natural resources is an essential step for a better understanding of genetic resources for the implementation of *in situ* and *ex situ* conservation activities [13]. So adequate knowledge about the plants is necessary for planning sustainable development of any region like India, where the flora is rich in diversity and endemism [14] Variation is the basic resource to be explored for genetic improvement in any species and hence play a key role in plant improvement programmes [15-17]. Many researchers have studied the genetic variability in *inter* and *intra*-populations on natural ecosystems for the purposes of gene pool conservation [18,19]. The result contained in this study identifies the degree of genetic diversity based on isozyme and RFLP data in *Plumbago*. The similarity and variation between *Plumbago zeylanica*, *Plumbago rosea* and *Plumbago auriculata* are reported with respect to the enzymes esterase, peroxidase, polyphenol oxidase and RFLP (Fig. 1 A to D). The present study reveals that the three selected species are easily separable isozymically, besides revealing the affinities. In electrophoresis, each zone is occupied by a particular isozyme in the form of band and is representative of the appearance of a particular gene locus coding for that isozyme. In certain enzyme system, more than one distinct band could be resolved in a particular zone. These bands could represent allelic isozymes, coded by different alleles of the same gene at locus and thus occupy that particular zone on the gel [20]. In the present study also the similar kind of banding profiles are

observed in all enzyme systems indicating the presence of multiple alleles. Isozymes such as esterase and peroxidase have been utilized to assess the genetic similarity and differences at the various taxonomic levels [21-30]. Similarly in the present study also, these isozymes are used as biochemical marker for the systematic study of *Plumbago* species. Unique banding profiles of esterase, peroxidase, polyphenol oxidase and RFLP were observed in *Plumbago zeylanica*, *Plumbago rosea* and *Plumbago auriculata,* which represent the fingerprint of that particular species. Such finger printing is useful in differentiating the species and act as biochemical markers for these species in plant systematic studies. From the study, it is understandable that the species *P. auriculata* is distinct from *P. zeylanica* and *P. rosea* which show greater affinity towards each other. The cladogram shown that two clusters, of which cluster 2 includes only one species viz., *P. auriculata* showed 100% of divergence with other two species. High levels of genetic variation would bestow the population or species more flexibility to fit a variable environment, or to occupy new ecological sites and new habits [31]. In the present study we observed, *P. auriculata* showed high percentage variation compared to other two species. The present study results were directly accordance with Huenneke's observations, *P. auriculata* is more flexible one to fit with a variable environment, or to occupy new ecological sites and new habits in nature. Cluster 1 (C_1) showed two nodal (N) branches (C_1N^1 and C_2N^2). C_1N^1 was *P. zeylanica* and C_1N^1 was *P. rosea* (Fig. (**2**)). Information on the levels and distribution of genetic diversity of any plant species may contribute to the knowledge of their evolutionary history and potential, and is critical to their conservation and management [32, 33]. The isozymic and genetic profiles describes that the *P. zeylanica* and *P. rosea* may be originated from same mother plant. The enzymatic variation between the three species shows that this gene pool is a good resource for breeding studies. This profile system can use for identifying the correct species for future breeding and Pharmaceutical studies. In conclusion it can be stated that the availability of isozyme loci has substantially increased our knowledge of the genetics of plant populations. These results have important implications for the conservation strategy.

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