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Biochemical Variation Studies in *Aegle marmelos* (L.) Corr - A medicinally important plant

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ABSTRACT

The present study was aimed to evaluate the biochemical polymorphism of Aegle marmelos (L.) Corr - A medicinally important plant using the isoperoxidase and protein pattern. For the electrophoresis studies, young leaves were harvested from different localities viz., Thrissur, Dharmapuri, Tenkasi, Salem, Attur and Kolli Hills. The isolation and separation of protein and isoenzyme was performed by the standard methods. The protein gel system revealed a total of forty six bands with eight active zones / regions (PB1 to 8) and twenty one positions were observed in the protein system of A. marmelos. Multiple zones of activity were obtained for iso-peroxidase system PRX1 to 5. A total of twenty nine bands in twelve different positions of expression were observed in the iso-peroxidase system of A. marmelos. Pairing affinity or similarity indices analysis revealed the similarity and evolutionary relationship among the selected accession of A. marmeols. Highest percentage (60%) of similarity was observed between the accession from Tenkasi and Salem. The cladogram of A. marmelos revealed the genetical similarity and variation, evolutionary relationship among between the selected six the selected accession. The cladogram shown that two major clusters, of which cluster 2 include only one accession viz., Thrissur. This profile system can be used as biochemical marker for selection superior genotype of the medicinally important plant Aegle marmelos.

Keywords: Isoperoxidase, Protein, Cladistics, *Aegle marmelos*, Biochemical Marker.

INTRODUCTION

Aegle marmelos (L) Corr. (Family: Rutaceae), commonly known as the "Bael Tree" is an important tree species with multiple utility. The species is used in the Indian system of medicine for treating various ailments. The unripe fruit is an astringent, a digestive and stomachic, and is used to cure diarrhea and dysentery [1]; the ripe fruit is used for curing dyspepsia [2, 3]. The roots and bark are used in the treatment of fever and to control pain in the abdomen [4]. The leaves possess anti-inflammatory and analgesic properties [5]. The alkaloid aegeline present in the leaf is a potent antiasthmatic agent [6]. Various parts of the tree, including the fruit, possess medicinal properties. The roots are useful for treating diarrhoea, dysentery, and dyspepsia [7]. The leaf is used for opthalmia, diabetes, and asthmatic complaints. The aqueous extracts of the stem and root bark are used to treat malaria, fever, jaundice, and skin diseases such as ulcers, urticaria, and eczema [8]. In pharmacological trials, both the fruit and root showed antiamoebic and hypoglycaemic activities [9, 10]. The plant is rich in alkaloids, among which aegline, marmesin, marmin, and marmelosin are the major ones. Aqueous leaf extract and methanolic extract of the root bark of *A. marmelos* showed preventive effects on myocardial diseases [11, 12]. The compounds luvangetin and pyranocoumarin, isolated from the seeds of *A. marmelos*, showed significant antiulcer activity [13]. Essential oil isolated from the leaf has antifungalactivity [14]. The leaves are astringent, febrifuge, expectorant, and are reported to have hypoglycaemic, antiasthmatic and antispermatogenic properties [15, 16] and to cure jaundice [17]. It also enhances the wound healing activity [18]. *A. marmelos* root is one of the ingredients of the popular ayurvedic preparations such as *Dasamula* and *Vilvadi lehya*. Different parts of the tree also contains certain biochemical constituents namely alkaloids, aegelinol, coumarin, steroid [19], terpenoid [20] and tannin [21]. The plant has been widely used for its having antibacterial [22], antifungal [20], antioxidant [23], antidiarrhoetic [24], pesticidal, antidote, anti-inflammatory, hepatitis, tuberculosis, dyspepsia and also beneficial for heart and brain [19]. Various parts of the plants are also used for treating anaemia, wound healing, high blood pressure, asthma, jaundice, and troubles during pregnancy, typhoid [21] and diabetes [25]. The ripe fresh fruits are eaten and its juice is used as soft drinks, for making candy, squash, pulp powder and nectar [19] unripe fruits are used for making marmelle oil [26] and baelshut [27] which may be valuable for medicine. The edible portion of the flesh contain water, protein, starch, fat, mineral salt, carotene, niacin, vitamin B1, vitamin B2, vitamin C, calcium and iron [28]. Also, the tree yields quality timber for making pestles, posts, shafts, and furniture [29]. Plant regeneration via i*n vitro* methods has been reported in A. marmelos from different explants, i.e., cotyledonary node [30], root segments [31], nucellus [32], and single-node segments [29]. Pati et al., [33] demonstrated the in vitro clonal propagation of bael (*Aegle marmelos* Corr.) CV. CISH-B1 through enhanced axillary branching and genetic fidelity of the in vitro raised plants using 13 RAPD, 3 ISSR and 2 DAMD primers. *A. marmelos* is a medium spreading, deciduous, highly heterogygous woody fruit tree [34] in which polyembryony is a common phenomenon [35]. The bael fruit is commonly multiplied by seed in nurseries and the seedlings show great variation in morphological and biochemical characters due to heterozygous nature of plant. Trees are genetically variable in their natural population and the amount of variation is dependent on the species level. Sufficient genetic variability is needed to improve forest trees since genetic variation within a population is the raw material upon which evolutionary changes occur. Knowledge on genetic diversity among and within species is needed for all conservation purposes. Information on the baseline diversity, either measured or

predicted, is essential in deciding what and how to conserve, accession of genetic changes and genetic parameters that are relevant in conservation plan. In usually quantified in terms of number of polymorphic loci per species, the effective number of alleles per locus and the number of heterozygotes loci per individual. Genetic markers derived from electrophoretic analysis can be used to survey the level of genetic diversity within and among populations and also for taxonomic purpose [36]. Isozyme analysis is a highly appropriate method for identifying genomic allele components as well as supplementing DNA analysis. Since the 1930s, electrophoresis in conjunction with the zymogram technique has been used as a tool for the study of heritable variation. Isozymes are widely used because of their relative efficiency and cost effectiveness, particularly in studies of intra- and inter-specific variation Johnson *et al*. [37], Siva and Krishnamurthy [38], Johnson et al. [39] and Smila *et al*. [40]. There is no report on the isozyme and protein pattern studies on wood apple from South India. The main objective of this study is to evaluate genetic variations (protein and iso-peroxidase level) of *Aegel marmeols*.

EXPERIMENTAL SECTION

For the electrophoresis studies, young leaves were harvested from differ localities viz., Thrissur, Dharmapuri, Tenkasi, Salem, Attur and Kolli Hills and ground on ice old mortar and pestle with 0.1 M phosphate buffer (pH 7 .0). The slurry was centrifuged at 10,000 rpm at 4^{o} C for 10 min. and the supernatant were collected and separated by native poly acrylamide gel electrophoresis. The native (PAGE) and SDS – PAGE gel electrophoresis was performed by Anbalagan [41] method. For isoperoxidase, the gel was stained with O - dianisidine (100mg) acetate buffer (90ml, pH 4.2), ethanol (5ml), 30% H_2O_2 (1ml) and distilled water (4ml) [40]. The banding patterns were documented and Rf values were calculated using Biogene Software. Variation in banding pattern was determined by the migration from the origin towards the anode. Isozymes region were designated to define the general area on the zymogram with in which the bands migrated.

RESULTS AND DISCUSSION

The protein gel system revealed a total of forty six bands with eight active zones / regions (PB1 to 8) and twenty one positions were observed in the protein system of *Aegle marmelos* (Fig. 1. A. and Table -1). By the unique and shared expression the accessions showed their evolutionary relationships. In the protein system, the $PB1^2$ (0.04) was shared by accessions from Dharmapuri, Tenkasi, Salem, Attur and Kolli Hills, $PB6^2$ was shared by accessions from Tenkasi, Salem, Attur and Kolli Hills, $PB8^1$ was showed its expression in accession from Dharmapuri, Tenkasi, Salem and Kolli Hills. The accession from Thrissur showed its uniqueness by the presence of following bands MW-Rf 0.02, 0.59 and 0.65. Next to that, the accession from Tenkasi showed the individuality by the expression of MW-Rf. 0.08 and 0.37 in the protein system. The MW-Rf. 0.75 was restricted its availability with only Attur accession. The accession from Kolli Hills also showed its distinctiveness by the presence of 0.47 and 0.64 proteins. The similarity indices of the six accessions based on the protein profiles were tabulated in Table 2.

Multiple zones of activity were obtained for iso-peroxidase system PRX1 to 5. A total of twenty nine bands in twelve different positions of expression were observed in the iso-peroxidase system of *Aegle marmelos* (Fig. 1.B and Table -1). $PRX1^1$ (0.03) showed its presence in all

selected accessions. Similarly, the MW-Rf 0.27 also showed its presence except the accession from Attur. Next to that, MW-Rf 0.33 was shared by the accessions from Dharmapuri, Tenkasi, Salem and Kolli Hills. $PRX2^1$ (0.11) and $PRX\ 4^3$ was restricted to the accession from Dharmapuri. The accession from salem showed its uniqueness by the presence of MW-Rf. 0.20 in the isoperoxidase system. The MW-Rf. 0.29 was present only in the accession from Attur. $PRX5^1$ (0.44) was expressed only in accession from Thrissur (Fig. 1. B). The similarity indices of the six accessions based on the iso-peroxidase patterns were tabulated in Table 2.

Pairing affinity or similarity indices analysis revealed the similarity and evolutionary relationship among the selected accession of *Aegle marmeols*. Highest percentage (60%) of similarity was observed between the accession from Tenkasi and Salem. Next to that, 59.26%, 57.14% and 56% of similarity was observed between the accession from Tenkasi and Kolli Hills, Tenkais and Dharmapuri and Salem and Kolli Hills respectively. Lowest percentage similarity (18.18%) and highest percentage (81.82%) of variation was observed between the accession from Thrissur and Salem. The cladogram of *A. marmelos* revealed the genetical similarity and variation, evolutionary relationship among between the selected six the selected accession.

Table 1: Biochemical profile of *Aegle marmeols*

MW- Rf	Positions	Thrissur	Dharmapuri	Tenkasi	Salem	Attur	Kolli hills
PROTEIN PROFILES							
0.02	$PB1^1$	+	-	-	-	-	-
0.04	$PB1^2$	-	+	+	+	+	+
0.08	$PB1^3$	-	-	+	-	-	-
0.11	$PB2^1$	-	-	+	+	-	-
0.27	$PB3^1$	+	+	+	-	-	-
0.29	$PB3^2$	-	-	+	+	+	+
0.37	$PB4^1$	-	-	+	-	-	-
0.40	$PB4^2$	-	-	+	+	-	-
0.47	$PB5^1$	-	-	-	-	-	+
0.48	$PB5^2$	-	-	+	-	+	-
0.51	$PB6^1$	+	+	-	-	+	-
0.53	$PB6^2$	-	-	+	+	+	+
0.59	$PB6^3$	+	-	-	-	-	-
0.60	$PB6^4$	-	-	-	+	-	-
0.63	$PB7^1$	-	+	+	-	-	-
0.64	$PB7^2$	-	-	-	-	-	+
0.65	$PB7^3$	+	-	-	-	-	-
0.67	$PB7^4$	-	-	-	+	+	-
0.73	$PB8^1$	-	+	+	+	-	+
0.75	$PB8^2$	-	-	-	-	+	-
0.80	$PB8^3$	-	+	+	-	+	+
Iso- Peroxidase Profiles							

0.03	PRX1[1]	+	+	+	+	+	+
0.11	PRX2[1]	-	+	-	-	-	-
0.17	PRX2[2]	-	-	+	-	-	+
0.20	PRX2[3]	-	-	-	+	-	-
0.22	PRX3[1]	-	+	-	-	+	+
0.27	PRX3[2]	+	+	+	+	-	+
0.29	PRX3[3]	-	-	-	-	+	-
0.33	PRX4[1]	-	+	+	+	+	-
0.39	PRX4[2]	-	-	-	+	+	-
0.40	PRX4[3]	-	+	-	-	-	-
0.44	PRX5[1]	+	-	-	-	-	-
0.49	PRX5[2]	-	-	-	+	-	+

The cladogram shows that two major clusters, of which cluster 2 include only one accession viz., Thrissur. Cluster 1 (C_1) showed two nodal (N) branches (C_1N^1 and C_2N^2). Nodal 1 showed two branches (B), $C_1N^1B_1$ was accession from Attur and $C_1N^1B_2$ was further divided in to two branches viz., $C_1N^1B_2b_1$ and $C_1N^1B_2b_2$. $C_1N^1B_2b_1$ was specific to Kolli Hills and *$C_1N^1B_2b2$* was shared by Tenkasi and Kolli Hills accession. Nodal 2 (C_1N^2) showed only one branch and unique to accession from Dharmapuri (Fig. 1 C and Table -2).

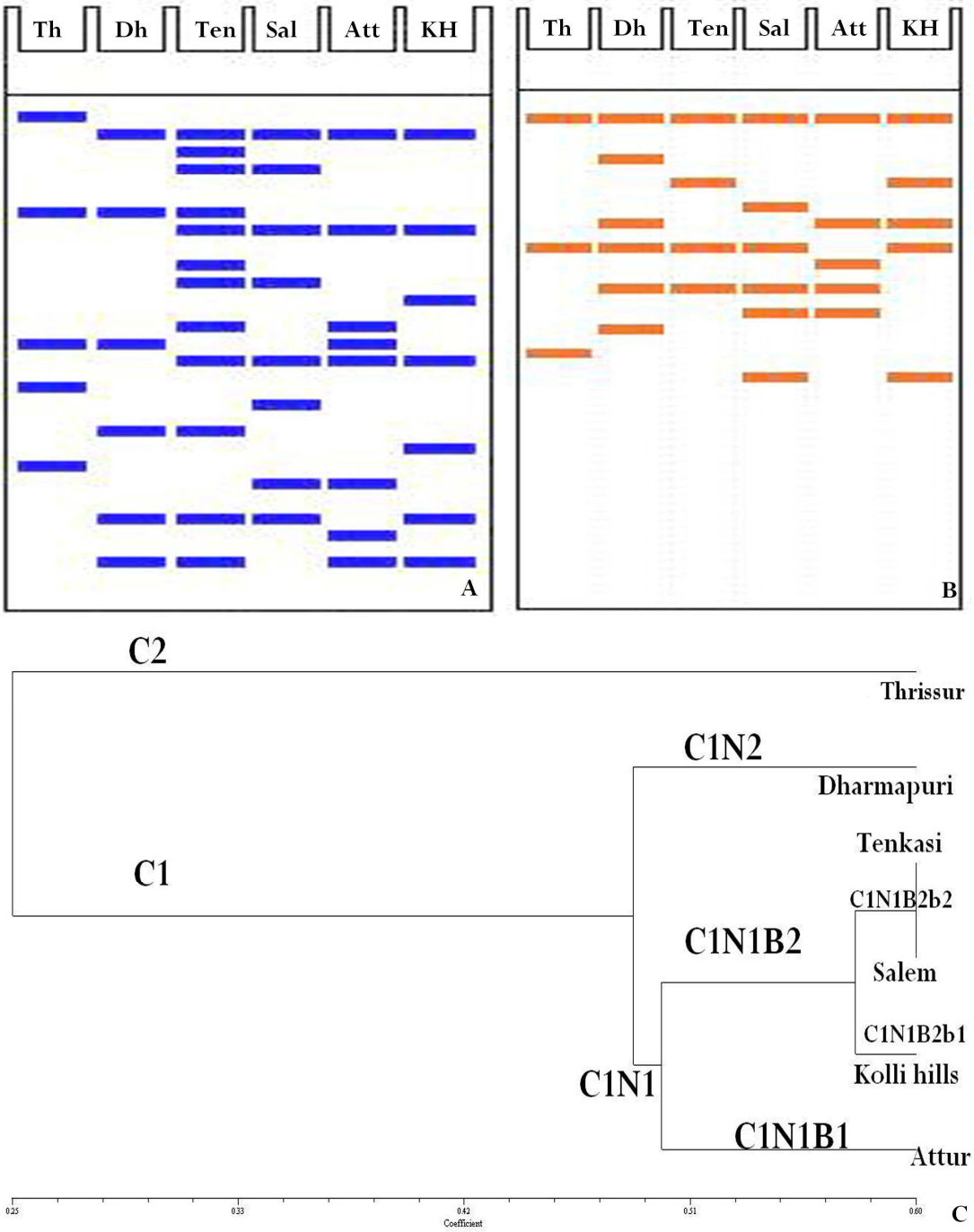

Fig. 1. Biochemical Profile of *Aegle marmeols*

A. SDS – PAGE Protein profile of Aegle marmeols; B – Isoperoxidase profile of Aegle marmeols; C- Cladogram of Aegle marmeols based on the protein and isoperoxidase pattern. (In fig C; C1 and C2 denotes the Clusters; N1 and N2 denotes the nodes; B1 and B2 denotes the branches)

Table 2: Protein and Isoperoxidase similarity indices of *Aegle marmeols*

Accessions	Thrissur	Dharmapuri	Tenkasi	Salem	Attur	Kolli hills
PROTEIN PROFILE						
Thrissur	1.0000					
Dharmapuri	0.3636	1.0000				
Tenkasi	0.1176	0.5555	1.0000			
Salem	0.0000	0.2857	0.6000	1.0000		
Attur	0.1538	0.4285	0.5000	0.5000	1.0000	
Kolli hills	0.0000	0.5000	0.5555	0.5714	0.5714	1.0000
ISO-PEROXIDASE						
Thrissur	1.0000					
Dharmapuri	0.4444	1.0000				
Tenkasi	0.5714	0.6000	1.0000			
Salem	0.4444	0.5000	0.6000	1.0000		
Attur	0.2500	0.5455	0.4444	0.5455	1.0000	
Kolli hills	0.5000	0.5455	0.6667	0.5455	0.4000	1.0000
PROTEIN and ISO-PEROXIDASE PROFILE						
Thrissur	1.0000					
Dharmapuri	0.4000	1.0000				
Tenkasi	0.2500	0.5714	1.0000			
Salem	0.1818	0.3846	0.6	1.0000		
Attur	0.1905	0.4800	0.4828	0.5185	1.0000	
Kolli hills	0.2105	0.5217	0.5926	0.5600	0.5000	1.0000

Here, the presence and absence of bands has been used to categorize the similarity and variation among the species by the biochemical compositions. The protein and isoperoxidase banding profile system revealed the biochemical variation and evolutionary relationship among the six accessions of *Aegle marmelos* viz., Thirussur, Dahrmapuri, Tenkasi, Salem, Attur and Kolli Hills. According Hamrick and Godt [36], isozymes are practical, useful genetic and biochemical markers as well as good estimators of genetic variability in plant populations. In the present study we used the protein and isoperoxidase patterns as a tool for intra specific variation studies. The protein profile and enzymatic pattern of *Aegle marmelos* shows that accession from Tenkasi, Salem and Kolli Hills are clustered in a single branch, indicates the evolutionary origin of the *Aegle marmelos*. The result contained in this study identifies the degree of genetic diversity based on protein and isozyme profile in *Aegle marmeols.* Although, a wide array of DNA-based molecular procedures introduced in the last two decades allow genetic diversity to be estimated with greater precision, isozyme studies still have numerous advantages like wider applicability, low cost and speed of estimation [42, 43].

Theory predicts that levels of genetic variability depend on population size [44], with small populations having lower levels of variability than larger ones. As pointed out by Gitzendanner and Soltis [45], it is usually considered that rare species have low levels of genetic variability

because of the generalized assumption that rare species have small population sizes. The present study also revealed the high levels of polymorphic banding patterns in agreement with Gitzendanner and Soltis [45] observations. The high level of polymorphic bands presence may be due to the cosmopolitan availability in all localities. One of the main goals of conservation geneticists is to quantify levels of genetic diversity, as well as the distribution of genetic variability within and between populations, since preservation of the evolutionary potential of endangered species is a primary aim in species conservation [46]. Knowledge of the genetic structure of the species will give us information about historical and contemporary patterns of gene flow among populations. The present study elucidated the distribution of genetic variability among the selected accession; this will help to identify the superior genotype of *Aegle marmelos*. This profile system can used as biochemical marker for the medicinally important plant *Aegle marmelos*. The electrophoretic separation constructed a pave for the further biochemical and molecular studies on *Aegle marmelos*. Loss of genetic variation has traditionally been considered to decrease both the short- and long-term adaptability of populations in variable and changing environments. Knowledge of genetic variability and its structure will provide a basis for the sustainable management and conservation of populations in threatened plants. In the present study, biochemical variability and similarity (protein and isoperoxidaaaase) of *Aegle marmelos* of populations from India was identified. However it is necessary to use molecular marker to know the genetic structure of the species. Further studies on the genetic analysis of *Aegle marmelos* with more accessions and with advanced molecular markers like SNPs, VNTRs, ISSRs will produce the more detailed genetic structure of *Aegle marmeols*.

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