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PHYLOGENETIC RELATIONSHIP OF TEN IPOMOEA JACQ. SPECIES BASED ON PROTEINS AND ISOZYME ANALYSIS

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ABSTRACT

The genotype of an organism expresses itself through the phenotype that is through proteins. Protein molecular markers provide indirect information about plant genome structure. Quantitative estimation of protein content from germinating seedlings was performed by Lowry assay. Total soluble protein analysis of all the species of *Ipomoea* under study was carried out by Native PAGE and SDS-PAGE, followed by isozyme analysis for Esterases (EST), Peroxidases (POX), Malate dehydrogenase (MDH), Ribulose bis-phosphate carboxylase-oxygenase (RUBISCO). The genetic similarity matrix was calculated using Jaccard's co efficient and the dendrogram was constructed using UPGMA. None of the ten species showed identical bands in the electrophoretic leaf protein banding pattern on SDS PAGE. On the basis of similarity index of Esterases isozyme analysis *I. aquatica* and *I. quamoclit* showed 100% affinity, while *I. triloba and I. violacea* exhibited minimum similarity with all the ten species. On the basis of similarity index Peroxidases isozyme analysis *I. hederifolia* and *I.turbinata* exhibited minimum similarity of 25%. The cluster analysis based on the combination of total soluble Proteins and the four isoenzymes showed that *I. pes-caprae* and *I. carnea* are closest with maximum similarity of 77.8%, whereas *I. quamoclit* and *I. violacea* has least similarity of 42.3%.

INTRODUCTION

The root of electrophoretic analysis of isozymes was laid down in 1957 (McMillin 1983), when Hunter and Mohler discovered the isozymes. In 1959, Markert and Moler introduced the concept of isozymes, which they defined as the different molecular forms in which proteins may exist with the same enzymatic specificity. This means that different variants on the same enzymes have identical or similar functions and are present in the same individual. Genetic polymorphism for isozymes within the same population was discovered by Wendel and Weeden in 1990, that revealed the possibility for population genetics to make precise quantitative estimates of genetic variability based upon one parameter of the molecular structure of the primary products of the genes themselves. The genetic markers provided by protein and enzyme polymorphism in plant population has been used widely to assess the degree of genetic variability and species relationship (Raj *et al.,* 2011). Polymorphism may be defined as simultaneous occurrence within or between populations of

multiple phenotypic forms of a trait attributable to the allels of a single gene or the homologs of a single chromosome. In natural populations recurrent mutations of genes produce variability. There are polymorphic loci, variable in the sense described above, and monomorphic or nonvariant. Inter-specific phenotypic variations can be analyzed using Native PAGE, SDS-PAGE protein profile and isozyme pattern, followed by determining the similarity index analysis and the construction of a phylogeny tree. Enzymatic activity staining after native PAGE is a widely used technique for isozyme analysis. It allows the detection and visualization of multiple isoforms of a specific enzyme present in a small amount of extract. The study of isoforms by native PAGE, however, is often limited by the availability of a specific and sensitive detection method. Isozymes arise in nature by two general mechanisms, i.e. genetic and epigenetic. The source of gene multiplicity is duplication through mutation, polyploidization and chromosomal aberrations. The relative staining strength of isozyme bands has been considered to represent the relative activity and content of corresponding isozymes. Generally, the higher the enzyme content and activity, the higher the staining

strength and the wider the band in the zymogram. However, isozymes represent a group of enzymes which catalyse the same reaction. They may have different structures and optimum conditions of reaction such as pH and temperature. (Dongxu *et al.,* 1992). Simple analytical procedures, allow some allozymes/ isozymes to be applied at relatively low costs, depending on the enzyme staining reagents used. isozymes are codominant markers that have high reproducibility. Zymograms (the banding pattern of isozymes) can be readily interpreted in terms of loci and alleles, or they may require segregation analysis of progeny of known parental crosses for interpretation. Sometimes, however, zymograms present complex banding profiles arising from polyploidy or duplicated genes and the formation of intergenic heterodimers, which may complicate interpretation. Isozyme analysis has been widely used for analysing genetic diversity and population structure in a large range of plant species (Hamrick and Godt, 1989; Fady-Welterlen, 2005). The analyses are cheap and relatively easy to perform, which makes it possible to study an excessive amount of individuals.

Some of the commonly investigated isozyme profiles for phylogenetic relationships between taxa are:

Esterases (EST): An esterase is a hydrolase enzyme that splits esters into an acid and an alcohol in a chemical reaction with water called hydrolysis. A wide range of different esterases exist that differ in their substrate specificity, their protein structure and their biological function.

Peroxidases (POX): Plant peroxidases (are monomeric heme-containing enzymes that are usually glycosylated and that calalyze a large variety of reactions:

Donor + $H_2O_2 \rightarrow$ Oxidized Donor + $2H_2O$
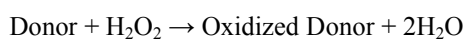
Isoperoxidases, arising from the transcripton of different genes or from post-translational modification, are widely distributed within both the intra- and extracellular environment. Peroxidases have been studied for their important role in lignification and suberization, for their active participation in the formation of diphenyl bridges, cross-linking of hydroxypropyline –rich proteins (extension) in the cell wall matrix and for their control function of redox state in apoplast.

Malate Dehydrogenase (MDH): Malate dehydrogenase is an enzyme of the Citric acid cycle that catalyses the reversible reaction of the conversion of malate to oxaloacetate It is one of the most active enzymes in the mitochondria, glyoxysomes, peroxisomes chloroplasts and the cytosol.

Ribulose-1,5-bisphosphate Carboxylase Oxygenase (RUBISCO): RUBISCO is involved in the Dark reaction of photosynthesis (Calvin Cycle) and so also in photorespiration. It catalyses either the carboxylation or the oxygenation of ribulose bisphosphate with CO_2 or O_2.

MATERIALS AND METHODS

Phenotypic variations in the ten species were analyzed using Native SDS-PAGE protein profile and isozyme patterns of Esterase (EST), Peroxidase (POX), Malate Dehydrogenase (MDH) and Ribulose bisphosphate Carboxylase Oxygenase (RUBISCO) and similarity index analysis was carried out to determine the relationship between the ten species. Scarified

and surface sterilized seeds were germinated in sterile coco-peat at room temperature. For the assay, fresh germinating seedling tissue was used.

Total Soluble Protein Analysis

Quantitative estimation of protein content from germinating seedlings was performed by Lowry assay (1951) which is an often-cited general use protein assay. 0.1g tissue was homogenized in 10ml D/W using mortar and pestle and filtered through 4 layers of muslin cloth. The filtrate was centrifuged to separate the total soluble proteins. The homogenates were diluted with 9 ml D/W before using for assay. Based upon the value of protein per gram of tissue, each sample prepared for electrophoresis was diluted accordingly.

Native PAGE

Total soluble proteins were extracted using 1.5 M Tris-HCl pH 8.0 (Heavers *et al.,* 1995) Electrophoresis was carried out using 15% Non-denaturing Poly-acrylamide gel (Garfin, 2003).

SDS PAGE: Total soluble proteins were extracted using 0.05M Tris-HCl pH 8.0, 12% Glycerol and 1% β-Mercaptoethnol as extraction buffer (Garfin, 2003). Electrophoresis was carried out in a discontinuous SDS-PAGE system of Laemmli (1970) using 15% acrylamide gel. Both the gels of Native PAGE and SDS PAGE were stained in 0.25% of Coomassie brilliant blue (R250) (Wilson, 1979) for 45mins followed by destaining until the background color disappeared and protein bands were clearly visible. The gel was photographed using white light and preserved in 7% Acetic Acid in D/W (Heavers *et al.,* 1995) for 1 month.

Isozyme analysis

Isozyme profile was analysed for Esterases (EST), Peroxidases (POX), Malate dehydrogenase (MDH), Ribulose bis-phosphate carboxylase-oxygenase (RUBISCO) from fresh 7 days old seedlings. Extraction was carried out in pre-cooled mortars and pestle in appropriate buffers. Each of the extract was filtered through 4 layers of muslin cloth. The extracted enzymes were separated by centrifuging in cold centrifuge at the rate of 1000rpm for 20mins. This supernatant was used for electrophoresis. Isozymes were separated by electrophoresis in 7.5% (Davis, 1964) polyacrylamide gels using sample loading buffer (McLellan, 1982). Followed by electrophoresis staining in appropriate staining solution was carried out as indicated for each of the isozymes in their respective sections below.

Esterases (EST): Esterases were extracted using 100mM Sodium phosphate buffer pH- 6.0, Electrophorese gel was immersed in 100ml of staining solution containing 100mM Naphthyl acetate (1ml), Fast blue salt (0.018gm/ml), 20 mM PO4 buffer pH- 6.0 (Sadasivam and Manickam, 1996) for 45 min. at room temperature. Dark brown bands of Esterases were observed in visible light and photographed using white background.

Peroxidases (POX): Peroxidases were extracted using Tris HCl pH -6.8 buffer. After electrophoresis the gel was immersed in staining solution composed of - Benzidine (2.08gm), Acetic Acid (18ml), 3% H2O2 (100ml) in 80ml D/W (Soltis *et. al.,*1989). The gel was observed in visible light

for bright blue coloured bands of Peroxidases and immediately photographed, as the colour is unstable.

Malate Dehydrogenase (MDH): MDH was extracted using 50mM Tris HCl pH- 8.5 buffer. Subsequently the gel was immersed in staining solution containing – 50mM Tris HCl pH-8.5 (50ml), 10mg/ml Nicotinamide Adenine Dinucleotide(NAD) solution (1ml), 50 mg/ml Malic acid (1 ml), 5mg/ml Phenazine Methosulphate (PMS) (0.4ml) (Wendel and Weeden 1990). The gel was observed in visible light for blue coloured bands of MDH and photographed using white background.

Ribulose bisphosphate Carboxylase Oxygenase (RUBISCO): RUBISCO was extracted using D/W, from fresh leaves of plants grown in bright sunlight at least for 15 days. After electrophoresis the gel was immersedin staining solution of Amido black (50 gm), Methanol (45ml), Glacial Acetic Acid (10ml), D/W (45ml). The gel was incubated for 1 hour. The gel was destained using the fixative - Methanol (45ml), Glacial Acetic Acid (10ml), D/W (45ml) (Soltis *et. al.*, 1989). The gel was observed in visible light for black colored bands of RUBISCO and photographed using a white background.

Data Analysis

Only clear and unambiguous bands were recorded based on their relative mobility and the bands were numbered serially as 1, 2, 3, ..., n, from the wells. The slowest band (nearest to the well) in a gel was numbered as the 1st band and the fastest (farthest from the well) in each gel was the last band number. Binary data matrix was generated taking 1 as the presence of the band on the gel and 0 as the absence of the band. The data was subjected to analysis using *MVSP-3.2* version software. The genetic similarity matrix was calculated using Jaccard's co efficient and the dendrogram was based on Jaccard's similarity Index is obtained using UPGMA.

RESULTS AND DISCUSSION

Total Soluble Protein Analysis

Total Soluble Protein quantities of each of the plant species estimated (Fig.-1) showed, *I. violacea* having maximum quantity of total soluble protein, while *I. nil* contained the least amount.
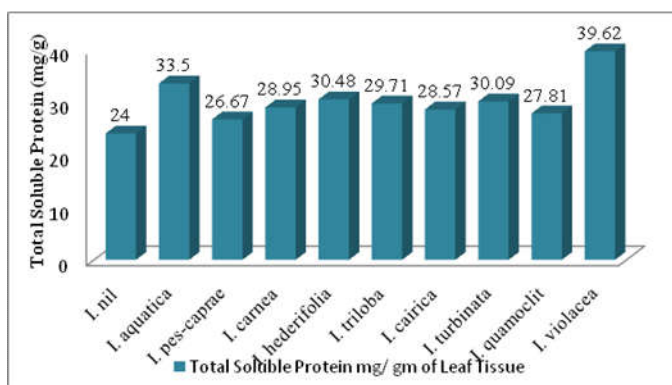

Fig. 1. Total Soluble proteins as mg/g of leaf tissue

The scoring for Native PAGE, SDS PAGE, Esterase, Peroxidase, Malate dehydrogenase and RUBISCO was done

for the presence or absence of bands, which are identical by their respective mobility and numbered in sequence from cathode origin. For each polypeptide / isozyme band two phenomorphs were scored, i.e. presence or absence of bands. Presence of each band on the gel was scored as 1 and absence as 0.
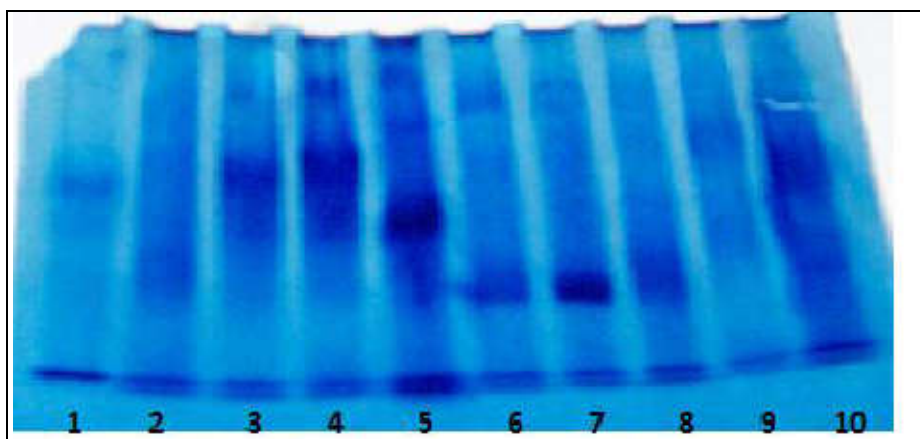
Native PAGE

The electrophoretic leaf protein banding pattern of the 10 species of *Ipomoea* carried out on Native PAGE, revealed a total of 10 electrophoretic bands (Fig. 2). In all the ten species studied, band 10 (*Rm* 0.96) was monomorphic, while bands 1 (*Rm* 0.06) and band 5 (*Rm* 0.4) were restricted to *I. nil,* the most primitive genus studied of sub genus *Ipomoea,* section *Pharbites,* series *Heterophylla* of Austin (1979). Band 7 (*Rm* 0.48), was present only in *I. nil* and *I. quamoclit*. *I. pes-caprae* and *I. carnea* and so also *I. triloba* and *I. cairica* showed identical bands. Maximum polymorphism (number of bands) was seen in *I. hederifolia* and *I.violacea*. Minimum polymorphism was seen in *I. quamoclit*. *I. pes-caprae* and *I. carnea* shared all their five bands with *I. violacea* but *I. violacea* showed an additional band with a *Rm* of 0.62. Similarly, *I. aquatica* too shared all its five bands with *I. violacea* and *I. violacea* showed an additional band with *Rm* of 0.42.

On the basis of similarity index calculated using UPGMA Jaccard's Coefficient (Table-1), *I. es-caprae* and *I. carnea* and so also *I. triloba* and *I. cairica* showed 100% similarity, followed by *I. violacea* with *I. aquatica, I. pes-caprae* and *I. carnea*. *I. nil* exhibitted minimum similarity with all the ten species. Based on UPGMA Jaccard's Coefficient of Similarity (Jaccard, 1901) Dendrogram was made, which showed four major clusters connected to each other at different nodes with variation in similarity index, as shown in Fig. 3. The first cluster is formed of *I. triloba* and *I. cairica* as a group/unit, because it has 100% similarity is connected to *I. turbinata* at Node – 6 with the similarity of 60 % (Table 2). The second cluster again has two plants with 100% similarity as a unit of (*I. pes-caprae* + *I. carnea*) connected to *I. violacea* at Node – 4. Third cluster connects *I. aquatica* and *I. hederifolia* at Node – 3, having the similarity of 83.3%. Last and the fourth major cluster is formed at the base of the dendrogram, which includes *I. nil* and *I. quamoclit.* Plants at Node -4[(*I. pes-caprae* + *I. carnea*) *I. violacea*] connects to plants of Node -3 (*I. aquatica* + *I. hederifolia*) at Node – 5 with the similarity of 67.1%, which in turn connects to plants of Node – 6 [(*I. triloba* + *I. cairica*) *I. turbinata*] forming Node – 7. Finally, all the plants at Node – 7 connects to plants of Node -8(*I. nil* + *I. quamoclit*) together with the least similarity of 22.3%.

SDS PAGE

The electrophoretic leaf protein banding pattern of the 10 species of *Ipomoea* carried out on SDS PAGE, revealed a total of 13 electrophoretic bands (Fig. 4). In all the ten species studied, band 13 (*Rm* 0.95) was monomorphic, while band 2 (*Rm* 0.08) was unique to *I. pes-caprae,* and bands 5 (*Rm* 0.5) and 8 (Rm 0.82) were restricted to *I.cairica*. Band 7 (*Rm* 0.48), was present only in *I. nil* and *I. quamoclit*. None of the ten species showed identical bands. Maximum polymorphism (number of bands) with 9 bands was seen in *I. cairica*. Minimum polymorphism (number of bands) 4 bands was seen in *I. quamoclit*.

KEY	1	2	3	4	5	6	7	8	9	10
	I. nil	*I. aquatica*	*I. pes-caprae*	*I. carnea*	*I. hederifolia*	*I. triloba*	*I. cairica*	*I. turbinata*	*I. quamoclit*	*I. violacea*

Fig.2. Native PAGE of Protein Complexes in *Ipomoea* Species

Table 1. Jaccard's Coefficient Similarity Matrix for Native PAGE protein Profile

On the basis of similarity index calculated using UPGMA Jaccard's Coefficient (Table-1), *I.*

Ipomoea species	*I. nil*	*I. aquatica*	*I. pes-caprae*	*I. carnea*	*I. hederifolia*	*I. triloba*	*I. cairica*	*I. turbinata*	*I. quamoclit*	*I. violacea*
I. nil	1									
I. aquatica	0.125	1								
I. pes-caprae	0.125	0.667	1							
I. carnea	0.125	0.667	1	1						
I. hederifolia	0.111	0.833	0.571	0.571	1					
I. triloba	0.143	0.5	0.286	0.286	0.667	1				
I. cairica	0.143	0.5	0.286	0.286	0.667	1	1			
I. turbinata	0.143	0.286	0.286	0.286	0.429	0.6	0.6	1		
I. quamoclit	0.4	0.333	0.333	0.333	0.286	0.4	0.4	0.167	1	
I. violacea	0.111	0.833	0.833	0.833	0.714	0.429	0.429	0.429	0.286	1

Table 2. Grouping of *Ipomoea* species into clusters based on Native PAGE Data

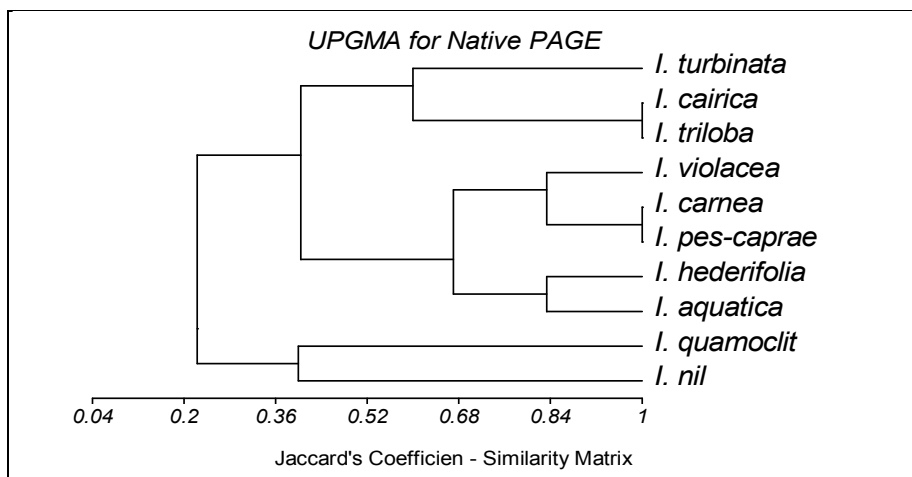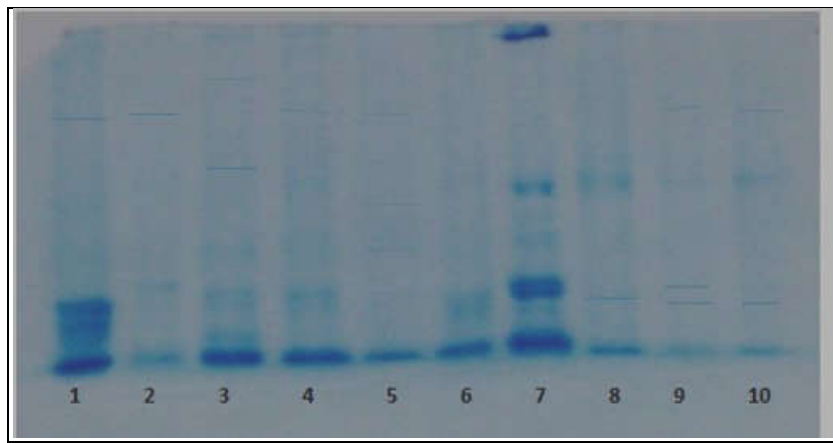| Node	Group 1	Group 2	Similarity	In group
1	*I. pes-caprae*	*I. carnea*	1	2
2	*I. triloba*	*I. cairica*	1	2
3	*I. aquatica*	*I. hederifolia*	0.833	2
4	Node 1	*I. violacea*	0.833	3
5	Node 3	Node 4	0.671	5
6	Node 2	*I. turbinata*	0.6	3
7	Node 5	Node 6	0.403	8
8	*I. nil*	*I. quamoclit*	0.4	2
9	Node 8	Node 7	0.223	10

Fig. 3. Dendrogram showing phylogenetic relationship of the ten species of *Ipomoea* based on Native PAGE data

KEY	1	2	3	4	5	6	7	8	9	10
	I. nil	*I. aquatica*	*I. pes-caprae*	*I. carnea*	*I. hederifolia*	*I. triloba*	*I. cairica*	*I. turbinata*	*I. quamoclit*	*I. violacea*

Fig.4. SDS-PAGE Protein profile of *Ipomoea* Species

Table 3. Jaccard's Coefficient Similarity Matrix for SDS-PAGE protein Profile

Ipomoea species	*I. nil*	*I. aquatica*	*I. pes-caprae*	*I. carnea*	*I. hederifolia*	*I. triloba*	*I. cairica*	*I. turbinata*	*I. quamoclit*	*I. violacea*
I. nil	1									
I. aquatica	0.556	1								
I. pes-caprae	0.6	0.4	1							
I. carnea	0.889	0.667	0.7	1						
I. hederifolia	0.625	0.375	0.3	0.556	1					
I. triloba	0.556	0.5	0.75	0.667	0.375	1				
I. cairica	0.545	0.25	0.545	0.5	0.273	0.5	1			
I. turbinata	0.625	0.375	0.625	0.556	0.429	0.833	0.556	1		
I. quamoclit	0.5	0.429	0.333	0.444	0.5	0.429	0.3	0.5	1	
I. violacea	0.667	0.444	0.364	0.6	0.5	0.3	0.455	0.333	0.375	1

Table 4. Grouping of *Ipomoea* species into clusters based on SDS PAGE Data

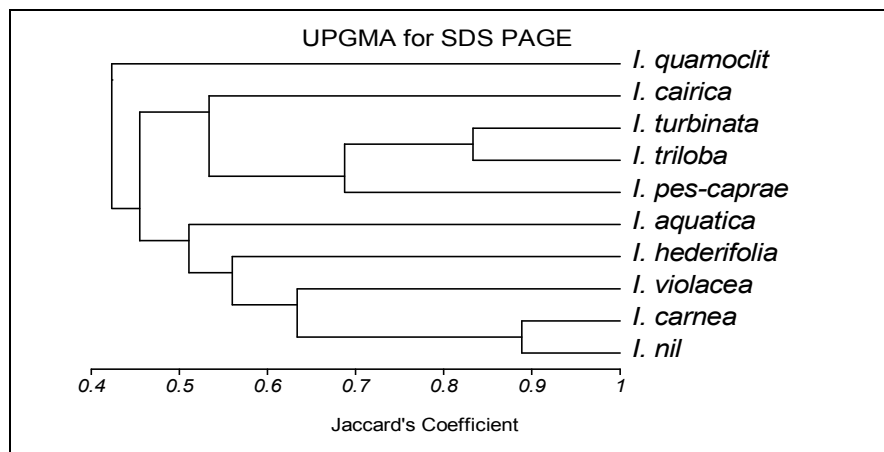| Node	Group 1	Group 2	Similarity	Objects in group
1	*I. nil*	*I. carnea*	0.889	2
2	*I. triloba*	*I. turbinata*	0.833	2
3	*I. pes-caprae*	Node 2	0.688	3
4	Node 1	*I. violacea*	0.633	3
5	Node 4	*I. hederifolia*	0.56	4
6	Node 3	*I. cairica*	0.534	4
7	Node 5	*I. aquatica*	0.51	5
8	Node 7	Node 6	0.455	9
9	Node 8	*I. quamoclit*	0.423	10

Fig. 5. Dendrogram showing phylogenetic relationship of the ten species of *Ipomoea* based on SDS PAGE data

KEY	1	2	3	4	5	6	7	8	9	10
	I. nil	*I. aquatica*	*I. pes-caprae*	*I. carnea*	*I. hederifolia*	*I. triloba*	*I. cairica*	*I. turbinata*	*I. quamoclit*	*I. violacea*

Fig.6. Isozyme Pattern for Esterases in *Ipomoea* Species

Table 5. Jaccard's Coefficient Similarity Matrix for Isozyme Pattern of Esterases

Ipomoea species	I. nil	I. aquatica	I. pescaprae	I. carnea	I. hederifolia	I. triloba	I. cairica L.	I. turbinata	I. quamoclit	I. violacea
I. nil	1									
I. aquatica	0.4	1								
I. pescaprae.	0.667	0.4	1							
I. carnea	0.5	0.5	0.5	1						
I. hederifolia	0.6	0.667	0.6	0.75	1					
I. triloba	0.667	0.4	0.667	0.8	0.6	1				
I. cairica	0.667	0.4	0.429	0.8	0.6	0.667	1			
I. turbinata	0.5	0.5	0.5	0.6	0.4	0.8	0.5	1		
I. quamoclit	0.4	1	0.4	0.5	0.667	0.4	0.4	0.5	1	
I. violacea	0.6	0.25	0.333	0.167	0.2	0.333	0.333	0.4	0.25	1

Table 6. Grouping of *Ipomoea* species into clusters based on Esterase PAGE Data

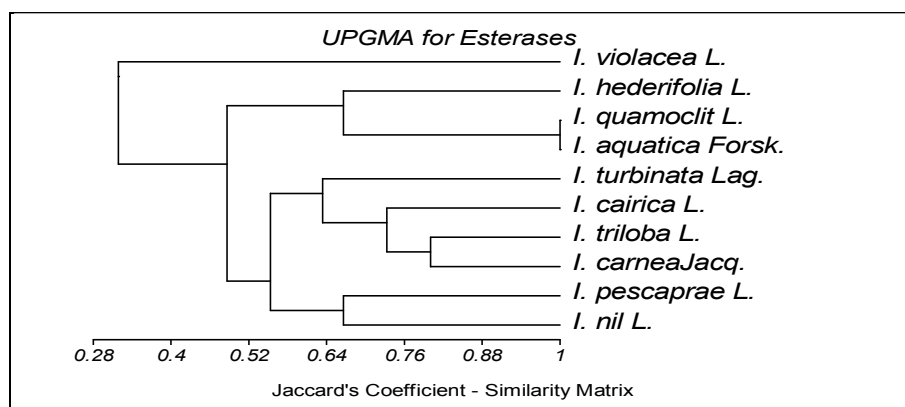| Node	Group 1	Group 2	Similarity	Objects in group
1	*I. aquatica*	*I. quamoclit.*	1	2
2	*I. carnea*	*I. triloba*	0.8	2
3	Node 2	*I. cairica*	0.733	3
4	*I. nil L.*	*I. pescaprae*	0.667	2
5	Node 1	*I. hederifolia*	0.667	3
6	Node 3	*I. turbinata*	0.633	4
7	Node 4	Node 6	0.554	6
8	Node 7	Node 5	0.486	9
9	Node 8	*I. violacea*	0.319	10

Fig. 7. Dendrogram showing phylogenetic relationship of the ten species of *Ipomoea* based on *Esterase* data

KEY	1	2	3	4	5	6	7	8	9	10
	I. nil	*I. aquatica*	*I. pes-caprae*	*I. carnea*	*I. hederifolia*	*I. triloba*	*I. cairica*	*I. turbinata*	*I. quamoclit*	*I. violacea*

Fig.8. Isozyme Pattern for Peroxidases in *Ipomoea* Species

Table 7. Jaccard's Coefficient Similarity Matrix for Isozyme Pattern of Peroxidases

Ipomoea species	*I. nil*	*I. aquatica*	*I. pescaprae*	*I. carnea.*	*I. hederifolia*	*I. triloba*	*I. cairica*	*I. turbinata*	*I. quamoclit*	*I. violacea*
I. nil	1									
I. aquatica	0.6	1								
I. pescaprae	0.75	0.8	1							
I. carnea	0.75	0.8	1	1						
I. hederifolia	0.333	0.429	0.5	0.5	1					
I. triloba	0.75	0.8	1	1	0.5	1				
I. cairica	0.75	0.8	1	1	0.5	1	1			
I. turbinata	1	0.6	0.75	0.75	0.333	0.75	0.75	1		
I. quamoclit.	1	0.6	0.75	0.75	0.333	0.75	0.75	1	1	
I. violacea	0.6	1	0.8	0.8	0.429	0.8	0.8	0.6	0.6	1

Table 8. Grouping of *Ipomoea* species into clusters based on Peroxidase PAGE Data

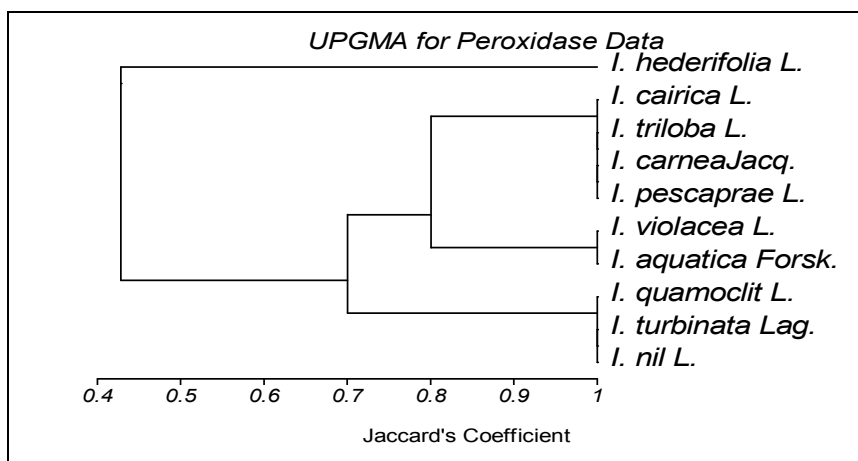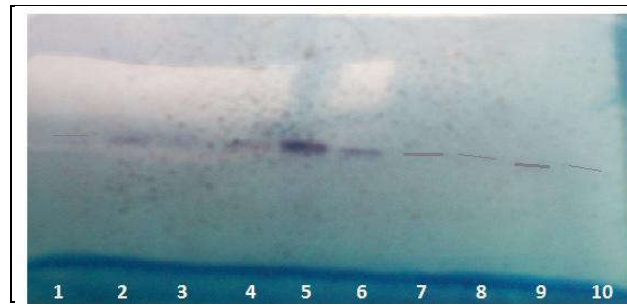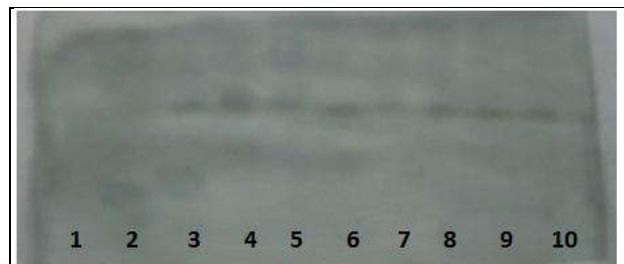| Node	Group 1	Group 2	Similarity	Objects in group
1	*I. nil*	*I. turbinata*	1	2
2	Node 1	*I. quamoclit*	1	3
3	*I. aquatica*	*I. violacea*	1	2
4	*I. pes-caprae*	*I. carnea*	1	2
5	Node 4	*I. triloba*	1	3
6	Node 5	*I. cairica*	1	4
7	Node 3	Node 6	0.8	6
8	Node 2	Node 7	0.7	9
9	Node 8	*I. hederifolia*	0.429	10

Fig. 9. Dendrogram showing phylogenetic relationship of the ten species of *Ipomoea* based on *Peroxidase* data

KEY	1	2	3	4	5	6	7	8	9	10
	I. nil	*I. aquatica*	*I. pes-caprae*	*I. carnea*	*I. hederifolia*	*I. triloba*	*I. cairica*	*I. turbinata*	*I. quamoclit*	*I. violacea*

Fig. 10. Isozyme Pattern for MDH in *Ipomoea* Species

KEY	1	2	3	4	5	6	7	8	9	10
	I. nil	*I. aquatica*	*I. pes-caprae*	*I. carnea*	*I. hederifolia*	*I. triloba*	*I. cairica*	*I. turbinata*	*I. quamoclit*	*I. violacea*

Fig.11. Isozyme Pattern for RUBISCO in *Ipomoea* Species

Table 9. Jaccard's Coefficient Similarity Matrix for combination of Total soluble Proteins and the four Isoenzymes

Ipomoea species	I. nil	I. aquatica	I. pes-caprae	I. carnea	I. hederifolia	I. triloba	I. cairica	I. turbinata	I. quamoclit	I. violacea
I. nil	1									
I. aquatica	0.448	1								
I. pes-caprae	0.533	0.571	1							
I. carnea	0.586	0.692	0.778	1						
I. hederifolia	0.433	0.577	0.5	0.607	1					
I. triloba	0.536	0.577	0.667	0.667	0.556	1				
I. cairica	0.533	0.467	0.548	0.6	0.5	0.731	1			
I. turbinata	0.538	0.462	0.556	0.556	0.444	0.773	0.615	1		
I. quamoclit	0.565	0.545	0.462	0.52	0.458	0.522	0.462	0.524	1	
I. violacea	0.5	0.654	0.567	0.621	0.517	0.467	0.516	0.464	0.423	1

Table 10. Grouping of *Ipomoea* species into clusters based on Total soluble Proteins and the four Isoenzymes Data

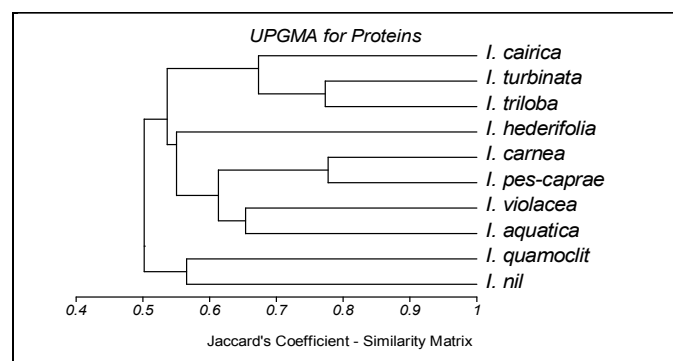| Node	Group 1	Group 2	Similarity	Objects in group
1	*I. pes-caprae*	*I. carnea*	0.778	2
2	*I. triloba*	*I. turbinata*	0.773	2
3	Node 2	*I. cairica*	0.673	3
4	*I. aquatica*	*I. violacea*	0.654	2
5	Node 4	Node 1	0.613	4
6	*I. nil*	*I. quamoclit*	0.565	2
7	Node 5	*I. hederifolia*	0.55	5
8	Node 7	Node 3	0.536	8
9	Node 6	Node 8	0.502	10

Fig. 12. Dendrogram showing phylogenetic relationship of the ten species of *Ipomoea* based on combination of Total soluble Proteins and the four Isoenzymes

On the basis of similarity index calculated using UPGMA Jaccard's Coefficient (Table-3), *I. nil* and *I. carnea* showed 94.1% affinity, while *I. hederifolia* and *I.cairica* exhibited minimum similarity 27.3%. Based on UPGMA Jaccard's Coefficient of Similarity, Dendrogram was made, which showed Two major clusters connected to each other at different nodes with variation in similarity index, as shown in Fig.5. First major cluster is made up of five species, wherein, *I. nil* and *I. carnea* are located closest at Node -1 with 88.9% similarity, further *I. violacea* is added to form Node – 4 (Table -4). Then *I. hederifolia* is connected at Node-4, forming Node – 5 with four species {[(*I. nil* + *I. carnea*) *I. violacea*] *I. hederifolia*} and finally, *I. aquatica* is added to this cluster at Node – 7. (Table-6). Second major cluster has *I. triloba* and *I. turbinata* at Node -2 having 83.3% similarity. *I. pes-caprae* connects to Node – 2, forming Node -3. These three species at Node -2 then gets connected to *I. cairica* at Node – 6 with similarity of 53.4%. These two major clusters are connected to each other at Node – 8 with similarity of 45.5%. *I. quamoclit* appears to be out grouped, as it is later added in the dendrogram as the last node connecting to Node – 8.

Analysis of Isozymes

Esterase: The electrophoretic esterase banding pattern of the 10 species of *Ipomoea* carried out for Esterase, revealed a total of 7 electrophoretic bands (Fig. 6). In all the ten species studied, bands 5 (*Rm* 0.47) and 6 (*Rm* 0.5) were monomorphic, while band 1 (*Rm* 0.35) was restricted to *I. pes-caprae*. *I. aquatica* and *I. quamoclit* showed identical bands. Maximum polymorphism , with five bands in each, was seen in *I. nil, I. pes-caprae, I. triloba* and *I.cairica*. Minimum polymorphism (number of bands) was seen in *I. aquatica* and *I. quamoclit.* They showed two bands each and these two bands were monomorphic to all the ten species. On the basis of similarity index (Table-5), *I. aquatica* and *I. quamoclit* showed 100% affinity, while *I. triloba and I. violacea* exhibitted minimum similarity with all the ten species. Based on UPGMA Jaccard's Coefficient of Similarity Dendrogram was made, which showed Three major clusters connected to each other at different nodes with varation in similarity index, as shown in Fig. 7. Since *I. aquatica* and *I.quamoclit* has 100% similarity, it is seen as a group forming the first major cluster by connecting to *I. hederifolia* at Node – 5 with a similarity of 66.7% (Table-6). Second major cluster is formed of *I. triloba* and *I. carnea* with 80% similarity at Node -2, then *I. cairica* is added at Node – 3 with 73.3% similarity. Finally, *I. turbinata* is added to this cluster at Node – 6. *I. nil* and *I. pes-caprae* connects at Node – 4, forming third cluster. Cluster two {[(*I. triloba* + *I. carnea*) *I. cairica*] *I. turbinata*} and cluster three (*I. nil* + *I. pes-caprae*) are inturn connected to each other at Node – 7 with 55.4% similarity, this is then connected to Cluster one [(*I. aquatica, I.quamoclit*) *I. hederifolia*] at Node – 8 with 48.6% similarity. Finally, *I. violacea* has been added to the dendrogram with least similarity 31.9%.

Peroxidase

The electrophoretic leaf peroxidase banding pattern of the 10 species of *Ipomoea* carried out, revealed a total of 7 electrophoretic bands (Fig. 8). In all the ten species studied, bands 4 (*Rm* 0.36) and 7 (*Rm* 0.82) were monomorphic, band 2 (*Rm* 0.31) was monomorphic for all species except *I. hederifolia*. *I. hederifolia* had two unique bands, band 1(*Rm* 0.30) and band 3 (*Rm* 0.34). *I. nil, I. turbinata* and *I. quamoclit*

with 3 bands each; and so also *I. pes-caprae, , I. carnea , I. triloba and I. cairica* with 4 bands each and *I. aquatica* and *I. violacea* with 5 bands each showed identical bands. Maximum polymorphism was seen in *I. hederifolia* and *I.violacea.* Minimum polymorphism (number of bands) was seen in *I. nil, I. turbinate* and *I. quamoclit.* On the basis of similarity index (Table-7), *I. nil, I. turbinata* and *I. quamoclit ; I. pes-caprae, , I. carnea , I. triloba , I. cairica* and so also *I. aquatica, I. violacea* showed 100% affinity. *I. hederifolia* and *I.turbinata* exhibited minimum similarity of 25%. Based on UPGMA Jaccard's Coefficient of Similarity Dendrogram was made, which showed Two major clusters connected to each other at different nodes with varation in similarity index, as shown in Fig. 9. Firstly, *I.nil, I, turbinata* and *I. quamoclit* formed a group, because of they did not show any variation in peroxidises isozyme pattern. In second cluster there are two groups of 100% similarity as (*I. violacea, I. aquatica*) and (*I.pes-caprae, I. carnea, I.triloba* and *I. cairica*) being connected to each other at Node – 7 with 80% similarity (Table-8). Cluster one and Cluster two is connected to each other at Node – 8 with similarity of 70% similarity. Finally, *I. hederifolia* is added to the dendrogram at Node – 9, and it appears to be out grouped with least similarity with other species. The electrophoretic leaf MDH and RUBISCO banding pattern of the 10 species of *Ipomoea* carried out, revealed a single unique band with an *Rm* of 0.49 for MDH (Fig. 10) And an *Rm* of 0.52 for RUBISCO (Plate XLV, Fig. 3.2.7a and Table. 3.2.7a). There was no polymorphism observed for these two enzymes. Nair and Keshavachandran (2006) for RUBISCO in *Gymnema sylvestric* accessions.

The cluster analysis carried out, based on the combination of total soluble Proteins and the four isoenzymes (Tables-9, 10) the dendrogram (Fig. 12) revealed four major clusters initially divided into two major clusters:

- **Cluster 1** comprising of 3 species of the most evolved sub-genus *Quamoclit* namely, *I. cairica, I. turbinata* and *I. triloba*. Within the cluster, *I. turbinata* and *I. triloba* are closer to each other with a similarity of 77.3% than with *I. cairica,* which is later being connected at Node – 3 (Table-10)
- **Cluster 2** comprising of the species *I. hederifolia* of section Mina of sub-genus *Quamoclit.* It showed a similarity of 53% with cluster 1 having members of the same sub-genus and 55% with cluster 3 having all the four members studied from sub-genus *Eriospermum.*
- **Cluster 3** comprising of all the four species *I. carnea, I. pes-caprae, I. violacea* and *I. aquatica* of the subgenus *Eriospermum*. Within the cluster, *I. pes-caprae* of section *Erpipomoea* and *I. carnea* of series *Jalapae* of section *Eriospermum* are connected to each other at Node – 1 with similarity of 77.8%. So also *I. violacea* and *I. aquatica* of section *Erpipomoea* are connected at Node –4 with 65.4% similarity. *hederifolia* of section Mina of sub-genus *Quamoclit* is also been added here at Node – 7 with 55% similarity with all the four species at Node – 5 species [(*I. carnea* + *I. pes-caprae*) (*I. violacea* + *I. aquatica*)]
- **Cluster 4** comprising of *I. quamoclit* of sub-genus *Quamoclit* and *I. nil* of the most primitive section *Pharbitis* of sub-genus *Ipomoea* with 56.5% similarity. Though distinctly different, they are in the same cluster because most of the bands of *I. quamoclit* are identical to those of *I. nil* and *I. nil* has additional bands.

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