Isozyme Polymorphism and Genetic Diversity Among *Swertia* Species – Endangered Medicinal Plants of Himalayas

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Esterase, catalase, peroxidase and malate dehydrogenase banding patterns of five different species of *Swertia* (Family – Gentianaceae) were analyzed in five species *viz.- S. chirata, S. bimaculata, S. cordata, S. peniculata* and *S. petiolata*. All the isozymes tested, showed remarkable variation in electrophoretic banding pattern on PAGE among different species and within species as well. The implications of the findings with reference to genetic diversity and the usefulness of the isozyme technique for germplasm characterization have been discussed.

Key Words: Catalase, Esterase, Malate Dehydrogenase, Peroxidase, *Swertia*

Swertia species (Family – Gentianaceae), included in the endangered medicinal plants list, has being used in the preparation of Ayurvedic medicine since ancient times. Plants are found to grow wild at high altitude (6,000 to 10,000 ft.) in the small pockets of Darjeeling, Kumaoun and Chotanagpur hills of India. Some species of Swertia have also been reported in Nepal, Japan and China. Indian Ayurvedic System of Medicine recommends the use of whole plant in various diseases. Traditionally, *Swertia* species are used as laxative, febrifuge, stomachic, livertonic, anti-malarial, expectorant and bitter digestive tonic. Plant contains various phenyl glycosides namely swertin, swertiomerin, gentiopicroside, amarogentin, ameroswerin, iso-swertianolin, swertianolin and norswertianolin. It also contains oleanolic acid, which has anti-ulcer and analgesic activities and inhibits 5-alpha-reductase which is related to oleopecin. It also has angiotelectase activity (Khetwal and Verma, 1984).

Isozymes are efficient tools for examining genetic variation, because they exhibit Mendelian inheritance, co-dominant expression, complete penetrance and an absence of pleiotropic and epistatic interactions; all of which facilitate genetic analysis (Sanchez *et al.*, 2000). They are used to characterize and identify cultivars, document the parentage of cultivars, or interspecific hybrids and examine the genetic relationship among cultivars in germplasm collections (Bhat and Chandel, 1991; Clement *et al.*, 1997).

Conventionally, different species of *Swertia* are classified on the basis of their morphological features. Existence of various morphological similarities among different species of *Swertia* is a major source of confusion in the collection and classification of genetic variability and maintenance of diverse germplasm collection. Although more than 10 species of *Swertia* are reported, their identity is not well established, which indeed is a pre-requisite for any molecular genetic approach for crop improvement. So far, there is no known report on the biochemical basis of species identification of this plant. This underlines the need to survey variation in the germplasm collections as a function of various isozymes and to depute molecular identification mark, so that the exact nature of species diversification can be demarcated. The present study was undertaken to evaluate the genetic diversity prevalent in five different *Swertia* species and to characterize them using the technique of electrophoresis of isozymes.

Materials and Methods

Five different species of *Swertia*, selected for present studies were – *S. chirata, S. bimaculata, S. cordata, S. peniculata and S. petiolata*. Mature seeds were collected from all the five wild growing *Swertia* species from surrounding areas of Darjeeling near Indo-Nepal border and were multiplied at Panchvati Greentech Research Society (PGRS) Nursery, Darjeeling under uniform environmental conditions. Fresh, soft apical leaves and fibrous roots from one year old plants of each species were collected separately in a closed ice-box. Samples were first powered with the help of pestle and mortar using liquid nitrogen and then homogenized with acetone to remove chlorophyll and other secondary metabolites. The homogenates were filtered through Whatman No.1 filter paper. The residue on the filter paper was homogenized with extraction buffer in a pre-chilled mortar and pestle in a cold room. The sample to buffer ratio was maintained

at 1:3 (w/v) for each sample. The homogenate was then centrifuged at 10,000 rpm for 20 min at 4°C and the clear supernatant was collected. Protein contents of each sample were estimated by Folin – Ciocaltau reagent method (Lowry *et al.*, 1951). The protein contents of each extracts were equalized by the addition of extraction buffer. For each sample 20 µg protein equivalent was loaded on the gel. On completion of electrophoresis, gels were incubated in the respective staining mixtures of different enzymes. After optimum staining, gels were fixed in 7% acetic acid. The relative migration velocity (Rm) was calculated for each band and zymograms were constructed from the Rm values. The process was repeated at least three times to confirm reproducibility of the results.

Results and Discussion

Composition of the extraction buffer is an important consideration to be taken into account for obtaining a better resolution of the enzyme systems. This depends upon various factors like tissues, plant types etc. Each taxon and tissue posses its own set of problems regarding the difficulty of cell breakage and associated problems caused by endogenous tannins, phenols, phenol-oxidases and other cellular constituents. Two more important considerations to be made are adequate cell breakage with minimum time and an optimal tissue to volume ratio. A trial with five different extraction buffers was made for the present study. Buffer containing 0.1M Tris-HCl (pH 7.5), 10% Sucrose, and 0.1% β-merceptoethanol was good for extraction of enzyme from leaf whereas, buffer containing 0.1 M Tris–HCl (pH 7.5), 10% Sucrose, 0.1% β-merceptoethenol and 5% PVP from roots, as these buffers resulted in the maximum number of clear and distinct bands. Zymograms of all the four enzymes drawn to the scale with Rm values are depicted in Fig 1.

Isozyme Polymorphism

Extension studies of polymorphism in isozymes gave reliable estimates of genetic relationship among the garlic genotypes (Bhat and Chandel, 1991). All the four isozymes tested in the present studies showed considerable polymorphism in the number and the pattern of electrophoretic bands produced on the gel.

a) *Esterase (E.C. 3.1.1.2)*

The electrophoretic banding patterns of esterase enzyme were divided into two zones (EstL1 & EstL2) for leaves and three (EstR1, EstR2 & EstR3) for roots. These enzyme zones were governed by two gene loci in leaves and three in roots. EstL1 exhibited dimeric isozymic forms of the enzyme in variable allelomorphic combinations. Lane 1 and 2 species exhibited protein products of gene loci in allelic forms like AA × Aa producing two possible forms of dimeric protein *i.e.* AA (Rm = 0.06) and Aa (Rm = 0.15). Estl1 of lane 3,4 and 5 species exhibited protein product of gene loci in possible two allelic forms in heterozygous conditions *viz.* Aa x Aa, producing 3 possible dimeric forms of proteins *i.e.* AA (Rm = 0.06), Aa (Rm = 0.15) and aa (Rm = 0.2). The banding pattern in EstL2 zone was same as that of EstL1 except for lane 2, clearly indicating that this gene locus also produced dimeric proteins. Lane 2 protein was possibly a product of homozygous gene 'C' as CC (say).

For the enzymes extracted from roots, banding pattern was divided into 3 zones. Zone 1 *i.e.* EstRl of lane 1 and 4 exhibited the same number of bands and banding pattern as that of EstL1 of lane 3, 4 and 5 of leaf samples. Lane 2, 3 and 5 of EstR1 zone exhibited only 2 bands as that of EstL1 of leaves, this was due to two different protein products of alleloform for gene loci 1 *viz.* aa x Aa. EstR2 was completely absent in leaf samples and lane 5 of root samples. This clearly showed an organ specific variation. Lane 1, 2 and 3 of root samples exhibited protein products of allelic combination like Bb × bb, producing dimeric protein products from gene like Bb (Rm = 0.30) and bb (Rm = 0.34). In EstR3 zone, lane 1 and 4 showed 2 bands whereas, lane 2, 3 and 5 showed only 1 band (Fig.1A).

b) *Catalase (E.C. 1.11.1.6)*

Banding patterns obtained from both leaves and the root samples were divided into two zones. All the species showed monomorphic form of proteins in CatL1 zone *i.e.* monomeric protein products of either 'a' or 'A' gene at the same Rm = 0.1. In CatL2 zone, all the species showed dimeric form of protein, but in various combinations of alleles. Bands in lane 1 and 3 of CatL2 zone were the protein products of homozygous gene of different alleles whereas; lane 2, 4 and 5 existed in dimeric protein forms as CC (Rm = 0.35), Cc (Rm = 0.4) and cc (Rm = 0.46).

CatR1 zone of root samples showed a single band in all the species at the same Rm = 0.1 except in lane 5, and exhibited monomorphic form of isozyme. In CatR2 zone, no band was detected in lane 1 whereas,

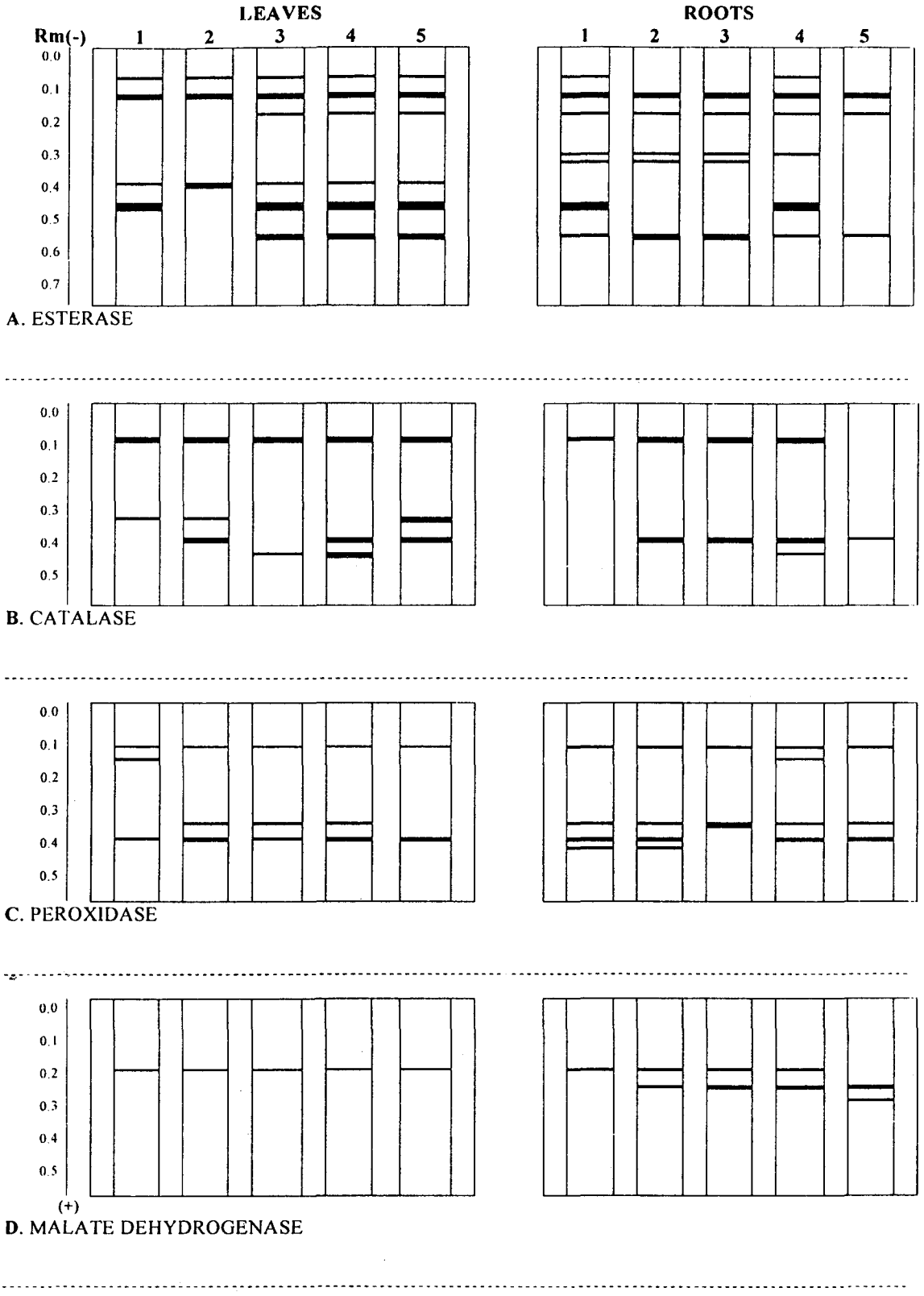

Fig. 1. Isozyme profiles of different Swertia species. (1) S. chitra; (2) S. bimaculata; (3) S. cordata; (4) S. paniculata (5) S. petiolata)

lane 2-5 exhibited dimeric form of proteins; bands of Rm = 0.42 (Cc, say) were present in heterozygous allelic product forms. Apart from this, lane 4 also exhibited protein products of homozygous allelic form at Rm = 0.46 (cc, say) (Fig. 1B).

C. Peroxidase (E.C. 1.11.1.7)

Enzyme from leaf samples of all the species exhibited dimeric nature of proteins in PerL1 zone, which was probably a protein product of homozygous gene AA (say) at Rm = 0.12 whereas, lane 1 had another band at Rm = 0.15 in heterozygous product form of gene Aa (say). In PerL2 zone, all the species showed a common band at Rm = 0.38, a dimeric protein product of heterozygous form of gene Aa (say); while bands at Rm = 0.34 appeared in lane 2, 3 and 4 depicted protein products of homozygous gene AA (say).

Bands appearing in the enzyme of root samples in PerR1 zone of lane 1, 2, 3 and 5 species were similar to that of PerL1 zone of lane 2, 3, 4 and 5 whereas, lane 4 in PerR1 zone was similar to that of lane 1 of PerL1. All the bands in PerR2 zone were similar in banding pattern and Rm values for the species of lane 1, 2 and 5. Protein in PerR2 was dimeric and alleles for a gene 'C' (say) existed in heterozygous from *i.e.* Cc, producing three different combinations to generate three bands. Lane 3 existed in homozygous product form of CC and lane 4 existed in two varied combinations of allele of a gene namely Cc × CC (say), producing two different dimeric proteins (Fig. 1C).

D. Malate Dehydrogenase (E.C. 1.1.1.37)

By comparing leaf and root isozyme banding patterns, we can conclude that all the species exhibiting a single band at Rm = 0.2 were of dimeric protein products of homozygous gene AA (say) in MdhL1 and MdhR1 zone. In MdhR1 zone lane 2-5 showing common banding pattern at Rm = 0.26 existed in hetero-dimeric product of gene form Aa (say); while bands at Rm = 0.2 and at Rm = 0.3 exhibited a homozygous protein product form of gene AA and aa (say) respectively (Fig. 1D).

For unambiguous identification of species, it was essential to use as many isozyme systems as possible for achieving some conclusive results. The data on isozyme systems that were monomorphic in a given set of species was also essential. This study indicated that considerable polymorphism was prevalent for isozymes analyzed. Although, it was not possible to clearly identify any of the species of *Swertia*, based on electrophoretic banding patterns of only one isozyme. However, when complete profile of all the four isozymes from both the root and the leaf were considered, each of the species studied could be distinguished from others.

Further study of the germplasm utilizing some more species with additional isozymes markers and improved separation techniques are necessary to draw a concrete interpretation of genetic variability among different species of *Swertia*.

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