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# ASSESSMENT OF GENETIC DIVERSITY OF *VIOLA SERPENS* WALL. IN HIMACHAL PRADESH USING MOLECULAR MARKERS

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## ABSTRACT

*Viola serpens* Wall. commonly known as "Banakasha" belongs to family *Violaceae*. It is one of the most useful medicinal plants and used as antipyretic, demulcent, diaphoretic and diuretic drug. It is useful in asthma, bleeding piles, cancer of throat, constipation, cough, fever, skin diseases and headache. In the present investigation an attempt has been made to assess the genetic diversity of *Viola serpens* Wall. using morphological and isozymic markers. Ten qualitative and seven quantitative characters have been studied but only quantitative morphological descriptors showed significant variation. The isozyme analysis was conducted for nine enzyme system and all of them showed significant activity producing typical banding patterns.The two enzymes systems i.e. Alkaline phosphatase and Acid phosphatase were found to be monomorphic in nature. Allele frequency in present study ranged from 0.5-1.0. Polymorphism in the selected thirty genotypes of *Viola serpens* was found to be 26.67 per cent.

**Keywords:** *Viola serpens*, Isozyme, Allele, Polymorphism.

## INTRODUCTION

*Viola serpens* belongs to family *Violaceae*. It is found throughout the temperate Himalayas up to an altitude of 2000 meters. In Himachal Pradesh, it is found in Chamba, Kangra, Kinnaur, Kullu, Mandi, Sirmaur, Bilaspur, Shimla and Solan districts. It is a small perennial herb with short tufted rootstocks. This plant has a small stem which is covered with scales.

Leaves of *Viola serpens* are ovate-cordate and leaf margins are crenate-serrate. Fruit is globose capsule and contains very few seeds. Flowers are lilac blue or white in colour. The flowering and fruiting occur between month of March and September .The roots of *Viola serpens* contains glucoside (methyle salicylate), alkaloid (violin) and a glycoside (viola quecitrin) (Anonymous, 1978). It also contains volatile oils such as rutin, cyamin and saponin etc. This plant has tremendous medicinal properties. In action, it is antipyretic, demulcent, diaphoretic, diuretic, emetic, emollient, expectorant, febrifuge and puragative. It is useful in asthma, bleeding piles, cancer of throat, constipation, cough, fever, skin diseases and headache. Its syrup is given in infantine disorders.

Keeping in view the importance of this medicinal important plant species, it was considered important to assess the genetic variability of the germplasm using molecular markers. Molecular methods provide valuable tools for reliable and precise identification of plants at any stage of their growth and development. Isozymes offer the most reliable single gene markers (Arulsekar and Parfitt, 1986) and polymorphism at enzyme loci has been shown to be stable under a number of environmental conditions. In the present study thirty genotypes of *Viola serpens* Wall. were analysed using isozymes analysis to estimate their genetic diversity.

## MATERIALS AND METHODS

The present investigations were carried out at Department of Biotechnology, Dr. Y.S. Parmar University of Horticulture and Forestry, Nauni, Solan in the state of Himachal Pradesh, India during winter season i.e. January and February of 2009. Thirty genotypes of *Viola serpens* Wall. were collected from the ten sites five districts viz. Mandi, Hamirpur, Bilaspur, Solan and Sirmaur of Himachal Pradesh. The geographical locations of ten selected sites along with their altitudes are given in table 1. The selected plants were given codes from $V_1$ to $V_{30}$ for further studies. Nine enzyme systems investigated were Peroxidase (PER), Acid phosphatse (ACP), Aspartate amino transferase (AAT), Esterase (EST), Catalase (CAT), Malate dehydrogenase (MDH), Alcohol dehydrogenase (ADH), Phosphorylase (PPH) and Alkaline phosphatase (ALP).

### Sample Preparation

Fresh, young and green leaves were excised from 30 genotypes of *Viola serpens* and then stored in liquid nitrogen till further use.Three hundred milligrams of leaves were washed thoroughly first under running tap water and then with distilled water and were grinded in

prechilled pestle and mortar containing 1.5 ml of Tris-citrate buffer, pH 8.3 (Trizma base-6.2 g/L and Citric acid-1.46 g/L) in the presence of crushed glass. The homogenate was filtered through two folds of muslin cloth and then centrifuged at 20,000 rpm for 20 minutes at 4°C. The supernatant was taken and stored at -20°C till further study.

**Electrophoresis**

Polyacrylamide gel electrophoresis was carried out to separate various isozymes due to its high resolving power, transparency and chemical inertness. Anionic system (Ornstein and Davis, 1964) was used for different enzyme systems. The bands of enzyme activity were revealed by immersing the gels in the histochemical stains. Staining recipes for ADH and AAT used were as Described by Shaw and Prasad (1970). Staining of MDH was done according to Shaw and Koen (1968) while the methods of Scandalios (1969) were employed for EST and ACP. Staining procedures for Graham *et al.* (1964) were employed for PER.

Staining of CAT was done according to Conkle *et al*. (1982) and methods of Tanksley and Ortan (1983) were employed for PPH and ALP. The genotypes were determined from the banding patterns obtained for each isozyme. The genotypes on the basis of isozymes were compared for each locus to assess genetic variability. Different genetic parameters were obtained by analysing the data. The allele frequency was calculated as proportion of a particular allele present at a given locus. Other genetic parameters studied to differentiate the genotypes included the proportion of polymorphic loci (Nei, 1975), the polymorphic index (Allard *et al*., 1978), the average and effective number of alleles per locus (Crow and Kimura, 1970) and the observed ($h_o$) and expected heterozygosity ($h_e$), according to Nei (1975). The observed and expected heterozygote frequencies were compared using F-statistics (Wright, 1965).

The inter relationships of genotypes could be found on the basis of the following parameter.

**Similarity index**

Similarity index was **c**alculated according to Hunter and Kannenberg (1971) on the basis of presence or absence of bands. The identical bands in the particular enzyme system were rated as zero. If the two individuals differed for the presence or absence of a band within the same locus, these were given a rating of one. Thus, a diversity index comprised of cumulative ratings obtained in all the enzyme systems for each pair of the genotypes.

**Dendrogram**

Dendrogram was constructed from genetic distance values by the unweighted pair group arithmetic average (UPGMA) method and NTSYS-pc software version 2.0 to analyse the pattern of cultivars relatedness.

**RESULTS AND DISCUSSION**

In the present investigation out the nine, seven enzyme systems viz. Peroxidase, Aspartate amino transferase, Esterase, Catalase, Malate dehydrogenase, Alcohol dehydrogenase and Phosphorylase produced polymorphism showing considerable and very useful variation among the thirty genotypes of *Viola serpens.* These enzyme systems successfully differentiated the sites of Mandi, Hamirpur and Bilaspur districts from the sites of Solan and Sirmaur districts whereas, the two enzymes viz, Acid phosphatase and Alkaline phosphatase produced monomorphic banding pattern.

The qualitative description of these enzymes has been depicted in the form of zymograms.

**Isozymic banding patterns**

Isozymes phenotypes of different enzyme systems have been described as under.

**Peroxidase (PER) (EC 1.1.1.1)**

Thirty genotypes of *Viola serpens* plant species produced three zones of enzyme activity. First the most cathodal zone, having locus PER I and it has two bands PER Ia with RM value 0.90 PER Ib with RM value 0.86. PER Ia was observed in first eighteen genotypes of Mandi, Hamirpur and Bilaspur districts but absent in genotypes of Solan and Sirmaur districts. PER Ib was present in all the genotypes from district Hamirpur, Solan and Kalwari site of district Bilaspur and absent in others. The second zone locus PER II showed single band PER IIa with RM value 0.81 and was present in all genotypes *Viola* serpens. The third zone showed single locus, PER IIIa with RM value 0.10 was also present in all the thirty genotypes from ten sites. Thus a marker PER I has been identified which can successfully differentiate among the genotypes of *Viola* serpens.

**Aspartate amino transferase (AAT) (EC 2.6.1.2)**

Studies of Aspartate amino transferase system with thirty genotypes of *Viola* serpens plant species produced three zones of enzyme activity. The most cathodal zone displayed a single locus AAT I with single band AAT Ia with RM value 0.30 was present in only three

genotypes of site AwahDevi of Hamirpur district and all the sites of Solan and Sirmaur districts. AAT II, the second locus, showed a single band AAT IIa with RM value 0.20. AAT IIa was present in all thirty genotypes. AAT III, the third locus had a single band AAT IIIa with RM value 0.08 was present in all the thirty genotypes of all the sites. Thus markers AAT Ia has been identified, which can successfully differentiate among the genotypes of *Viola* serpens.

**Esterase (EST) (EC 3.1.1.1)**

Esterase system displayed two zones of enzyme activity. The most cathodal zone having locus EST I showed a single band EST Ia with RM value 0.75 was present in genotypes of Mandi, Hamirpur and Bilaspur. Second zone having locus EST II showed two bands EST IIa with RM value 0.24 and EST IIb with RM value 0.10. Both these bands were found to be present in all the thirty genotypes. Thus, a marker EST Ia had been identified which can successfully differentiate among the genotype of *Viola* serpens.

**Catalase (CAT) (EC 1.11.1.6)**

Catalase system displayed a banding pattern with two zones of enzyme activity. The most cathodal zone having locus CAT I showed two bands CAT Ia with RM value 0.20, was present in genotypes of Solan and Sirmaur district. Second band CAT Ib with RM value 0.18 was present in all the thirty genotypes. The second zone having locus CAT II showed a single band, CAT IIa with RM value 0.10 was present in all the thirty genotypes *Viola* serpens. Thus, markers CAT Ia can successfully differentiate among all the genotypes of *Viola* serpens.

**Malate dehydrogenase (MDH) (EC 1.1.1.37)**

Studies of Malate dehydrogenase system with thirty genotypes of *Viola serpens* plant species produced two zones of enzyme activity. First the most cathodal zone, having MDH I showed two bands MDH Ia with RM value 0.26 and MDH I b with RM value 0.20. MDH Ia was observed in the genotypes of Mandi, Hamirpur and Bilaspur districts. The second band MDH Ib was present in all the thirty genotypes. The second zone locus MDH II showed single band MDH IIa with RM value 0.10. The bands of MDH IIa was present in all the genotypes of *Viola serpens* from ten sites. Thus, a marker MDH Ia has been identified, which can successfully differentiate among all the thirty genotypes of *Viola* serpens.

**Alcohol dehydrogenase (ADH) (EC 1.1.1.1)**

Thirty genotypes of *Viola serpens* plant species produced three zones of enzyme activity. First the most cathodal zone, having locus ADH I showed two bands, ADH Ia with RM value of 0.50 was present in all thirty genotypes. Second band ADH Ib with RM value 0.47 was present in genotypes of Solan and Sirmaur districts. The second zone locus ADH II showed a single band ADH IIa with RM value 0.20 was present in all thirty genotypes. The third zone showed locus ADH III with single band ADH IIIa with RM value 0.07 and was also present in all thirty genotypes. Thus, a marker ADH Ib has been identified which can successfully differentiate between the thirty genotypes of *Viola* serpens.

**Table 1 Geographical location of selected ten sites and the sample codes of *Viola* serpens**

| S.No. | Sites | Districts | Site code | Sample code | Altitude (m) |
|---|---|---|---|---|---|
| 1. | Sadhot | Mandi | $S_1$ | $V_1$ $V_2$ $V_3$ | 1100-1369 |
| 2. | Durgapur | | $S_2$ | $V_4$ $V_5$ $V_6$ | |
| 3. | AwahDevi | Hamirpur | $S_3$ | $V_7$ $V_8$ $V_9$ | 785 |
| 4. | Patta | | $S_4$ | $V_{10}$ $V_{11}$ $V_{12}$ | |
| 5. | Kalwari | Bilaspur | $S_5$ | $V_{13}$ $V_{14}$ $V_{15}$ | 350-670 |
| 6. | Ghumarwin | | $S_6$ | $V_{16}$ $V_{17}$ $V_{18}$ | |
| 7. | Nauni | Solan | $S_7$ | $V_{19}$ $V_{20}$ $V_{21}$ | 1365-1400 |
| 8. | Salogara | | $S_8$ | $V_{22}$ $V_{23}$ $V_{24}$ | |
| 9. | Rajgarh | Sirmaur | $S_9$ | $V_{25}$ $V_{26}$ $V_{27}$ | 900-1200 |
| 10. | Narag | | $S_{10}$ | $V_{28}$ $V_{29}$ $V_{30}$ | |

**Table 2 Genetic variation parameters between 30 genotypes of *Viola serpens***

| Parameters | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Total no. of loci** | 22 | 22 | 22 | 22 | 22 | 22 | 22 | 22 | 22 | 22 | 22 | 22 | 22 | 22 | 22 | 22 | 22 | 22 | 22 | 22 | 22 | 22 | 22 | 22 | 22 | 22 | 22 | 22 | 22 | 22 |
| **Total no. of Alleles** | 26 | 26 | 26 | 26 | 26 | 26 | 28 | 28 | 28 | 27 | 27 | 27 | 26 | 26 | 26 | 27 | 27 | 27 | 26 | 26 | 26 | 26 | 26 | 26 | 25 | 25 | 25 | 25 | 25 | 25 |
| **Proportion of Polymorphic loci** | 0.272 | 0.272 | 0.272 | 0.272 | 0.272 | 0.272 | 0.362 | 0.362 | 0.362 | 0.317 | 0.317 | 0.317 | 0.272 | 0.272 | 0.272 | 0.317 | 0.317 | 0.317 | 0.272 | 0.272 | 0.272 | 0.272 | 0.272 | 0.272 | 0.227 | 0.227 | 0.227 | 0.227 | 0.227 | 0.227 |
| **Polymorphic index** | 0.136 | 0.136 | 0.136 | 0.136 | 0.136 | 0.136 | 0.181 | 0.181 | 0.181 | 0.158 | 0.158 | 0.158 | 0.136 | 0.136 | 0.136 | 0.158 | 0.158 | 0.158 | 0.136 | 0.136 | 0.136 | 0.136 | 0.136 | 0.136 | 0.113 | 0.113 | 0.113 | 0.113 | 0.113 | 0.113 |
| **Average no. of Alleles per locus** | 1.181 | 1.181 | 1.181 | 1.181 | 1.181 | 1.181 | 1.272 | 1.272 | 1.272 | 1.227 | 1.227 | 1.227 | 1.136 | 1.136 | 1.136 | 1.227 | 1.227 | 1.227 | 1.181 | 1.181 | 1.181 | 1.181 | 1.181 | 1.181 | 1.136 | 1.136 | 1.136 | 1.136 | 1.136 | 1.136 |
| **Effective no. of Alleles per locus** | 0.056 | 0.056 | 0.056 | 0.056 | 0.056 | 0.056 | 0.050 | 0.050 | 0.050 | 0.048 | 0.048 | 0.048 | 0.058 | 0.058 | 0.058 | 0.048 | 0.048 | 0.048 | 0.058 | 0.058 | 0.058 | 0.058 | 0.058 | 0.058 | 0.063 | 0.063 | 0.063 | 0.063 | 0.063 | 0.063 |
| **Mean observed Heterozygosity ($h_o$)** | 0.272 | 0.272 | 0.272 | 0.272 | 0.272 | 0.272 | 0.362 | 0.362 | 0.362 | 0.317 | 0.317 | 0.317 | 0.272 | 0.272 | 0.272 | 0.317 | 0.317 | 0.317 | 0.272 | 0.272 | 0.272 | 0.272 | 0.272 | 0.272 | 0.227 | 0.227 | 0.227 | 0.227 | 0.227 | 0.227 |
| **Expected Heterozygosity ($h_e$)** | 0.136 | 0.136 | 0.136 | 0.136 | 0.136 | 0.136 | 0.181 | 0.181 | 0.181 | 0.158 | 0.158 | 0.158 | 0.136 | 0.136 | 0.136 | 0.158 | 0.158 | 0.158 | 0.136 | 0.136 | 0.136 | 0.136 | 0.136 | 0.136 | 0.113 | 0.113 | 0.113 | 0.113 | 0.113 | 0.113 |
| **F- statistics** | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 |

**Table 3 Summary of isozyme analysis obtained from 30 genotypes of *Viola serpens***

| | |
|---|---|
| Total number of enzyme studied | 9 |
| Total number of isozyme bands | 30 |
| Polymorphic isozyme bands | 8 |
| Monomorphic isozyme bands | 22 |
| RM value range | 0.06-0.90 |
| Percent polymorphism | 26.67% |

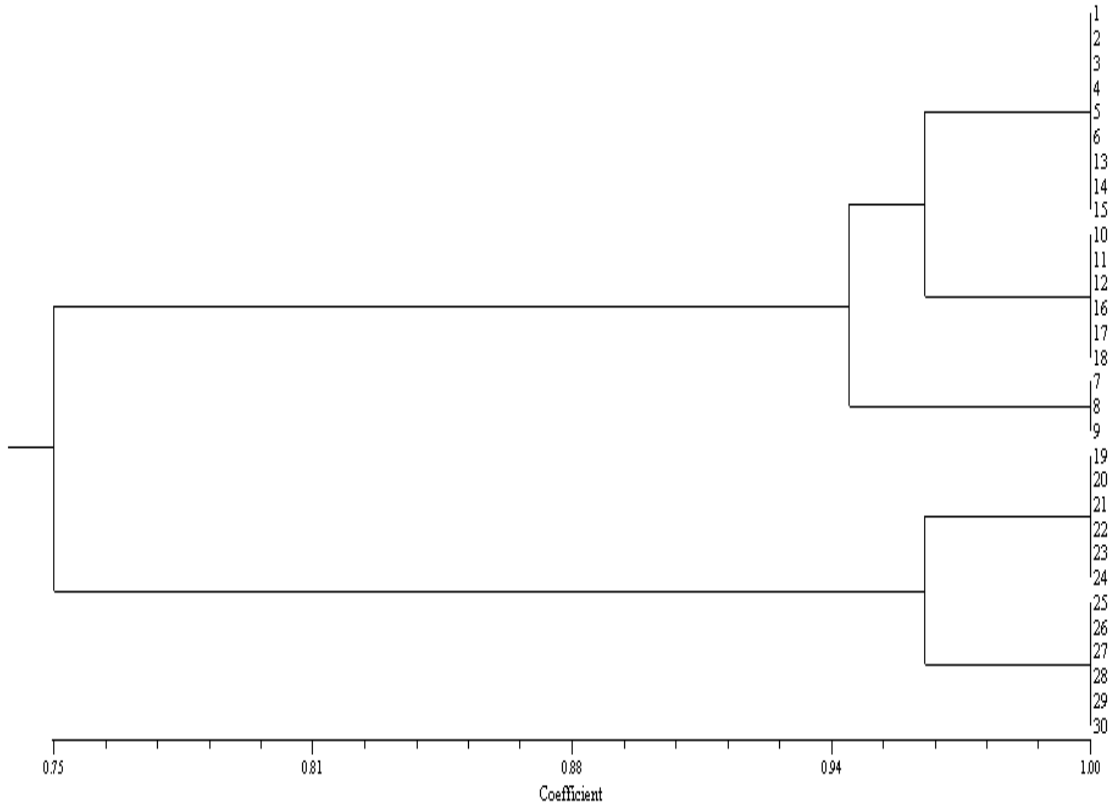


**Fig 2 Dendrogram showing genetic relatedness among thirty genotypes of *Viola serpens* Wall.**

F- statistics showed negative values, depicting more heterozygosity than the observed values (table 2). Goncharenko *et al*. (1994) also obtained negative value of weight fixation showing excess of heterozygosity between *Populus sylvestris* populations. However, such observations obtained in the present study may be biased due to very small sample size. Nei (1972) observed that in order to reduce the sampling errors of heterozygosity a large number of loci rather than a large number of individuals per locus must be examined and to study allele frequency distribution also, large number of individuals must be examined.

**Phosphorylase (PPH) (EC 2.4.1.1)**

Thirty genotypes of *Viola serpens* plant species produced three zones of enzyme activity in

Phosphorylase system. First the most cathodal zone having locus PPH I showed only one band, PPH Ia with RM value 0.34 and was present in genotypes Mandi, Hamirpur and Bilaspur districts. The second zone showed single locus, PPH II and single band PPH IIa with RM value 0.20 and was found to be present in all thirty genotypes of *Viola* serpens. The third zone having locus PPH III showed two bands, PPH IIIa with RM value 0.12 and PPH IIIb with RM value 0.10 respectively. Both these bands are present in all the thirty genotypes of *Viola* serpens. Thus, a marker PPH Ia has been identified which can successfully differentiate between the thirty genotypes of *Viola* serpens.

Genetic interpretation studies revealed that a total of 30 isozyme loci were scored coding for nine enzyme systems in thirty genotypes of *Viola* serpens. The number of loci coding for individual enzyme ranged from 3 to 5. A total of 25-28 alleles were identified (table 2).

Allele frequency varied between 0.5 to 1 (Nei, 1975) depending upon whether a locus displayed heterozygous or homozygous conditions. Various monomorphic loci identified were PER IIa, PER IIIa, ACP Ia, ACP IIa, ACP IIb, AAT IIa, AAT IIIa, EST IIa, EST IIb, CAT Ib, CAT IIa, MDH Ib, MDH IIa, ADH Ia, ADH IIa, ADH IIIa, PPH IIa, PPH IIa, PPH IIIa, PPH IIIb, ALP Ia, ALP Ib and ALP IIa. A locus was considered polymorphic if the frequency of the most common allele was not more than 0.99 (Nei, 1975). The proportion of polymorphic loci depicted the maximum value of 0.362 in three samples of AwahDevi site of Hamirpur district as compared to 0.317 in the samples of Patta site of Hamirpur and Ghumarwin site of Bilaspur districts, 0.272 in the samples of Kalwari site of Bilaspur,all sites of Mandi and Solan districts and 0.227 the lowest in the samples of Sirmaur district. In the present study, the average number of alleles per locus varied from 1.136 to 1.272. Expected heterozygosity ranged from 0.113 to 0.181.

**Interrelation of genotypes**

Isozymes polymorphism data was analysed considering all the bands obtained from nine enzymes. The similarity index revealed that all the thirty genotypes fell in the range of 0.73 to 1.00. Since these observations are based on nine enzyme systems only, therefore it is necessary to study more enzymes to get a clear picture about interrelationships. According to the dendrogram thirty genotypes were divided in two major clusters (fig 2). The upper cluster demarcated the genotypes in two sub clusters. Cluster-I consisted of genotypes of all the sites of Mandi, Hamirpur and Bilaspur districts and cluster-II included all the genotypes from Solan and Sirmaur districts. In the present investigation total number of isozymes bands

obtained were 30 and RM values ranges from 0.06-0.90 for all the thirty genotypes (table 3). These, samples are not sufficient to characterize the individual genotypes.

## CONCLUSION

From the present study, it is concluded that thirty genotypes from ten sites of Himachal Pradesh when evaluated with nine isozymes systems have depicted a 26.67 % polymorphism and thus low variation in *Viola serpens* Wall. exists. Therefore it is concluded that *V. serpens* Wall. plant species is not in a good status and the plant species falls in the endangered group. To the best of our knowledge this is the first report of isozyme analysis in *Viola serpens* Wall.

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