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Effect of altitude on the morphological and biochemical parameters of *Viola* species

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ABSTRACT

Viola species has been recognized as an important medicinal plant due to its role in the treatment of respiratory disorders, cancer, tumor and many pathological conditions. In the present study, the effects of altitude on morphological and biochemical parameters especially on Tyrosine Ammonia Lyase (TAL) and Phenylalanine Ammonia Lyase (PAL) were investigated. The results revealed a significant difference in plant height, root length, leaf width and leaf shape. The extent and magnitude of variation can be attributed to the difference in the altitudinal ranges occupied by Viola species. A dendrogram was constructed on the basis of distance matrix to find the correlation between Viola canescens and Viola ordarata and on clustering, one group was found to consist of only Viola canescens and the other group contains rest of the Viola ordorata. Present study also revealed that there was a significant variation in the enzymatic activity viz. PAL and TAL, which are considered to be important branch point enzymes in the phenylpropanoid pathway mechanism of plants. The maximum PAL activity was observed in the samples collected from Naranag and Doodhpathri regions, while the least activity was observed from Kashmir University and Wanpoh samples. Similarly, maximum TAL activity was observed in the samples collected from Yusmarg and Daksum samples, while least activity was observed in the samples collected from Doodhpathri and Wanpoh. In conclusion, the present study showed that the Viola species growing at higher altitude exhibits higher enzyme activity and morphological adaptations that combats the oxidative stress.

Keywords: Morphology, PAL, TAL, Viola canescens, Viola ordorata.

I INTRODUCTION

The genus *Viola* is an assemblage of many species and is commonly distributed throughout the world. In India, the plant has been found in States like Himanchal Pradesh, Uttarakhand, Utter Pradesh, Meghalaya and Jammu & Kashmir (Chandra et al. 2015). *Viola ordorata* and *Viola canescens* are the two important species that has been predominantly seen in the State of J&K. *Viola odorata* is a species of the genus *Viola* native to Europe and Asia, but has also been introduced to North America and Australia. It is commonly known as wood violet, sweet violet, English violet, common violet, florist's violet or garden violet (Mittal et al. 2015). In India, the plant is commonly known as '*Banafsa'* or '*Banaksa'*. '*Nun-Posh*' is its vernacular name in the state of Jammu and Kashmir. *Viola* belongs to family Violaceae and is hardy herbaceous flowering perennial

plant growing about 10cm and prefers sandy, loamy or clay well drained moist soil. The flowers are produced in the late winter and early spring. It grows around shrubberies, forest clearings, rocky and shady areas, mud walls, hedge banks (Rasool et al. 2016). The species of Viola usually grows at high altitudes usually ranging from 1600 to 2500 meters. The family Violaceae is very important ethnomedicinally and has been widely used in the traditional system of medicine (Chandra et al. 2015). Therefore its species has been found very effective in the treatment of various diseases.

Phenylalanine ammonia lyase (PAL; EC 4.3.1.25), the key enzyme linking primary metabolism of aromatic amino acids with secondary metabolic products in plants and other organisms. Besides, it plays a key regulatory role in controlling biosynthesis of all phenylpropanoid products. PAL has been reported to be stimulated under the stressful conditions like drought, temperature, radiation etc. Another enzyme Tyrosine ammonia lyase (TAL, EC 4.3.1.23), which also behave like PAL has been reported in number of organisms and are having greater catalytic efficiency for L-tyrosine (Sija et al. 2016). Both these enzymes has been studied in details in numbers of organisms.

The objective of the present study was to evaluate morphological changes that the plants exhibit in response to high altitudinal ranges, besides the activities of important biochemical enzymes PAL and TAL were also studied due to their involvement in the biosynthesis of important secondary metabolites under variable set of environmental conditions in *Viola* species occurring in Kashmir valley.

II MATERIAL AND METHODS

2.1. Plant Material

The important sites in which the species of *Viola* grows were identified initially and then the plant material was collected from these sites of Kashmir valley at the appropriate period of growth and development. The date of collection and coordinates of these sites were also noted down for future reference (Table 1).

Table 1. Collection sites of *Viola* species

S. No.	Collection site	Species Collected	Sample No.	Date of Collection	Altitude (amsl)
1.	Kashmir University Botanical Garden, Srinagar	*V. ordorata*	KUBG 01	29/08/2017	1800 mts
2.	Yousmarg, Budgam	*V. ordorata*	YSMG 06	24/05/2017	2396 mts
3.	Daksum, Anantnag	*V. ordorata*	DKSM 07	25/05/2017	2438 mts
4.	Doothpathri, Budgam	*V. ordorata*	DOPR 04	09/08/2017	2730 mts
5.	Upper Doothpathri, Budgam	*V. ordorata*	DOPR 05	09/08/2017	2790 mts
6.	Naranag, Ganderbal	*V. ordorata*	NRNG 03	30/07/2017	2128 mts
7.	Domal, Naranag, Ganderbal	*V. canescens*	NRNG 02	30/07/2017	2800 mts
8.	Wanpoh, Anantnag	*V. ordorata*	WNPH 08	25/05/2017	1671

2.2. Morphological Analysis

Immediately after collection, the plant material was assessed for fresh/dry weight measurements in order to determine the biomass content. Subsequently the plant material was subjected to morphological analysis by

taking into account the following trait descriptors like plant length, root length, leaf length, petiole length, leaf diameter and leaf shape.

2.3. Biochemical Analysis

Enzyme extraction: 0.5 g of fresh leaf material was homogenized in 3ml of ice cold sodium borate buffer (pH 8.5) containing 1.4 mM 2-mercaptoethanol and 0.1g of insoluble polyvinylpyrrolidone. The extract was then centrifuged at 10000 rpm for 25 minutes.

2.3.1. Phenylalanine Ammonia Lyase (PAL) Assay

PAL activity was analyzed by following the methodology of Rosler et al., 1997. It involves the rate of conversion of L-phenylalanine into trans-cinnamic acid at 290nm in UV-VIS spectrophotometer. The mixture containing 0.1 ml of enzyme extract was treated with 0.5 ml of sodium borate buffer (pH 8.5) and 0.5 ml of phenylalanine in the same buffer. The final volume was made up to 3 ml by adding deionized water and kept it for incubation at 37^{o}C for 30 minutes. After incubation period was over, the tubes were mixed by inversion and the increase in absorbance was recorded at 290 nm for 5 minutes.

The samples were prepared in triplicate for each analysis and the mean value of the $\Delta A_{290nm/min}$ was obtained using the maximum linear rate for both the test and the blank.

One unit will deaminate 1.0 µmole of L-phenylalanine to trans-cinnamate and ammonia per minute at of 37^{o}C for 30 minutes at pH 8.5.The amount of trans-cinnamic acid synthesized was calculated by using extinction coefficient of 9630 $M^{-1}cm^{-1}$

2.3.2. Tyrosine Ammonia Lyase (TAL) Assay

TAL activity was analyzed by following the methodology of Rosler et al., 1997. It involves the rate of conversion of L-tyrosine into p-coumaric acid at 335 nm in the UV-VIS spectrophotometer. The mixture containing 0.1 ml of enzyme extract was treated with 0.5 ml of sodium borate buffer (pH 8.5) and 0.5 ml of L-tyrosine in the same buffer. The final volume was made up to 3 ml by adding deionized water and kept it for incubation at 37^{o}C for 30 minutes. After incubation period was over, the tubes were mixed by inversion and the absorbance was measured at 335 nm for 5 minutes.

The samples were prepared in triplicate for each analysis and the mean value of the $\Delta A_{335nm/min}$ was obtained using the maximum linear rate for both the test and the blank.

2.4. Data Analysis

The data generated was analyzed using the software programs which includes "**DendroUPGMA: A dendrogram construction utility"** for the construction of dendrogram and distance based matrix from the morphological data generated from the analysis of *Viola* species from Kashmir Himalaya. Besides, the data obtained from the biochemical analysis of *Viola* species was analyzed by using "**GraphPad Prism Programme (Version 6.0.1)"**.

2.5. Morphological Analysis of *Viola ordorata* and *Viola canescens*

In the present study, the initial data clearly revealed the amount of variation in the biomass content exhibited by the *Viola* species inhabiting varied altitudinal ranges. The maximum amount of fresh/dry weight was observed in the sample NRNG03 (*Viola ordorata*) and the least was found in the samples NRNG02 and NRNG07 (*Viola ordorata*). This gives an indication about the role of altitude in determining the biomass content in the *Viola* species. However, the fresh/dry weight content was found to be comparatively less in case of NRNG02 (*Viola canescens*) amongst all the samples collected (Table 2).

Sample No.	Species Name	No. of Plants Taken	Fresh Weight (g)	Dry Weight (g)
KUBG 01	*V. ordorata*	20	24.90	2.39
NRNG 02	*V. canescens*	20	3.39	0.34
NRNG 03	*V. ordorata*	20	39.33	3.89
DOPR 04	*V. ordorata*	20	29.60	2.96
DOPR 05	*V. ordorata*	20	33.77	3.20
YSMG 06	*V. ordorata*	20	22.85	2.91
DKSM 07	*V. ordorata*	20	3.39	0.34
WNPH 08	*V. ordorata*	20	20.90	2.00

Table 2. Fresh/Dry Weight measurement of *Viola* species

III RESULTS AND DISCUSSION

The plant material was also subjected to morphological analysis by taking into account the following standard trait descriptors like plant length, root length, leaf length, petiole length, leaf diameter and leaf shape (Table 3). From the data, it was observed that there was a significant amount of variation amongst the samples collected from different altitudinal ranges. The group of samples KUBG01, DOPR04, DOPR05 and WNPH08 were found to be more or less similar. Whereas, the group of samples NRNG03, YSMG06 and DKSM07 were found to be more or less similar, so far as their morphological characteristics were concerned. However, NRNG02 was found to be entirely different in terms of its morphological characteristics (Table 3). These results again suggested the role of altitude in determing the morphological features in *Viola* species.

Table 3. Morphological analysis of *Viola* species using trait descriptors

Sample No.	Plant height (cm)	Root length (cm)	Leaf length (cm)	Petiole length (cm)	Leaf diameter (cm)	Leaf shape
KUBG01	24.33	7.33	5	9	3	Heart
NRNG02	32.57	12.71	5.1	13.41	6.7	Ovate-Heart
NRNG03	17.5	9.0	2.5	6	3.2	Heart
DOPR04	20.5	9.0	3.5	8	3	Heart
DOPR05	23.80	7.0	2.8	14	4.5	Orbicular
YSMG06	17.5	9.0	1.5	7	1.5	Heart
DKSM07	17.0	7.0	1.5	8.5	1.8	Heart
WNPH08	19.5	7.0	3.5	10	3.2	Heart

A distance based matrix was constructed based on the morphological trait descriptors revealed the amount of variation existing within and between the samples of *Viola* species (*V. ordorata* and *V. canescens*). The results indicated that that samples NRNG03 and YSMG06 were closely related, whereas, the samples NRNG02 and DKSM07 were distantly related amongst all the samples (Table 4). A dendrogram was constructed on the basis of distance matrix to find the correlation between *Viola canscens* and samples of *Viola* ordarata and on clustering one group was found to consist of only *Viola canscens* and the other group contains rest of the samples of *Viola ordorata* (Fig 1). Thus morphological analysis helped in the taxonomic delineation of *Viola* species collected from the various sites of Kashmir Valley. The extent and magnitude of variation can be attributed to the difference in the altitudinal ranges inhibited by *Viola* species.

Table 4. Distance matrix based on Euclidean coefficient

	KUBG 01	**NRNG 02**	**NRNG 03**	**DOPR 04**	**DOPR 05**	**YSMG 06**	**DKSM 07**	**WNPH 08**
KUBG01	0	11.401	8.045	4.551	5.699	8.242	8.233	5.170
NRNG02		0	17.742	14.317	10.954	17.943	18.333	15.161
NRNG03			0	3.747	10.463	2.211	3.669	5.000
DOPR04				0	7.323	4.031	4.684	3.007
DOPR05					0	10.168	9.245	6.056
YSMG06						0	2.567	4.888
DKSM07							0	3.803
WNPH08								0

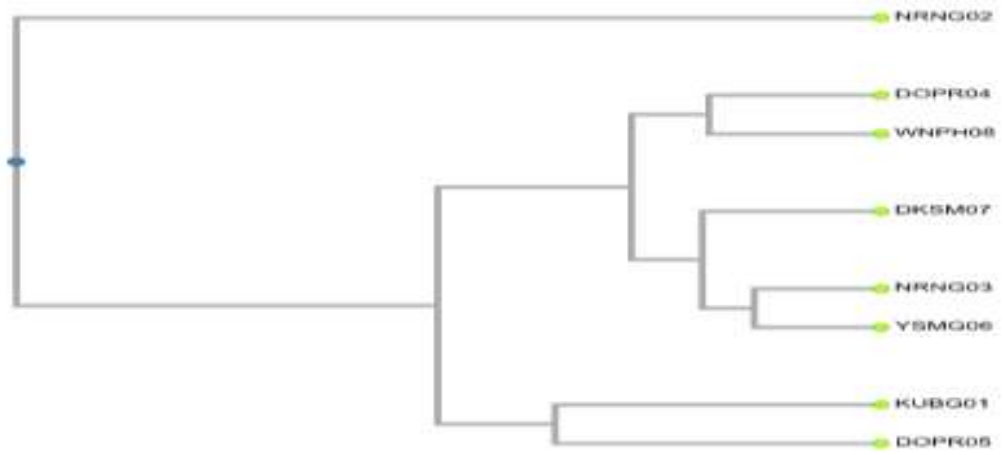

Figure 1. Dendrogram based on distance matrix showing relationship in *Viola* species.

In the same way, phenylalanine ammonia lyase (PAL) and tyrosine ammonia lyase (TAL) activity was analyzed amongst the different samples of *Viola* species collected from Kashmir valley. It was observed that the PAL and TAL activity showed significant variation between the *Viola* samples collected from different sites. The maximum PAL activity was observed in the samples collected from Naranag and Doodhpathri sites, while the least activity was observed from the samples collected from Kashmir University Botanical Garden and Wanpoh sites (Fig 2). Moderate PAL activity was observed in the rest of the samples. On the other hand, maximum TAL activity was observed in the samples collected from Yusmarg and Daksum sites, while least activity was observed in the samples collected from Doodhpathri and Wanpoh sites. Moderate TAL activity was observed in the rest of the samples (Fig 3). The activity of PAL and TAL was found to be directly linked with the altitudinal ranges in which the samples of *Viola* species were growing.

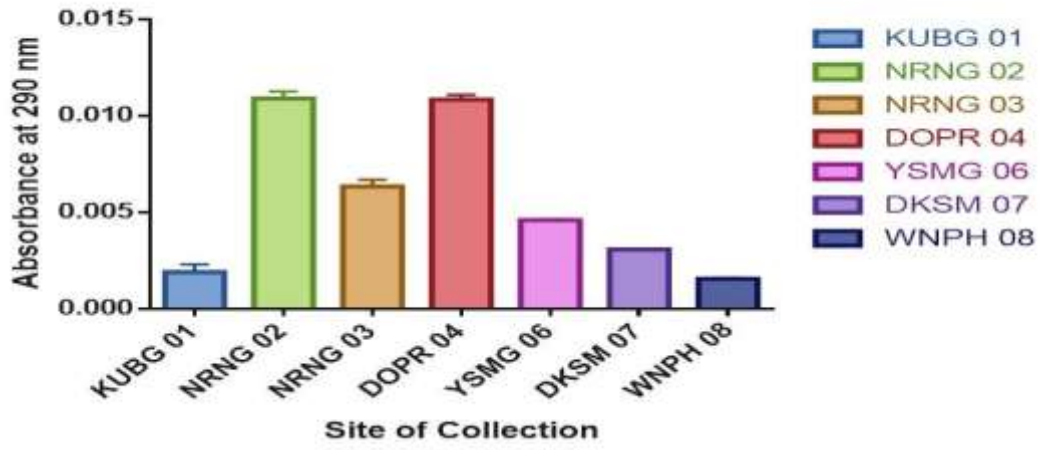

Figure 2. PAL activity in the *Viola* samples collected from Kashmir valley

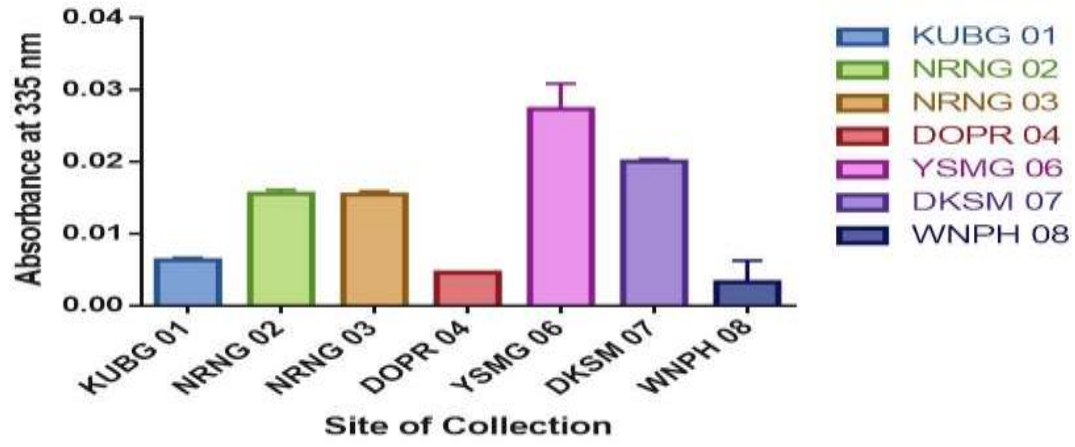

Figure 3. TAL activity in the *Viola* samples collected from Kashmir valley

IV CONCLUSIONS

The present study clearly depicted the influence of altitudinal ranges on the morphological and biochemical parameters of the *Viola* species. It was concluded that with the increase in altitude, there was a significant variation in the morphology and biomass content of the samples under study. Besides, the analysis helped in the taxonomic delineation of *Viola canescens* from *Viola ordorata*. It was also concluded that there was a significant amount of variation in their enzymatic activity, especially PAL and TAL, which are considered to be important branch point enzymes in the phenylpropanoid pathway mechanism of plants.

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Conflict of interest
The authors declare that there is no conflict of interest.

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