Journal of Hill Research
20(2) : 46-52, 2007

Biochemical Diversity in Some Rhododendron Species from the Darjeeling and Sikkim Himalayas

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(Received 19 April 2007; revised version received 11 November 2007; accepted 19 November 2007)

ABSTRACT

*Biochemical analysis of eight Rhododendron species occurring in Darjeeling and Sikkim Himalayas showed a high level of soluble protein content in the species distributed over a wide range of altitudinal gradient (*Rhododendron arboreum, R. cinnabarium *etc.), while the amino acid content was high in the species growing in the highest region (*R. maddenii*) in this study. The proline content was more pronounced in the species growing under water stress conditions. A high level of carbohydrate content as well as catalase and peroxidase activities seemed to be an adaptive feature in some of the species.*

INTRODUCTION

Rhododendrons are natural forest species and important horticultural plants. The genus is represented by 36 species in the Darjeeling-Sikkim Himalayan region (Pradhan and Lachungpa, 1990). Certain low temperatures are critical in defining the range of evergreen *Rhododendron* species (Vetaas, 2002). This group of plants constitute characteristic vegetation of the Himalayan region have a very slow growth rate, with size ranging from small scrubs (15-20 cm tall) in the alpine regions to trees over 25 m tall. Of the Indian species 98% occur in the Himalayas among which 72% occurs in Darjeeling-Sikkim Himalayas Singh *et al.*, 2003).

Many ethnobotanical and medicinal uses of Rhododendrons have been recorded so far. The leaves of *Rhododendron anthopogon* are used in making incense by the Tibetan people. The dried flowers of *R. arboreum* are used by Nepalese people for curing dysentery and diarrhoea. A medicinal brew called 'Gurans ko raxi' is fermented from the flowers of *R. arboreum* in the Singalila National Park area in Darjeeling Hills. *R. campanulatum* leaves are used in hemicrania, rheumatism and sciatica (Jain 1991). Besides, the leaves of *R. arboreum* and *R. falconeri* are used as fish poison (Chopra, 1992).

Such a useful genus as *Rhododendron* is being destroyed indiscriminately. The major threats for Rhododendrons are deforestation and unsustainable extraction for firewood by local people (Krishna *et al.*, 2002). Use of leaves and twigs as traditional incense has also caused its depletion.

Anthropogenic activities are the main threats for the existence of Rhododendrons. Potentials of this important group of plants have not been evaluated so far from pharmacological point of view. Moreover, at least some of the species of this plant may prove horticulturally important and even some hitherto unexplored perfumes may be formulated from Rhododendrons. Such commerce directed activities may ensure the survival of the species, but before that, a thorough knowledge of the physiology and biochemistry of this species is a must. The present work is one such attempt towards understanding the basic metabolism of this group of plants available in this part of the Himalaya.

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MATRIALS AND METHODS

Fresh juvenile actively growing leaves (2nd and 3rd leaf from the top) of 8 different species of Rhododendrons namely, *R. arboreum* Smith., *R. dalhousiae* Hook. f., *R. grande* Wight., *R. griffithianum* Wight. *R. triflorum* Hook. f., *R. cinnabarium* Hook. f., *R. maddenii* Hook. f., *R. falconeri* Hook. f. were collected were collected during March-April in the morning in an ice-box and brought to the laboratory for analysis. All the analytical work was done at 20 °C ± 2 °C with a RH of 78% ± 2%.

For the estimation of protein 1 g of leaf tissue was homogenized in 19 ml of 62.5 mM Tris-HCl buffer (pH 6.8) with neutral sand. The sample was centrifuged at 15,000 rpm for 15 minutes. The pellet discarded and the protein was estimated from the supernatant with Folin-Ciocalteau reagent (Lowry *et al.*, 1951) as modified by Bollag *et al.* (1996). For the estimation of free amino acids 500 mg of tissue was homogenized in 5 ml of boiling 80% ethanol and centrifuged at 3,000 rpm for 10 minutes. The supernatant was collected in a watch glass and evaporated to dryness. The chlorophyll was washed with and drained carefully from the surface of the watch glass with the help of solvent ether. The inner surface of the watch glass was then washed carefully with 80% ethanol and the residual solution was collected and made up to 5 ml with 80% ethanol. This extract served as the source for free amino acids. The estimation was done with Ninhydrin reagent as per the method of Moore and Stein (1948). For the extraction of proline, 200 mg fresh leaf tissue was homogenized with 10 ml 3% sulphosalicylic acid and centrifuged at 4, 000 rpm for 10 min; the supernatant was used as the source for proline which was estimated as per Sadashivam and Manickam (1996).

Carbohydrate was extracted from 100mg of leaf tissue from each species which were homogenized separately with 5ml 80% ethanol and centrifuged at 3,000 rpm for 10 minutes. The supernatant was taken in a watch glass and evaporated to dryness and the chlorophyll was removed by washing with solvent ether. Residual material was taken in a test tube by washing the inner surface of the watch glass (three times) with 80% ethanol and making up the volume to 5 ml with the same. The sample served as the source of soluble carbohydrate. For insoluble carbohydrate the residue from the above extraction was dissolved in 5 ml of 25% H_2SO_4 at 80% °C for 30 minutes (Rai *et al.*, 1992) and centrifuged at 5,000 rpm for 15 minutes. The supernatant was collected as a sample for insoluble carbohydrate estimation. Estimation of both types of carbohydrates was done following the method of McCready *et al.* (1950).

Extraction of nucleic acids (DNA and RNA) was made from the leaf tissues of the test plants (100 mg each) following the method described by Cherry (1962) and the quantitative estimation of the same was done following the method of Choudhuri and Chatterjee (1970).

For the extraction of catalase 500 mg plant material was homogenized with 10 ml 0.1 M chilled Na-phosphate buffer (pH-7.0) and centrifuged at 5,000 rpm for 10 minutes. The pellet was discarded and the supernatant was used as crude source of the enzyme extract. The enzyme assay was carried out according to the method of Snell and Snell (1971). For the estimation of peroxidase activity 100 mg tissue was homogenized with 5 ml 300 µm Na-phosphate buffer (pH-6.8) and centrifuged at 1,000 rpm for 10 minutes. The supernatant was used as the crude enzyme source and the enzyme activity was estimated following the method of Kar and Mishra (1976). The extraction of protease was done by homogenizing 500 mg tissue in 5 ml of chilled 0.1 M Na-phosphate buffer (pH-6.5) followed by centrifugation of the homogenate for 10 minutes at 10,000 rpm. The supernatant was used as the crude enzyme source. The assay mixture contained 1 ml of the enzyme extract, 0.1 ml $MgSO_4$, $7H_2O$ (0.1 M) and 1 ml 500 µg/ml BSA (Chhetri *et al.*, 2003).

For the estimation of RNAase activity 500 mg leaf tissue of each species was homogenized in 10 ml of chilled 0.1 M phosphate buffer (pH-6.5) and centrifuged at 5,000 rpm for 10 minutes. The supernatant was taken, the volume of which was made up to 10 ml with the same phosphate buffer. This served as the crude source of RNAase. To 1 ml of above extract 1 ml of yeast RNA (500 µg/ml) was added and incubated for 30 minutes. The reaction was terminated with perchloric acid (35%). Subsequently, 5 ml BSA (500 µg/ml) and 2 ml gelatine (0.1%) were added and the turbidity was measured at 420 nm against a control set minus the yeast RNA.

The estimation of IAA-oxidase was done from 100mg of leaf material which was homogenized with 10 ml of 0.1M K-phosphate buffer (pH-5.8) and centrifuged for 10 minutes at 6,000 rpm. The supernatant was taken as the enzyme source. The reaction mixture for IAA oxidase assay contained 0.5 ml 1 mM 2,4-DCP, 0.5 ml 1 mM $MnCl_2$, 0.5 ml 2 mM IAA, 1 ml crude enzyme extract and 3 ml Citrate buffer (pH-6.5). After incubation for 30 minutes at 30°C, the residual IAA was estimated by Salkowski's reagent. The activities of all the enzymes were determined according to Fick and Qualset (1975).

Photosynthetic pigments (chlorophyll a, b and caroteinoids) were extracted and estimated from fresh leaf samples of different species. For the estimation of photosynthetic pigments 50 mg leaf tissue was homogenized with 5 ml 96% ethanol and centrifuged for 10 minutes at 5,000 rpm. The estimation was done following the method of Lichtenthaler (1987).

RESULTS AND DISCUSSION

In the present study, a high level of protein content was found in all the species of Rhododendron analyzed.

However, the highest level of protein was found in the leaves of *R. arboreum* and *R. cinnabarium*. Interestingly enough, *R. arboreum*, *R. cinnabarium* and *R. maddenii* were the species with the widest altitudinal range of distribution (Singh *et al.*, 2003). Protein synthesis is necessary for the development of freezing tolerance and several distinct proteins accumulate during acclimation to cold as a result of changes in gene expression (Guy, 1990). These proteins are thought to protect cells dehydrated by water shortage or freezing by stabilizing other proteins and membranes (Taiz and Zeiger, 1998). Besides the simple indication of overall vigor, the higher level of protein in some species of Rhododendron may be explained as a plant response towards meeting the environmental stress conditions (De and Mukherjee, 1996). The free amino acid content was again found to be highest in the species bearing the widest altitudinal gradient of distribution and it was found to be lowest in the species having the narrowest altitudinal gradient of distribution indicating that the protein metabolism (both anabolism and catabolism) was minimal in the in the species having a narrow altitudinal range of distribution (Table 1).

The present finding indicates a higher level of proline accumulation in *R. dalhousiae* and *R. maddenii*, the species having epiphytic and lithophytic habit respectively and hence always in water stress conditions (Table 1). At the same time, a high level of proline in *R. cinnabarium* may be an indication of its accumulation in plant cells during cold acclimation. Increased osmotic pressure as a consequence of gradual water stress may also tend to enhance low temperature tolerance (Bhattacharya and Mukherjee, 1995).

An appreciable level of soluble and insoluble carbohydrates has been detected in all the species studied (Table 2). The highest levels both in terms of the soluble and insoluble carbohydrate content have been found in *R. falconeri* during the present studies. Incidentally, among the species of *Rhododendron* analyzed here, *R. falconeri* is the species growing in the highest altitudinal range with the narrowest belt of occurrence (Singh *et al.*, 2003). It would be worthwhile to mention here that the syntheses of low molecular weight solutes such as glucose, fructose, sucrose and raffinose family oligosaccharides are accumulated during the process of cold-acclimation (Zuther *et al.*, 2004).

DNA content was found to be almost uniform in all the species studied. However, the RNA level showed a wide variation in the different species studied. (Table 2). In general, the RNA content is much higher than that of DNA. A high requirement of proteins during cold acclimation may have necessitated a high content of RNA. Cold acclimated spinach and cabbage were found to accumulate proteins that have potent cryoprotective activity (Volger and Heber, 1975). The activity of the free radical scavenging enzymes catalase and peroxidase may be an indication of the vigor and the capacity for stress tolerance of plants. In the present study, the highest level of both catalase and peroxidase activity has been recorded in *R. arboreum*, *R. cinnabarium* and *R. maddenii* (Table 3). All these species among the studied lot showed the widest range of distribution across different altitudinal gradient. May be, a high level of such enzyme activity is an adaptive feature acquired by the plants during the course of evolution.

Table 1. Variations in the content of protein, free amino acids and proline in eight *Rhododendron* species from Darjeeling and Sikkim Himalayas

Plant Species	Protein (mg/g fr. wt.)	Free amino acids (mg/g fr. wt.)	Proline (mg/g fr. wt.)
R. arboreum	48.2 ± 0.805	16.1 ± 0.235	35.1 ± 0.672
R. triflorum	22.0 ± 0.605	7.8 ± 0.779	70.5 ± 0.749
R. griffithianum	23.8 ± 0.432	13.2 ± 0.070	65.3 ± 2.148
R. grande	23.4 ± 0.586	17.2 ± 0.353	80.2 ± 0.521
R. dalhousiae	11.3 ± 0.379	5.1 ± 0.349	145.5 ± 2.497
R. cinnabarium	43.1 ± 0.504	28 ± 0.216	118.5 ± 2.638
R. maddenii	34.2 ± 0.271	32.0 ± 0.178	114.2 ± 1.885
R. falconeri	32.6 ± 0.750	20.6 ± 0.414	37.5 ± 0.512

[Values are mean ± SE, n = 3 replicates]

Table 2. Variations in the content of soluble carbohydrates, insoluble carbohydrates, DNA and RNA in eight *Rhododendron* species from Darjeeling and Sikkim Himalayas

Plant Species	Soluble carbohydrates (mg/g fr. wt.)	Insoluble carbohydrates (mg/g fr. wt.)	DNA (mg/g fr. wt.)	RNA (mg/g fr. wt.)
R. arboreum	55.2 ± 0.923	16.2 ± 0.334	19.7 ± 0.709	56.3 ± 1.405
R. triflorum	60.7 ± 1.716	21.5 ± 0.093	17.9± 0.268	42.9 ± 0.409
R. griffithianum	68.5 ± 0.161	23.7 ± 0.319	15.3 ± 0.531	39.68 ± 1.616
R. grande	64.3 ± 0.736	24.3 ± 0.187	12.7 ± 0.227	29.9 ± 1.105
R. dalhousiae	59.8 ± 1.259	39.18 ± 0.185	13.7 ± 0.178	50.4 ± 0.374
R. cinnabarium	45.2 ± 2.187	13.8 ± 0.334	12.6 ± 0.380	36.1 ± 0.178
R. maddenii	65.5 ± 0.402	27.3 ± 0.517	13.6 ± 0.865	41.8 ± 0.531
R. falconeri	72.8 ± 0.788	41.5 ± 0.612	12.4 ± 0.349	69.1 ± 0.460

[Values are mean ± SE, n = 3 replicates]

Table 3. Variations in Catalase, Peroxidase, RNAase and Protease activities in eight *Rhododendron* species from Darjeeling and Sikkim Himalayas

Plant Species	Enzyme activity (units/h/g of fresh tissue)			
	Catalase	Peroxidase	RNAase	Protease
R. arboreum	7.8± 0.212	18.9 ± 0.442	6.9 ± 0.204	8.2± 0.497
R. triflorum	5.3 ± 0.147	13.2 ± 0.330	6.0 ± 0.108	4.5 ± 0.225
R. griffithianum	2.5 ± 0.141	7.0 ± 0.227	9.1 ± 0.178	8.8 ± 0.147
R. grande	3.6± 0.108	5.4 ± 0.070	7.7 ± 0.108	4.2 ± 0.147
R. dalhousiae	5.1 ± 0.070	4.6 ± 0.040	6.7 ± 0.147	9.7 ± 0.057
R. cinnabarium	6.9 ± 0.040	18.6 ± 0.216	7.1 ± 0.108	6.5 ± 0.114
R. maddenii	6.2 ± 0.178	19.2 ± 040	9.9 ± 0.108	7.1 ± 0.141
R. falconeri	6.6 ± 0.057	6.4 ± 0.187	12.3 ± 0.464	7.9 ± 0.108

[Values are mean ± SE, n = 3 replicates]

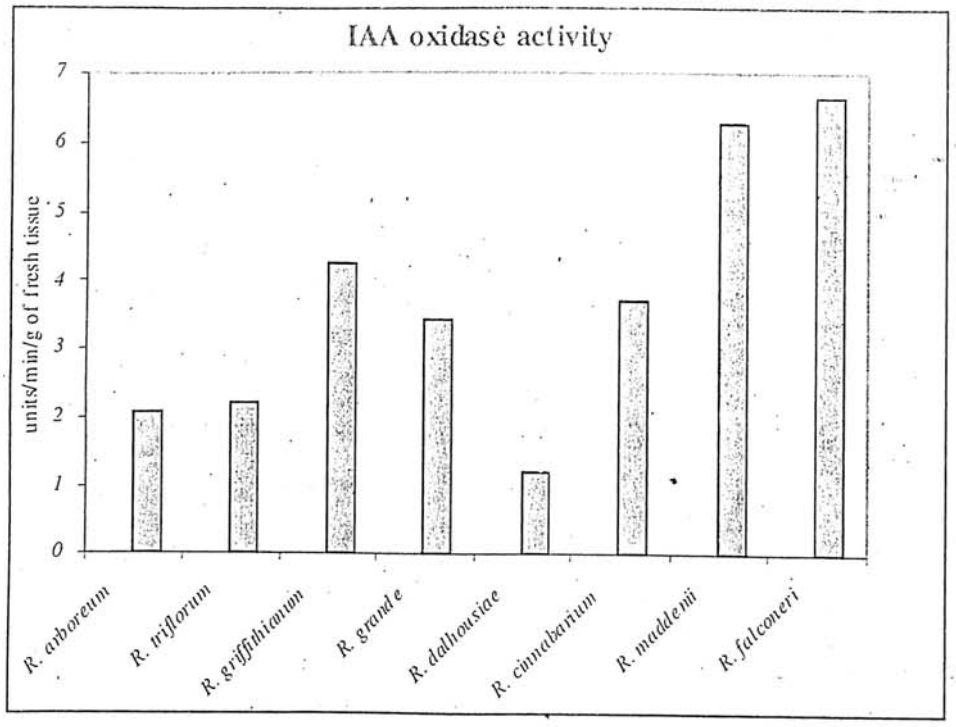

Fig-1. IAA oxidase activity in eight *Rhododendron* species from Darjeeling and Sikkim Himalayas.

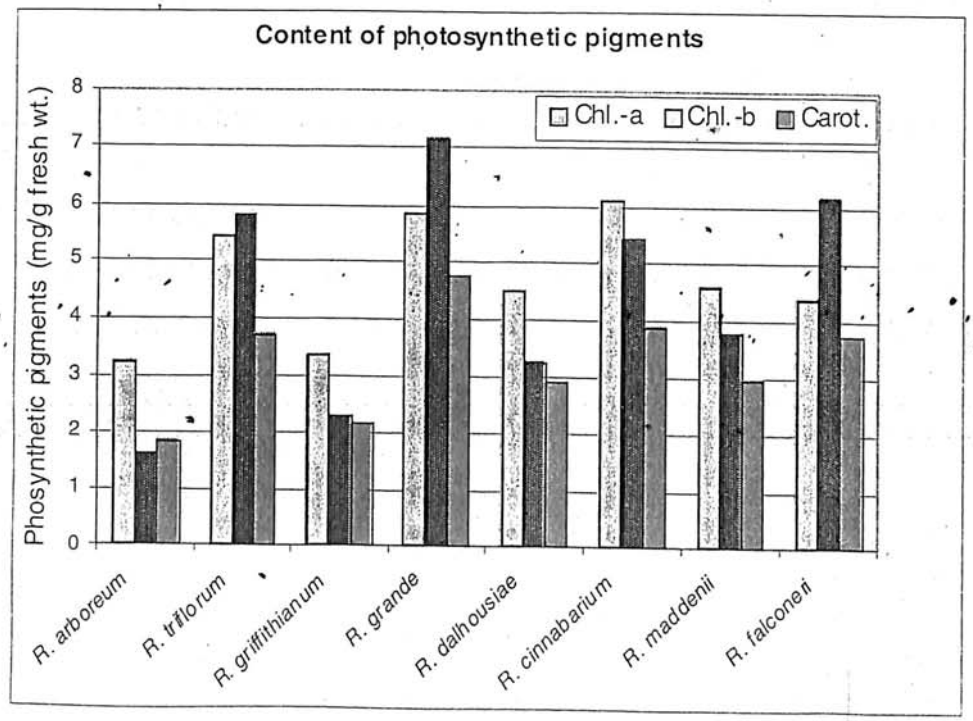

Fig-2. Variations in the content of photosynthetic pigments: Chlorophyll-a, Chlorophyll-b and Carotenoids in eight *Rhododendron* species from Darjeeling and Sikkim Himalayas.

The activities of protease and RNAase responsible for the catalytic break down of protein and RNA respectively may be an indication of the metabolic state of the plant. In the present study the highest level of protease activity has been recorded from *R. dalhousiae* (Table 3). These results may be co-related with the lowest level of protein content in the same species (Table 1). However, such simple co-relation has not been observed in case of RNAase and RNA pair. The highest activity of this enzyme was found in *R. falconeri*, which also possesses the highest content of RNA (Table 2 & 3).

The IAA-oxidase activity may be an indication of the physiological status of a plant or its parts. The lesser the activity of this enzyme, the more is supposed to be the physiological vigor of the plant. In the present study the least activity of this enzyme was found in *R. dalhousiae* and *R. arboreum* respectively. This fact points towards the adaptability of the plants in the lithophytic habitat and distribution over a wide altitudinal gradient respectively. Among the photosynthetic pigments, the lowest level of chlorophyll a, chlorophyll b and caroteinoids have been recorded from *R. arboreum*, while the highest level of chlorophyll a was found in *R. cinnabarium*. Chlorophyll b and caroteinoids were highest in *R. grande* (Fig 2). Impaired chlorophyll development may be due to interference with the synthesis of proteins, the structural components of chloroplasts (Nag *et al.*, 1981)

ACKNOWLEDGEMENTS

The authors are grateful to the anonymous reviewers for their constructive suggestions. Mr. Jaganath Adhikari is acknowledged for his help during the collection of the samples.

REFERENCES

Bhattacharjee, S. and Mukherjee, A. K. (1995). Chilling induced physiological and biochemical responses in a tropical leaf crop, *Amaranthus lividus* Linn. *Indian Journal of Experimental Biology* **33:** 529-532.

Bollag, D. M., Rozycki, M. D. and Edelstein, S. J. (1996). *Protein Methods* (2nd *edition)*, Wiley-Liss, New York, p.415.

Cherry, J. H. (1962). Nucleic acid determination in storage tissue of higher plants. *Plant Physiology* **37:** 670-678.

Chhetri, D. R., Ahmed, S. and Modak, S. (2003). Heavy metal induced physiological and biochemical responses in a temperate legume crop, *Vigna umbellata* T. *Indian Journal of Applied and Pure Biology* **18 (2):** 145-153.

Chopra, R. N., Nayar, S. L. and Chopra, I. C. (1992). *Glossary of Indian Medicinal Plants.* Publication and Information Directorate, CSIR, New Delhi, p.330.

Choudhuri, M. A. and Chatterjee, S. K. (1970). Seasonal changes in the levels of some cellular components in the abscission zone of *Coleus* leaves of different ages. *Annals of Botany* **34:** 275-287.

De, B. and Mukherjee, A. K. (1996). Mercury induced metabolic changes in seedlings and cultured cells of tomato. *Geobios* **23:** 83-88.

Fick, N. G. and Qualset, C. O. (1975). Genetic control of endosperm amylase activity and GA response in standard height and short statured wheat. *Proceedings of National Academy of Sciences, U.S.A.* **72:** 892-895.

Guy, C. L. (1990). Cold acclimation and freezing stress tolerance: Role of protein metabolism. *Annual Review of Plant Physiology and Molecular Biology* **41:** 187-193.

Jain, S. K. (1991). Dictionary of Indian Folk Medicine and Ethnobotany. Deep Publications, New Delhi, p.311.

Kar, M. and Mishra, D. (1976). Catalase, peroxidase and polyphenol oxidase activities during rice root senescence. *Plant Physiology* **57:** 315-320.

Krishna, A. P., Chhetri, S. and Singh, K. K. (2002). Human dimension of conservation in the Khangchenjunga Biosphere Reserve. *Mountain Research and Development* **24:** 328-331.

Lichtenthaler, H. K. (1987). Chlorophylls and carotenoids: Pigments of photosynthetic biomembranes. *Methods of Enzymology* **148:** 350-382.

Lowry, O. H., Rosebrough, J. Farr, A. L. and Randall, R. J. (1951). Protein measurement with the Folin-phenol reagent. *Journal of Biological Chemistry* **193:** 265-275.

McCready, R. N., Guggloz, J., Silviera, V. and Owens, A. S. (1950). Determination of starch and amylase in vegetables. *Analytical Chemistry* **22:** 1156-1158.

Moore, S. and Stein, W. W. (1948). Photometric Ninhydrin method for the use in chromatography of amino acids. *Journal of Biological Chemistry* **176:** 367-368.

Nag, P., Paul, A. K. and Mukherjee, S. (1981). Heavy metal effects in plant tissues involving chlorophyll, chrophyllase, Hill reaction activity and gel-electrophoresis pattern of proteins. *Indian Journal of Experimental Biology* **19:** 702-706.

Pradhan, U. C. and Lachungpa, S. T. (1990). *Sikkim Himalayan Rhododendrons.* Primulaceae Books, Kalimpong, p.130.

Rai, A. S., Chhetri, D. R. and Bhattacharjee, A. (1992). Effect of Sodium-Dikegulac on maintenance of viability of a few crop seeds under adverse storage conditions. *Environment and Ecology* **10(4):** 814-824.

Sadashivam, S. and Manickam, A. (1996). *Biochemical Methods (2nd edition).* New Age International (P) Ltd. New Dehi and Tamil Nadu Agricultural University, Coimbatore. p.256.

Singh, K. K., Kumar, S., Rai, L. K. and Krishna, A. P. (2003). Rhododendron conservation in the Sikkim Himalaya, *Current Science* **85 (5):** 602-606.

Snell, F. D. and Snell, C. T. (1971). *Calorimetric Method of Analysis.* Van Nostrad Reinhold Co. New York.

Taiz, L. and Zeiger, E. (1998). *Plant Physiology.* Sinauer Associates Inc. Publishers, Massachusetts,Vetas, O. R. (2002). Realized and potential climate niches, comparisons of four Rhododendron tree species. *Journal of Biogeography* **25:** 545-554.

Volger, H. G. and Heber, H. (1975). Cryoprotective leaf proteins. *Biochimica et Biophysica Acta* **412:** 335-349.

Zuther, E., Büchel, K., Hundertmark, M., Still, M., Hincha, D. K. and Heyer, A. G. (2004). The role of raffinose in the cold acclimation response of *Arabidopsis thaliana. FEBS Letters* **576:** 169-173.