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Impact of Altitudinal Gradient on Ammonium Assimilatory Enzymes in *Rauvolfia teraphylla* L. (Apocyanaceae) – A Perennial Medicinal Herb

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Abstract

Seedlings of *Ravuolfia tetraphylla* L. were grown, transplanted and acclimatized for 60 days at different altitudes gradient (250, 400 and 1600 m) in Yercaud, Salem, Tamil Nadu, India. Response to shift in altitude was observed in the test plants. Shoot length decreased with the increase in the altitude, while increase in the root length was directly proportional to the increase in the altitudinal gradient. Biomass accumulation in roots of *R. tetraphylla* recorded the maximum at high altitude at the same time shoot biomass was maximum at an intermediate height (400m), thereafter reduction in biomass was observed with the increase in the altitude. Total soluble protein content was significantly high at low altitude in the

shoot while it followed a reverse trend in the roots. Likewise, free tissue ammonia level in this species showed positive correlation with increase in the altitude. Ammonium assimilatory enzymes viz., glutamine synthetase (GS), glutamate synthase (GOGAT) and glutamate dehydrogenase (GDH) were analyzed. GS/ GOGAT activity and specific activity were altitude sensitive, whereas GDH activity exhibited inverse trend. A positive shift in ammonium assimilatory pathway in test plants growing at high altitude was observed in *R. tetraphylla*.

Key Words: Altitudinal gradient, *Ravuolfia tetraphylla*, Ammonium Assimilatory Pathway; Glutamate Dehydrogenase (GDH).

Introduction

Nitrogen metabolism in plants is a complex process, and is regulated by the form of nitrogen that is available to the plant (Magalhaes and Huber, 1989). Major source of nitrogen to plants is ammonium, and is largely assimilated by the roots (Yoneyama and Kumazawa, 1974). Generally, plants prefer ammonium (NH_4^+) compared to nitrate (NO_3^-) and nitrite (NO_2^-) as ammonia is the starting point for nitrogen assimilation in higher plants. Internal source of ammonia is photorespiration and amino acid catabolism (Srivastava and Singh, 1987). However, high level of ammonia is toxic, therefore it has to be converted to amino acids in plants to maintain its low level (Miflin and Lea, 1980). Enzymes namely Glutamine synthetase (GS) (EC 6.3.1.2)/ Glutamate synthase (GOGAT) (EC 1.4.1.14) and Glutamate dehydrogenase (GDH) (EC 1.4.1.2-4) play a vital role in ammonia assimilation, detoxification and regulation of nitrogen metabolism in plants. Among three enzymes, GDH occupies a key role in plant metabolism.

The ability of plant to acquaint to new environmental conditions depends upon its morphological adaptation and physiological response. In mountainous environment, variation in altitude offers wide variety of environmental conditions. In general, with increase in elevation, stressors such as temperature, pressure, light intensity, rainfall, partial pressure of metabolic gases are known to influence plant metabolism (Woodcock, 1976; Purohit, 1977). Like any other metabolic process, nitrogen metabolism in plants is significantly influenced by variation in the altitude. Increased nitrogen content has been reported in plants with the increase in the altitude (Korner, 1989). This implies that plants which are least influenced by altitudinal changes are much resistant to multiple stressors.

Several workers have compared morpho-physiological response of plants to the change in the altitude (Bhadula *et al.,* 1985; Rajasekaran *et al.,* 1998; Rajasekaran, 2000). Ammonium assimilation

in plants plays an important role in growth and development. Therefore it has been extensively studied in various plant species. However, studies on ammonium assimilation in plants from the various climatic zones have been limited and no studies have been undertaken in Shervaroyan hills, part of Eastern Ghats, Tamil Nadu, India. The present work is an attempt to study growth behavior and analyze the activity of ammonium assimilation enzymes in *R. tetraphylla* L. a perennial medicinal plant, grown at different altitudes in Shervaroyan hills.

Materials and Methods

Seeds of *R. tetraphylla* L. (Apocyanaceae) were sown in farmyard manure and garden soil in a ratio of 1:2 at Salem (250m) to raise seedlings. 40 day-old seedlings were transferred to experimental sites: Salem (250m), Kurumbapatti (400m) and Yercaud (1600m) in Shervaroyan hills of Salem, Tamil Nadu. Experimental plants acclimatized for 60 days at respective sites and growth performance was analyzed (shoot and root length and dry weight). For biochemical analysis, plant samples were frozen in liquid nitrogen after collection. Samples were homogenized in 0.1 M, Tris-HCl pH 7.5 containing 2 mM EDTA and 0.1% -mercaptoethanol, PMSF was added to prevent proteolysis. All the studies were conducted at 4°C. Total soluble protein was estimated using Bradford (1976) method.

Free tissue ammonia level was estimated using method of Chaney and Marbach (1983). GS activity was determined by the method of Truax *et al.* (1994). One hundred µl of supernatant was incubated with 900 µl of assay buffer (0.1 M - imidazole-HCl, 65 mM - L- Glutamate, 4 mM - $MnCl_2$, 0.75 ADP, 33 mM sodium arsenate and 17mM hydroxylamine, pH 6.8) at 30°C for one hour. The reaction was terminated by adding 1ml of stop solution (0.37 M $FeCl_3$, 0.2 M TCA in 0.67 N HCl). After centrifugation, absorbance was determined at 540nm. Glutamylhydroxamate (Sigma Chemical Co, USA) was used to develop standard curve.

A modified method of Dougall (1974) for the assay of NADH-GOGAT and NADH-GDH by following the rate of reduction of NADH at 340nm for 5 min. GOGAT assay mixture contain 20 mM Tricine, pH 7.5, 12.5 mM, α- ketoglutarate, 12.5 mM L-Glutamine and 0.15 mM NADH. Assay mixture for GDH contained 20 mM Tricine, pH 7.5, 1 mM α-ketoglutarate, 1 mM $CaCl_2$, 100 mM NH_4Cl and 0.15 mM NADH.

All the estimations were carried out in triplicate (n = 3) following standard methods.

Results and Discussion

Plant growth, biochemical and enzymes of ammonium assimilation in *R. tetraphylla* were estimated. In *R. tetraphylla* shoot and root length from three different altitudes is summarized in Fig. 1. Shoot length decreased with increase in altitude, while the root length increased with increase in the altitude. Similar observation with regards to the shoot has been reported for different plant species by Nautiyal and Purohit (1980), Rajasekaran *et al.,* (1998), Rajasekaran (2000). Likewise, increase in the root length with increase in the altitude has previously been reported by Bhatt and Purohit (1984) and Pankaj Prasad (1997). This could be due to non-availability of water in higher altitude and other factors such as soil, temperature. Sharma, (1980) reported dwarfism as an environment stress at high altitudes. Decrease in cumulative height and growth rate was steep in low land species than high land species (Todaria, 1980). Whereas shoot and root dry weight increased with the increase in the altitude (Fig 2). This could be due to higher concentration of carbon dioxide in the atmosphere (Purohit, 1998).

Fig. 1. Changes in shoot-root growth patterns of *R. tetraphylla* acclimatized at different altitudes.

Fig. 2. Changes in shoot-root dry weight of *R. tetraphylla* acclimatized at different altitudes

Total soluble protein (TSP) content in the shoot decreased with increasing altitude while reverse trend was observed in the root system (Fig 3). However, free tissue ammonia (FTA) in the shoot and the root system of *R. tetraphylla* increased with increasing altitude (Fig 4). Interestingly, the FTA level in the shoot was comparatively high than the root system. In roots maximum FTA was recorded at high altitude and minimum at low altitude. The present observations are in agreement with previous studies on *Selinum vaginatum* (Rajasekaran, 2000; Rajasekaran et al., 2009).

Fig. 3. Changes in TSP of shoot and root of *R. tetraphylla* acclimatized at different altitudes.

Specific and total activities of GS are presented in Fig 5-6. GS specific and total activities of both the parts showed an inverse relation with the increasing altitude. Maximum activity was observed at the low and minimum at high altitudes. Variation in activity of GS under certain environmental conditions has been attributed to reassimilation of ammonia released during photorespiration (McNally *et al.,* 1983; Wallsgrove *et al.,* 1983). However, seasonal variation in GS of temperate deciduous tree leaves strongly indicated that decline in light intensity and temperature in late season accounted for drop in GS activity (Pearson and Ji, 1994), similar has already been reported in

G. max and *S. vaginatum* (Rajasekaran *et al.,* 1998; Rajasekaran, 2000; Rajasekaran *et al.,* 2009). Likewise, decrease in GS activity under water stress in *Albizzia stipulata* and *Oeugenia dalbergioides* indicate that GS in both the plants is sensitive to water stress (Pankaj Prasad, 1997; Purohit, 1998), although in some plant species GS has been insensitive to water stress (Becana *et al.,* 1984). FTA accumulation reflected in decreased GS activity in shoot and root (Figs. 4-6). Kamachi *et al.*, (1992) reported that environmental conditions may induce FTA accumulation.

Fig. 4. Changes in shoot- root FTA levels of *R. tetraphylla* acclimatized at different altitudes

Fig. 5. Changes in shoot -root GS specific activity of *R. tetraphylla* at different altitudes.

that may affect GS activity at high altitude. This is in accordance with previous reports (Cren and Hiral, 1999; Rajasekaran, 2000). This could be due to the fact that GS is less effective under high ammonia accumulation

Fig. 6. Changes in shoot-root GS activity of *R. tetraphylla* acclimatized at different altitudes.

Both, specific and total activity of NADH-GOGAT at three different altitudes is shown in Fig 7-8. NADH-GOGAT specific and total activity in shoot and root followed similar trend as in GS. NADH-GOGAT activity in shoots showed similar trend in low and middle altitudes. A decrease of 31% was observed at high altitude. Likewise, with the increase in the altitude decrease in shoot and root GOGAT specific and total activity was observed (Fig. 7-8). This indicates that FTA content in the tissues has complex regulatory effects on GOGAT activity as several stresses have been reported to be operative along different altitudes (Bhadula and Purohit, 1994; Pankaj Prasad, 1997; Purohit, 1998; Rajasekaran, 2000; Rajasekaran et al., 2009). Specific and total activities of GDH-amination in *R. tetraphylla* (shoot and root) showed a positive correlation with increase in altitude (Fig. 9-10).

Fig. 7. Changes in shoot-root GOGAT specific activity of *R. tetraphylla* at different altitudes.

Fig. 8. Changes in shoot-root GOGAT activity of *R. tetraphylla* acclimatized at different altitudes.

Increased NADH-GDH activity has been reported along an altitudinal gradient (Pankaj Prasad, 1997; Rajasekaran *et al.,* 1998; Rajasekaran, 2000). However, increased GDH activity is an indicator of detoxification of ammonia released during the breakdown of proteins and amino acids (Becana *et al.,* 1984). In the present investigation, GDH-amination activity increased with the increase in the altitude (Fig. 9-10). Srivastava and Singh, (1987) reported that GDH pathway is active under certain nutritional and environmental conditions. Although, GS/GOGAT pathway is predominant in ammonia assimilation in higher plants, plants do switch over to GDH pathway under certain conditions of low energy and high ammonia (Yamaya, 1999; Rajasekaran *et al.,* 1998; Rajasekaran, 2000; Rajasekaran et al., 2009).

Fig. 9. Changes in shoot-root GDH specific activity of *R. tetraphylla* at different altitudes.

Fig. 10. Changes in shoot-root GDH activity of *R. tetraphylla* acclimatized at different altitudes.

Specific activity of GS decreased at high altitude (Fig. 5), whereas at these altitudes GDH specific activity increased (Fig. 9). GDH plays a complementary role to GS/GOGAT cycle in synthesis of glutamate (Srivastava and Singh, 1987; Rajasekaran *et al.,* 1998; Rajasekaran, 2000) or could catalyze the oxidation of glutamate to provide carbon-skeletons to the TCA cycle. At high altitude, FTA levels also were found to increase (Fig. 4). Increased levels of ammonia may have resulted in increase in *de novo* synthesis of GDH (Srivastava and Singh, 1987; Loulakakis and Angelakis 1990a; Watanebe *et al.,* 1992). This may be one of the reasons for enhancement of GDH activities at high altitudes. However, investigations on isoforms, isoform patterns of ammonium assimilatiory enzymes, *in-vitro* studies using inhibitors of ammonium assimilatory enzymes along an altitudinal gradient is required to elucidate mechanism for this behavior.

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References

Becana M, Aparicio-Tejo PM and Sanchex-Diaz M. 1984. *Plant Physiol.,* 61:653.

Bhadula SK and Purohit AN. 1994. Adaptational strategies of plants at high altitudes and future prospects for the conservation of biodiversity. *Adv Plant Sci Res* 1:1-24.

Bhadula, SK, Pandey, OP and Purohit, AN. 1985. Thermostbility and energy of activation of P-glycollate phosphatase and 3-PGA phosphatase in *Selinum vaginatum* Clarke grown at two altitudes *Photosynthetica* 19(3):417-420.

Bhatt, RM and Purohit, AN. 1984. Morpho-physiological behaviour of two *Anaphalis* species from contrasting environments along and altitudinal gradient *Ind J Plan Physiol*, 27(2):130-137.

Bradford, MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem,* 72:248-257.

Chaney, AL and Marbach, EP. 1983. Modified reagents for determination of urea and ammonia. *Chemical Biochem,* 8:130-132.

Cren, M and Hiral, B. 1999. Glutamine synthetase in higher plants: regulation of gene and protein expression from organ to cell. *Plant Cell Physiol,* 40:1187-1193.

Dougall, DK 1974. Evidence for the presence of glutamate synthase in extracts of carrot cell cultures *Biochem Biophys Res Comm* 58:639-646.

Kamachi, K, Yamaya, T, Hayakawa, T, Mae, T and Ojima, K 1992. Changes in cytosolic glutamine synthetase polypeptide and its mRNA in a leaf blade of rice plants during natural senescence. *Plant Physiol* 98:1323-1329.

Korner, CH 1989. The nutritional status of plants from high altitudes, a worldwide comparison *Oecologia* 81:379-391.

Loulakakis, CA and Roubelakis-Angelakis, KA 1990. Immuno-characterization of NADH-glutamate dehydrogenase from *Vitis vinifera* L. *Plant Physiol,* 94:109-113.

Magalhaes, JR and Huber, DM 1989. Ammonium assimilation in different plant species as affected by nitrogen form and pH control in solution culture *Fert Res* 21:1-6.

McNally, SF, Hirel, B, Gadal, P, Mann, AF and Stewart, GR 1983. Glutamine synthetase of higher plants. Evidence for a specific isoform content related to their possible physiological role and their compartmentation within the leaf. *Plant Physiol* 72:22-25.

Miflin, BJ and Lea, PJ 1980. Ammonium assimilation. In: *The Bio-chemistry of Plants. A*

comprehensive treatise: Amino acids and their derivatives (Eds PK Stumpf and EE Conn). 5:169-202. Academic Press. NY.

Nautiyal, BP and Purohit, AN 1980. Morphophysiology of C_3, C_4 and CAM plants at different altitudes. *Indian J Plant Physiol* 23:220-230.

Pankaj Prasad 1997. Eco-physiological aspect of fertilizer application and uptake in some nitrogen fixing tree species during early growth. *DPhil Thesis* HNB Garhwal University Srinagar (Garhwal).

Pearson J and Ji YM 1994. Seasonal variation of leaf glutamine synthetase isoforms in temperate deciduous trees strongly suggests different functions for enzymes *Plant Cell and Environ* 17:1331-1337.

Purohit, AN 1977. Exploratory survey of floristic pattern in Garhwal Himalayas and a possible adaptability mechanism. *The Himalaya* 1:14.

Purohit, I 1998. Eco-physiology and biochemistry of nitrogen fixing tree species of Garhwal Himalaya. *D Phil Thesis* HNB Garhwal University Srinagar (Garhwal)

Rajasekaran, C 2000. Changes in the activities of enzymes of ammonium assimilation in plants along an altitudinal gradient. *DPhil Thesis* HNB Garhwal University. Srinagar.

Rajasekaran, C, Kalaivani, T, Jayakumararaj, R, Abhijeet Singh, Pusalkar VR and Marimuthu R. 2009. Studies on the Impact of Altitudinal Gradient on Ammonium Assimilatory Metabolism in *Glycine max* L. (Fabaceae) *Ethnobotanical Leaflets*.

Rajasekaran C, Kuniyal CP, Prasad P and Bhadula SK 1998. Growth and ammonium assimilation in *Glycine max* L. and *Selinum vaginatum* Clarke. Seedlings growth at three different altitudes of Garhwal Himalaya *Physiol Mol Biol Plants* 4:33-37.

Sharma MC 1980. Growth, development and productivity of *Glycine soja*, *Dolichos lablab* and *Dolichos biflorus* at different altitudes in Garhwal Himalayas *DPhil Thesis*, HNB Garhwal University. Srinagar (Garhwal).

Srivastava HS and Singh RP 1987. Role and regulation of L-glutamate dehydrogenase activity in higher plants. *Phytochem* 26(3):597-610.

Todaria NP 1980. Growth behaviour and functional dynamics of plant species from different altitudes in Garhwal Himalaya. *DPhil Thesis* HNB Garhwal University, Srinagar (Garhwal).

Truax B, Lambert F, Gagnon D and Chevrier N 1994. Nitrate reductase and glutamine synthetase activities in relation to growth and nitrogen assimilation in red oak and red ash seedlings; effects of N-forms, N concentration and light intensity. *Trees* 9:12-18.

Wallsgrove RM, Keys AJ, Lea PJ and Miflin BJ 1983. Photosynthesis, Photorespiration and nitrogen metabolism. *Plant Cell Environ* 6:301-309.

Watanabe M, Nakayama H, Watanabe Y and Shimada N 1992. Induction of a specific isoenzyme of glutamate dehydrogenase during isolation and the first 48h of culture of *Brassica napus* leaf protoplasts. *Physiol Plant* 86:231-235.

Woodcock RG 1976. *Weather and Climate*. M & E Hand Book. Eatover, Plagmouts.

Yamaya T 1999. Molecular physiology of nitrogen utilization in plants. *Jap J Soil Sci Plant Nutr* 70:255-258.

Yoneyama T and Kumazaya K 1974. A kinetic study of the assimilation of ^{15}N-labelled ammonium in rice seedling roots. *Plant Cell Physiol* 15:655-561.