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BRIEF COMMUNICATION

Altitude-related changes in activities of carbon metabolism enzymes in *Rumex nepalensis*

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Abstract

Activities of some enzymes related to carbon metabolism were studied in different ecotypes of *Rumex nepalensis* growing at 1 300, 2 250, and 3 250 m above mean sea level. Activities of ribulose-1,5-bisphosphate carboxylase/oxygenase, phospho*enol*pyruvate carboxylase, aspartate aminotransferase, and glutamine synthetase increased with altitude, whereas activities of malate dehydrogenase, NAD-malic enzyme, and citrate synthase did not show a significant difference with change in altitude.

Additional key words: aspartate aminotransferase; citrate synthase; glutamine synthetase; malate dehydrogenase; NAD-malic enzyme; phospho*enol*pyruvate carboxylase; ribulose-1,5-bisphosphate carboxylase/oxygenase.

Plants display enormous plasticity to survive under changing environmental variables with altitude (Clements *et al.* 1950, Billings *et al.* 1961, Tranquillini 1964). This plasticity in species response to environmental variables may involve changes at anatomical, morphological, physiological, and biochemical levels to enable plants to combat 'harsh' climatic conditions at high altitude and maintain a reasonably efficient carbon harvesting system to compensate for the relatively short growing period (Tranquillini 1964, Larcher 1995, Purohit 2003). Plasticity in each of these responses could be of special significance under specific environment. Morphological plasticity is related to high competition in productive environments, whereas species acclimate through physiological plasticity in unproductive environments (Cordell *et al.* 1998).

Mountain plants evolved in response to their particular altitude environment differ in physiological response to the respective ecotypes from lowland areas (Billings *et al.* 1961, Hiesey *et al.* 1971, Mächler *et al.* 1977, Körner and Diemer 1987, Cordell *et al.* 1998, Hovenden and Schimanski 2000, Hovenden and Schoor 2003, Kumar *et al*. 2005, 2006, Vats and Kumar 2006). However, there is little information on altitude related changes in enzymatic activities related to carbon metabolism except for enzymes such as ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPCO). Higher RuBPCO activity was reported in high altitude ecotypes of *Selinum vaginatum* (Pandey *et al.* 1984). Lower activation state of RuBPCO but higher total carboxylase activity in barley, pea, and wheat at high elevation suggested responsiveness of the enzyme to low partial pressures of CO_2 at high altitude (Kumar *et al.* 2004). While low temperature could stimulate RuBPCO activity in C_4 plant *Atriplex* (Osmond *et al.* 1982), neither high altitude nor chilling could enhance RuBPCO capacity in C_4 plants *Bouteloua gracilis* and *Muhlenbergia montanum* (Pittermann and Sage 2000, 2001). Increased phospho*enol*pyruvate carboxylase (PEPC) activity was reported in C_3 species *Glycine soja* with increase in elevation from about 500 to 3 650 m, though the implication of this fact was not discussed (Pandey and Purohit 1980). Earlier, we reported

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Abbreviations: Asp – aspartate; AspAT – aspartate amino transferase; CS – citrate synthase; GS – glutamine synthetase; MDH – malate dehydrogenase; NAD-ME – NAD-malic enzyme; NADP-ME – NADP-malic enzyme; PEPC – phospho*enol*pyruvate carboxylase; RuBPCO – ribulose-1,5-bisphosphate carboxylase/oxygenase.
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that crop plants like barley and wheat when grown at high altitude significantly increased carboxylase and oxygenase activities of RuBPCO and activities of PEPC, aspartate aminotransferase (AspAT), and glutamine synthetase (GS) as compared to those grown at low altitude (Kumar *et al.* 2006). The objective of the present study was to find the response of these enzymes in altitudinal ecotypes of wild species, using *Rumex nepalensis* as the target plant that has its natural distribution spread along a wide altitudinal gradient in Himalaya (Bahar 2002).

Leaf samples of *R. nepalensis* were collected for enzymatic studies from three different altitudinal locations, *viz.* Palampur (1 300 m, 32°06'32"N, 76°33'43"E), Palchan (2 250 m, 32°17'41"N, 77°10'76"E), and Marhi (3 250 m, 32°20'47"N, 77°13'17"E). Fully developed young leaves were harvested between 09:00 and 10:00 h on a clear sunny day and stored in liquid nitrogen for further use. All the assays were performed in the Institute's laboratory at Palampur. The range for photosynthetic photon flux density varied from 1 500–1 700, 2 000–2 300, and 2 200–2 500 μmol m^{-2} s^{-1} for Palampur, Palchan, and Marhi, respectively. Mean monthly day temperatures during the month of data recording at these localities were 19.2±2.2, 17.5±1.7, and 12.2±3.2 °C, respectively.

For enzyme assays of RuBPCO, PEPC, NADP-malic enzyme (NADP-ME), and NAD-malic enzyme (NAD-ME), frozen leaf samples were ground with a mortar and pestle in extraction buffer containing 50 mM Tris-Cl buffer (pH 7.5), 1.0 mM $MgCl_2$, 5.0 mM dithiothreitol (DTT), 1.0 mM phenylmethylsulfonyl fluoride (PMSF), 0.02 g cm^{-3} polyvinylpyrrolidone (PVP), 10 % (v/v) glycerol, and 0.1 % (v/v) *Triton X-100*. The extract was centrifuged at 12 000×*g* for 10 min at 4 °C, and the supernatant was used for the determination of enzyme activity. For estimating total carboxylase activity of RuBPCO, the enzyme was activated using 20 mM $NaHCO_3$ and 10 mM $MgCl_2$ in 50 mM Tris-Cl (pH 8.0) for 10 min and assayed by measuring the incorporation of acid-stable ^{14}C from $NaH^{14}CO_3$ as described by Pierce *et al.* (1982). Oxygenase activity was monitored by measuring O_2 uptake using an oxygen electrode (*Hansatech*, UK) at 25 °C. All the solutions were saturated with O_2 using a CO_2 free-O_2 gas. The activated enzyme was assayed in the medium containing 100 mM Bicine buffer (pH 8.2), 0.2 mM EDTA, 0.5 mM RuBP, and 20 mM $MgCl_2$ (Pierce *et al.* 1982). PEPC was assayed spectrophotometrically at 340 nm in the presence of excess malate dehydrogenase (MDH) and lactate dehydrogenase (Ashton *et al.* 1990). The reaction mixture, final volume 0.5 cm^3, contained 50 mM Tris-Cl (pH 8.0), 5 mM $MgCl_2$, 5 mM DTT, 1 mM $NaHCO_3$, 5 mM glucose-6-phosphate, 0.2 mM NADH, 2 units of MDH (*Sigma*, USA), 0.1 units of lactate dehydrogenase (*Sigma*, USA), and crude extract. The reaction was initiated by the addition of 5 mM phospho*enol*pyruvate. NADP-ME was assayed spectrophotometrically by following $NADP^+$ reduction at 340 nm (ε = 6 200) as detailed by Ashton *et al.* (1990). The assay mixture contained 50 mM Tricine-KOH buffer (pH 8.3), 5 mM malate, 0.1 mM EDTA, 2 mM $MgCl_2$, and crude extract. Reaction was started by the addition of 0.5 mM NADP.

NAD-ME was assayed in the direction of malate decarboxylation by measuring NADH formation at 340 nm (ε = 6 220) as detailed by Ashton *et al.* (1990). Reaction mixture contained 50 mM HEPES-KOH buffer (pH 7.2), 5 mM malate, 4 mM $MnCl_2$, 0.2 mM EDTA, 5 mM DTT, 0.1 mM coenzyme A, and crude extract. The reaction was initiated by the addition of 2 mM NAD. Extraction buffer for AspAT consisted of 200 mM Tris-Cl buffer (pH 7.5), 2.0 mM EDTA, and 20 % (v/v) glycerol. The enzyme was assayed in an MDH-coupled reaction essentially as described by Ireland and Joy (1990). GS was extracted in the grinding medium containing 50 mM Tris-Cl buffer (pH 7.8), 1 mM EDTA, 10 mM $MgSO_4$, 5 mM sodium glutamate, 10 % (v/v) glycerol, and insoluble PVP (2 % m/v). Enzyme assay was performed as described by Lea *et al.* (1990) and the activity was calculated from the standard curve prepared with c-glutamylhydroxamate. Citrate synthase (CS) was extracted in 250 mM of Tris-Cl buffer (pH 8.0) and 0.5 M sucrose and assayed as described by Bogin and Wallace (1969). Extinction coefficient for mercaptide (ε = 13 600) was used to calculate the enzyme activity. MDH was extracted in 0.2 M potassium phosphate buffer (pH 7.4) and assayed by monitoring oxidation of NADH (ε = 6 220) as described by Davies (1969).

Carboxylase activity of RuBPCO increased with increase in altitude and was higher by 80 and 170 % at Palchan and Marhi, respectively, than in plants grown at Palampur (Fig. 1*A*). Similar trend was shown by oxygenase activity of RuBPCO (Fig. 1*H*). PEPC activity also showed similar trend with almost double activity at Marhi as compared to Palampur (Fig. 1*B*). Three out of four probable enzymes of oxaloacetate (OAA, a product of PEPC catalysed reaction) metabolism, viz. MDH, NAD-ME, and CS exhibited lower activities at Palchan and Marhi compared to Palampur (Fig. 1*C–E*). The fourth probable enzyme of OAA metabolism, AspAt, showed higher activity by 144 and 819 % at Palchan and Marhi compared to Palampur (Fig. 1*F*). The enzyme involved in nitrogen metabolism, GS, exhibited higher activity by 43 and 57 % at Palchan and Marhi as compared to Palampur (Fig. 1*G*).

RuBPCO activity, which is associated with the capacity of carbon fixation, may increase with altitude (Chabot *et al.* 1972). Temperature drops consistently with altitude and may impart a major impetus to shape leaf's photosynthetic response at high elevation. Response of RuBPCO to temperature is largely explained by the function of its activating enzyme RuBPCO activase, which has a low temperature optimum (Robinson and Portis 1989, Crafts-Brandner *et al.* 1997). RuBPCO activase is instrumental in maintaining high RuBPCO activity at low temperatures (Pearcy 1977).

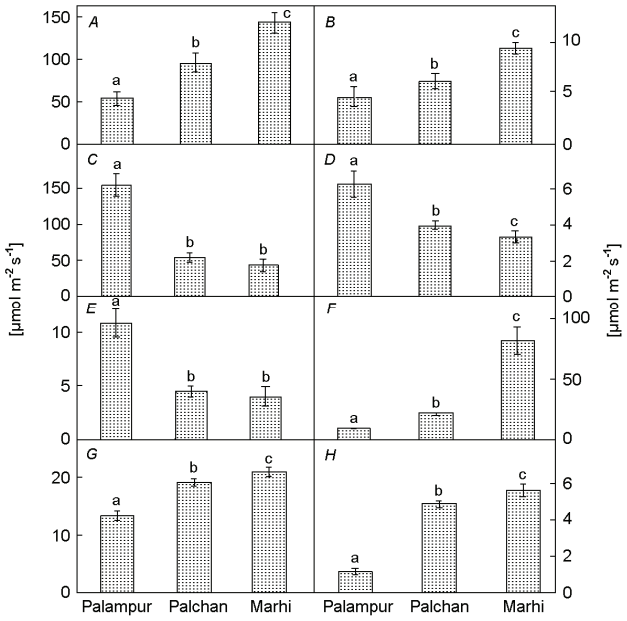

Fig. 1. Activities of ribulose-1,5-bisphosphate carboxylase/ oxygenase (*A* – carboxylase and *H* – oxygenase), phospho-*enol*pyruvate carboxylase (*B*), malate dehydrogenase (*C*), NAD dependent malic enzyme (*D*), citrate synthase (*E*), aspartate aminotransferase (*F*), and glutamine synthetase (*G*) in *Rumex nepalensis* at Palampur (1 300 m), Palchan (2 250 m), and Marhi (3 250 m). Means ± SE ($n = 4$). *Values indicated by different letters above the bar* show significant difference at $p<0.05$.

RuBPCO is also a good measure of capacity and efficiency of leaf photosynthesis under high irradiance (Walters 2005). Leaves acclimated to high irradiance are less susceptible to photoinhibition or photodamage, and the advantage arises largely due to increased electron transport, increased capacity to assimilate, or capacity to dissipate energy through various mechanisms including enhanced photorespiration (Streb *et al.* 1998, Savitch *et al.* 2000). High irradiance in combination with low temperature have the potential to induce chronic photoinhibition of photosystem 2 (Allen and Ort 2001), and have exclusive significance in high altitude locations where plants are mostly exposed to this combination of stresses. Photorespiration is a useful strategy at high elevation for barley and wheat, which showed increased oxygenase activity of RuBPCO with altitude (Kumar *et al.* 2006). Similar response of enhanced oxygenase activity of RuBPCO in *R. nepalensis* suggests its possible role to protect against photooxidative damage.

We found in *R. nepalensis* an increase in activity of yet another carboxylating enzyme, PEPC, at high altitude. This is in contrast to reported decrease in PEPC activity with altitude in C_3 plant *Fagopyrum esculentum* (Pandey and Purohit 1980) and *Salsola australis* (Pyankov *et al.* 1997), suggesting that where it increased, it may be related to an altered carbon metabolism for optimal photosynthetic performance at high altitude. PEPC probably could play an important role in capturing environ'-mental or photorespired CO_2 at high altitude in C_3 plants (Kumar *et al.* 2006).

PEPC in C_3 plants can provide carbon skeleton for synthesis of aspartate, malate, or citrate (Melzer and O'Leary 1987). Since activities of enzymes MDH, NAD-ME, and CS did not increase along with altitude, it was likely that CO_2 uptake catalyzed by PEPC, supported by its enhanced activity, lead to formation of AspAt at high altitude. The higher GS activity of *R. nepalensis* at high altitude suggested the role whereby it supported the reactions catalysed by enhanced AspAT activity by way of regenerating glutamate as a donor of amino group leading to Asp synthesis (Kumar *et al.* 2006). Higher RuBPCO oxygenase activity, indicative of photorespiratory cycle, could be a possible source of ammonia for GS catalysed reaction. These enzymatic alterations could provide adaptive advantage to plant in order to conserve carbon and nitrogen at high elevation.

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