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Antioxidative responses to different altitudes in *Plantago major*

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Abstract

The contents of antioxidants were investigated in leaves and in roots of *Plantago major*, which were collected from different altitudes of Mahan Mountain. A strong correlation between antioxidant content and elevation was observed. As altitude increased, the content of glutathione (GSH) increased both in leaves and in roots, while the activities of guaiacol peroxidase (POX) and superoxide dismutase (SOD) increased only in roots, and declined in leaves. Analyses of isozyme of POX and SOD showed that at high altitude (3300 m) the POX isozyme 5 and the SOD isozyme 8 were induced in roots, but not in leaves. It seemed that the POX isozyme 5 and the SOD isozyme 8 were required for adaptation as the oxidant level increased with lower temperature and higher light intensity as altitude increased. There were no significantly meaningful differences in SOD isozyme profiles and POX isozyme profiles in leaves among three altitudes. These results indicated that *Plantago major* could not grow at much higher altitude than 3300 m. The present study also suggested that high altitude had different effects on antioxidant system in leaves and in roots.

Keywords: Adaptation; Alpine plant; Altitude; Antioxidative system; Isoenzyme; *Plantago major*

1. Introduction

Elevational limits on plant distribution have long been attributed to specific physical factors of the alpine environment, which are characterized by short, often cold and unpredictable growing seasons.

At increasing altitudes, plants are exposed to decreasing mean temperatures and increasing light intensities, so they must have developed mechanisms, by which to prevent damage caused by chilling, by freezing or by photodestruction. Photosynthesis in high alpine plants seems to be adapted to these conditions, as has been found in the field by Larcher and Wagner (1976) and Körner and Diemer (1994). The study on *Carex curvula* showed that the light environment in a canopy was more important than temperature for CO_2-dependent photosynthetic yield. There normally seems to be no impairment of photosynthesis by low temperature and high light intensity under field conditions, which indicated a very well

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regulated system of carbohydrate turnover together with a functional system of antioxidants (Körner, 1982). Numerous ecophysiological studies on alpine plants described regeneration of metabolism and photosynthetic activities after climatic extremes, which seems to occur much faster in alpine vegetation than in cold-exposed and photoinhibited cultivated plants such as *Hordeum vulgare* or *Spinacia oleracea*. Recently, Wildi and Lütz (1996) reported that the leaves of high alpine plant species contained enhanced amount of antioxidants to protect them against oxygen radicals which should be increased with increasing altitude.

Mountain climate can easily change to cold conditions, which potentially result in photoinhibition or photodestruction (Somersalo and Krause, 1990). It has been suggested that chilling and increasing light intensities caused elevated levels of active oxygen species (Knox and Dodge, 1985; Wise and Naylor, 1987; Prasad et al., 1994; Hodges et al., 1997), and the production of active oxygen exceeded the capacity of the scavenging systems, resulting in oxidative damage. Plants, as well as other organisms, have evolved a complex antioxidative system in order to protect cellular membranes and organelles from the damaging effects of toxic concentrations of activated oxygen species (Salin, 1988; Walker and McKersie, 1993). Essentially, antioxidant defenses falls into two general classes, including: (1) low molecular weight antioxidants which consists of the lipid soluble membrane-associated antioxidants (e.g. α-tocopherol and β-carotene) and the water soluble reductants (e.g. glutathione and ascorbate); and (2) enzymic antioxidants (e.g. superoxide dismutase (SOD; EC1.15.1.1), catalase (CAT; EC1.11.1.6) and enzymes of the ascorbate/glutathione cycle; reviewed by Foyer et al. (1991)). SOD is a group of enzymes, which scavenges in cytosol, chloroplasts, and mitochondria. CAT is the primary H_2O_2 scavenger in the glyoxysomes, peroxisomes and mitochondria (Prasad et al., 1994, 1995), and H_2O_2 generated in the chloroplasts is removed from biological systems by the various forms of peroxidase through an ascorbate-glutathione cycle (Foyer and Halliwell, 1976; Gossett et al., 1994). In this system, ascorbate is oxidized to the monodehydroascorbate radical in the presence of H_2O_2 by ascorbate peroxidase (APX; EC 1.11.1.11) (Hossain et al., 1984). This radical can regenerate at the expense of NADPH, and dehydroascorbate reductase (DHAR; EC 1.8.5.1) regenerates ascorbate utilizing GSH to form GSSG (Jahnke et al., 1991). Subsequent GSH regeneration occurs by glutathione reductase (GR; EC 1.6.4.2.) in another NADPH-requiring reaction (Halliwell and Foyer, 1978). Ultimately, this cycle results in H_2O_2 being reduced by photosynthetically generated NADPH (Salin, 1988). Although this cycle is known to be responsible primarily for H_2O_2 scavenging in chloroplasts, its importance in the cytosol and other nonphotosynthetic tissues is also becoming apparent (Alscher, 1989). These low molecular weight antioxidants and the enzymatic antioxidants that synthesize and recycle them, have been associated with the overall tolerance and ultimate survival of a plant during periods of environmental stress (Salin, 1988; Foyer et al., 1991; Elstner, 1982). It was hypothesized that alpine plants might have developed protective mechanisms against low temperatures combined with high light intensities (e.g. high UV-B radiation). Moreover, the adjustments of enzymic activities and antioxidant levels in roots might be different from that in leaves.

To add more evidence to the excellent ecophysiological studies already carried out on high alpine plant adaptation, it is of importance to understand the molecular, biochemical and physiological means which protect high alpine plants from such environmental adversities.

In the present study, glutathione, SOD and POX were analyzed in order to show: (a) the changes of antioxidative system as altitude increases; (b) adaptation of the plant to high altitude environmental situation; and (c) what role does the antioxidant system play in high alpine plants in protecting them from environmental stress. To the authors' knowledge, the changes of antioxidative system of high alpine plant species have only been examined by Wildi and Lütz (1996) recently, and the changes of antioxidative system in roots of alpine plants have not been examined before.

2. Materials and methods

2.1. Plant material and study sites

The sampling sites, Mahan Mountain, near Lanzhou City, range in altitudes between 1500 and 3300 m. The climate at all the three sites is summarized in Table 1. These sites differ mainly in temperature. The length of the growing season of *Plantago major* L. varies from 8 months at 1600 m to about 7 and 5 months at 2600 and 3300 m, respectively.

For antioxidant assays and enzyme activity measurements, three samples, each consisting of five to seven youngest, fully expanded leaves and roots (0.5 g fresh weight) were collected from fully green plants in September. The samples were frozen in liquid N_2 immediately at the sites and taken to the laboratory for further analysis.

2.2. Determination of lipid peroxidation and GSH

Lipid peroxidation was determined according to the method of Dhindsa and Matowe (1981). This involved measuring the amount of malondialdehyde (MDA), a product of lipid peroxidation, by the thiobarbituric acid (TBA) reaction. Frozen samples (0.5 g) of leaf and root was ground in 10 ml distilled water using a pestle and mortar and a small amount of sand. Ten ml 0.5% TBA in 20% trichloroacetic acid were added. The mixture were heated at 95°C for 30 min and then quickly cooled in an ice-bath. After centrifuging at $10\,000 \times g$ for 10 min the absorbance of the supernatant at 532 nm was read and the value for non-specific absorption at 600 nm was subtracted. The concentration of MDA was calculated using its extinction coefficient of 155 mM^{-1} cm^{-1} (Heath and Packer, 1968).

GSH concentrations were measured by a modification of the method outlined by Ellman (1959). Frozen leaf tissue (0.5 g) and root tissue was homogenized with 3.75 ml of ice-cold 3% trichloroacetic acid (pH 7.0) containing 0.2 mM EDTA. After centrifugation at $20\,000 \times g$ for 30 min at 4°C, the supernatant was used for the analysis of GSH. Each test tube contained 0.25 ml of sample solution, 2.75 ml of phosphate buffer (pH 8.0), and 0.03 ml of 10 mM of dithiobis-2-nitrogenzoic acid (DTNB). The optical densities for each set of test tubes were determined at 412 nm from a standard curve of known concentrations of GSH. A test tube (one for each set) containing all the above ingredients and concentrations, but without the reagent DTNB, was used as blank.

2.3. Measurement of enzyme activities and protein analysis

Total POX activity was measured spectrophotometrically by monitoring the formation of tetraguaiacol ($\varepsilon = 26.6$ mM^{-1} cm^{-1}) from guaiacol at A_{470} in the presence of H_2O_2 as described by Chance and Maehly (1955). Total SOD activity was determined spectrophotometrically by measuring the inhibition of the O_2^- dependent reduction of cytochrome *c* at A_{550}, according to the method of McCord and Fridovich (1969). One unit of SOD was defined as the quantity of enzyme required to inhibit the reduction of cytochrome *c* by 50% in a 1 ml reaction volume.

Protein concentration was determined spectrophotometrically at A_{595} using the method of Bradford (1976) with bovine serum albumin (BSA) as standard.

2.4. Isozymes assay

About 0.5 g of liquid nitrogen powered samples were homogenized with five volumes of 0.1 mol l^{-1} Tris–HCl buffer (pH 7.5) containing 0.1% (v/v) 2-mercaptoethanol, 1 mmol l^{-1} EDTA, 10 mmol l^{-1} KCl, 10 mmol l^{-1} $MgCl_2$, 10% polyvinylpolypyrrolidone (PVP-40T), and 0.2 mol l^{-1} ascorbic acid. The homogenates were centrifuged at $18\,000 \times g$ for 20 min at 4°C, and the supernatant fractions were used for isozyme analysis.

Isozymes were resolved on 1.0 mm thick native polyacrylamide gels according to the method of Laemmli (1970), using a solution containing 25 mmol l^{-1} Tris and 192 mmol l^{-1} glycine (pH 8.3) as the electrode buffer. Electrophoresis was carried out at 15 mA constant current at 4°C till the bromophenol blue dye front reached the bottom (anode) end of the separating gel.

Table 1
Climatic data for the three study sites at Mahan Mountain near Lanzhou City, China[a]

Altitude (m)	Mean annual temperature (°C)	Mean temperature			Annual precipitation (mm)	Snow-free months
		Warmest month (°C)	Coldest month (°C)	In September (°C)		
1600	9.3	22.1	−6.1	15.7	316	April–December
2600	2.8	13.0	−8.6	8.9	623	April–November
3300	0.0	9.6	−10.1	5.3	560	May–October

[a] 1600 m (30 year mean), 2600 m and 3300 m (14 year mean).

Isozymes of POX were separated on nondenaturing polyacrylamide gels (7% T, 2.5% C). After electrophoresis, gels were stained in 100 mmol l^{-1} sodium phosphate buffer, containing 1.61% benzidine hydrochloride, 4.83% ammonium chloride and 0.013% H_2O_2 (Bapat et al., 1992), and then transferred to 7% (v/v) acetic acid to stop the reaction.

SOD isozymes were separated on nondenaturing polyacrylamide gels (10% T, 2.5% C). After electrophoresis, gels were stained with the method similar to that of Anderson et al. (1995). Gels were soaked in 50 mmol l^{-1} sodium phosphate buffer, pH 7.5, containing 2.45 mmol l^{-1} 2,2′-di-*p*-nitrophenyl-5,5′-diphenyl-3,3′-(3,3′-dimethoxy-4,4′-diphenylene) ditetrazolium chloride in darkness for 20 min, followed by soaking in 50 mmol l^{-1} sodium phosphate buffer, pH 7.5, supplemented with 26.5 mmol l^{-1} *N*,*N*,*N*′,*N*′-tetramethylethylenediamine and 26.5 μmol l^{-1} riboflavin in darkness for 40 min. Gels were then exposed to low light (9 μmol m^{-2} s^{-1}) for approximately 1 h, followed by transferred to 1% (v/v) acetic acid to stop the reaction.

The gels after staining for the respective enzymes were scanned on a CS-910 dual-wavelength TLC Scanner (Shimadzu, Japan) at sample 590 nm and reference 750 nm for POX and at sample 750 nm and reference 615 nm for SOD.

2.5. Statistical analysis

Analysis of variance (one-way ANOVA) was performed on experimental data. All statistical analysis was performed using software developed by Statistica/w 5.0 (StatSoft, USA). The data shown are means and standard deviation, S.D., of three independent replicates. The statistical significance for all tests was set at the $P \leq 0.05$ confidence level.

3. Results

3.1. Response of lipid peroxidation and GSH

With increase of altitude, the level of lipid peroxidation (MDA concentration) increased slightly in leaves, but increased significantly ($P < 0.05$) in roots (Fig. 1). At 3300 m, the concentration of MDA in roots of *P. major* was more than 1.6 fold higher than that found at 1600 m. The amount of GSH also increased with the elevation (Fig. 2), both in leaves and in roots; at high altitude it was approximately 1.3 times as high as lower altitude.

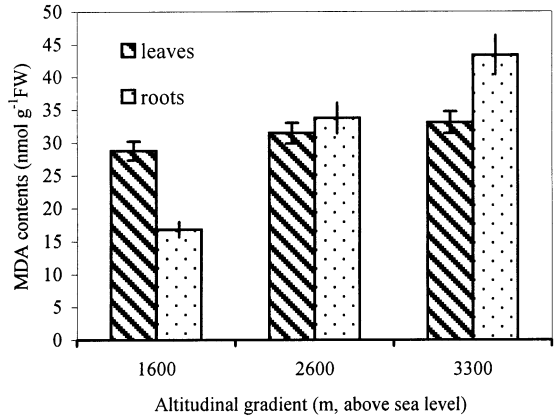

Fig. 1. Concentrations of malondialdehyde (MDA) in the youngest, fully expanded leaf and roots of *Plantago major* from different altitudes. Significance level at $P < 0.05$. Each point represents the mean ($\pm$ S.D.) for three replicates.

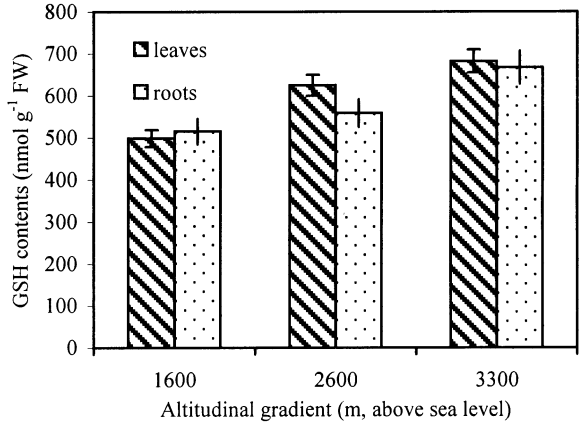

Fig. 2. Concentrations of GSH in the youngest, fully expanded leaf and roots of *Plantago major* from different altitudes. Significance level at $P < 0.05$. Each point represents the mean ($\pm$ S.D.) for three replicates.

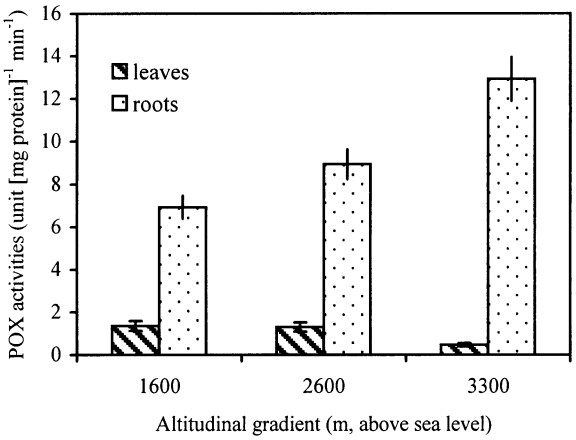

Fig. 3. Activities of POX in the youngest, fully expanded leaf and roots of *Plantago major* from different altitudes. Significance level at $P < 0.05$. Each point represents the mean ($\pm$ S.D.) for three replicates.

3.2. Antioxidant enzymes activities at different altitudes

The activities of SOD, POX in leaves and in roots at three different altitudes were compared. The two enzyme activities responded very differently to the increasing altitude in leaves and in roots. In leaves, POX activities declined with increasing altitude, and the activity of POX at 1600 m was 1.9 fold higher than that at 3300m (Fig. 3). It was interesting that the roots of *P. major* exhibited a significant ($P < 0.05$) increase in the activities of POX with increasing altitude. The POX activity of high altitude (3300 m) was nearly 0.8 fold higher than that of lower altitude (1600 m).

The same phenomenon could also be observed in SOD activity. The activities of SOD declined with increasing altitude in leaves (Fig. 4), but elevated in roots. SOD activity in leaves of high altitude was approximately 1.5 fold lower than that in leaves of lower altitude. But in roots, it elevated approximately 1.9 fold from 1600 to 3300 m.

3.3. POX and SOD isozyme

On non-denaturing polyacrylamide gels, peroxidases exhibited a total of four isozyme forms in leaves and eight isozyme forms in roots (Fig. 5). Isoperoxidase 6, 7 and 8 were expressed both in leaves and in roots of three different altitudes. Their activities decreased with increasing altitude (Fig. 5). Isoperoxidase 1, 2, 3, 4 and 5 were expressed only in roots. The POX isozyme bands 2, 3 and 4 were constitutively expressed in roots of plants at three different altitudes, but isoforms 1 was only expressed in roots at the altitude of 1600 and 2600 m, and disappeared in roots of high altitude (3300 m). However, isoform 5 was only expressed in roots of plants at the highest altitude (3300 m).

SOD revealed a total of seven isoenzymes in leaves and eight in roots, the isoforms 3, 7 and 8 were prominent and the isoforms 1, 4 and 6 were very faint. SOD isozyme bands 1, 2, 3, 5, 6 and 7 were constitutively expressed in leaves and in roots of three altitudes (Fig. 6). Among them, isoforms 3 and 7 were bands with higher enzyme activities, whereas the activities of isoforms 1, 2 and 5 diminished both in leaves and in roots as altitude increased (Fig. 6). The activity of SOD isoform 4 was greatly diminished both in leaves and in roots with increasing altitude, and disappeared at high altitude (3300 m) (Fig. 6). Whereas, isoforms 8, the slowest migrating, appeared only in roots of high altitude. In results, the net effect was the decrease in total SOD activities (Fig. 4).

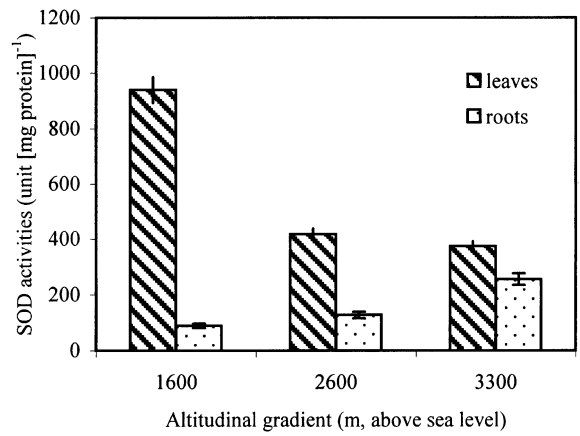

Fig. 4. Activities of superoxide dismutase (SOD) in the youngest, fully expanded leaf and roots of *Plantago major* from different altitudes. Significance level at $P < 0.05$. Each point represents the mean ($\pm$ S.D.) for three replicates.

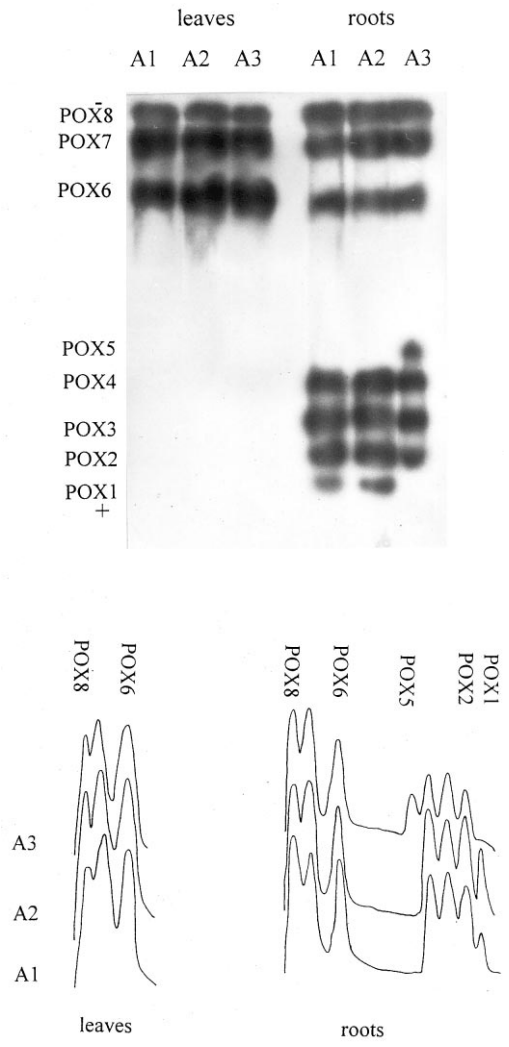

Fig. 5. POX enzyme profile in the youngest, fully expanded leaf and roots of *Plantago major* from different altitude. A1, 1600 m; A2, 2600 m; A3, 3300 m.

4. Discussion

The climatic environments of high alpine ecosystems can be characterized by extreme condition, e.g. low temperature and high irradiation. High alpine plants have evolved adaptation which avoid freezing damage and photodestruction (Lütz, 1996). The present study was intended to increase the understanding of the adaptation and survival mechanisms of alpine plants under natural environmental stress.

GSH is likely to play a protective role under chilling conditions (Anderson et al., 1995), high light conditions, and also under high UV radiation (Smith et al., 1990). Intense irradiation may generate oxygen radicals, such as singlet oxygen, in chloroplasts, which activate a defense system (Knox and Dodge, 1985). However, alpine plants have developed protective mechanisms against chilling and UV-B (Smith et al., 1990). Similar results were observed in the present study that the amount of GSH increased significantly both in leaves and in roots of *P. major* as altitude increased. These results, combined with the increasing MDA level, enable one to conclude that the

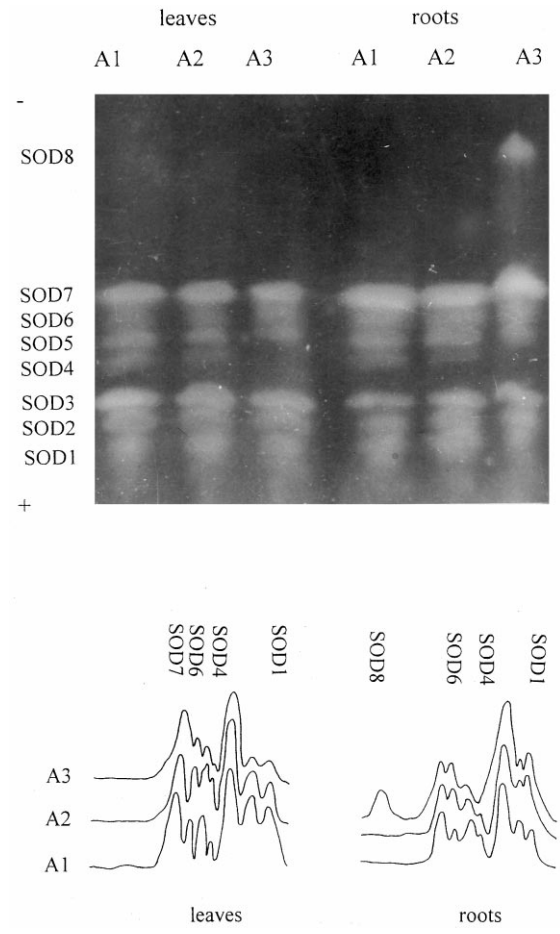

Fig. 6. Superoxide dismutase (SOD) enzyme profile in the youngest, fully expanded leaf and roots of *Plantago major* from different altitude. A1, 1600 m; A2, 2600 m; A3, 3300 m.

accumulation of GSH appeared to be regulated for the purpose of protection as the oxidant level increased with weather changes (e.g. a decrease in temperature, followed by strong irradiation). Grill et al. (1987) observed higher glutathione accumulation in light-exposed needles than in their shaded counterparts. Wildi and Lütz (1996) also found that the total of antioxidants in the leaves of high alpine plants was higher than that in the leaves of plant collected from lower sites.

Measurement of antioxidant enzyme activities in leaves and in roots of *P. major* at three different altitudes demonstrated that a strong correlation between antioxidant enzyme activities and elevation evidently existed. In the present study, activities of antioxidant enzymes increased with increasing altitude in roots. It has been reported that antioxidants and antioxidant enzymes are associated with overall tolerance and ultimate survival of plants during periods of environmental stress (Foyer et al., 1991), almost all kinds of stress can induce or enhance levels of SOD, GR and APX (Walker and McKersie, 1993; Foyer et al., 1994; Anderson et al., 1995; Hodges et al., 1997). But, in this study, the activities of SOD and POX declined significantly in leaves of *P. major* with increasing altitude, especially at 3300 m. The isozymes of POX and SOD were significantly affected by increasing altitude.

P. major is a perennial plant whose leaves scorches and dies in autumn, while the roots can survive during winter. Hence the roots might have developed stronger tolerance to chilling than the leaves. The lower activities of SOD and POX in leaves at 3300 m indicates that *P. major* can not grow much higher at Mahan Mountain because the radical defense system is less effective at higher altitudes. This may be also one of the reasons why *P. major* was not found at altitudes higher than 3300 m. Wildi and Lütz (1996) also reported that the much higher light intensities could even break the higher antioxidant pool as found in acclimated alpine plants.

In addition to higher irradiation and lower temperature, many other factors (increased UV radiation, the sum of average temperature during summer, lower air pressure etc.) also influence the plants at higher altitude. Ozone-stressed spruce trees showed higher accumulation of tocopherol and ascorbate than trees that were not stressed (Schmieden and Wild, 1994).

In conclusion, the present study demonstrated an altitude-dependent change of antioxidants in *P. major*. It can be presumed that the accumulation of antioxidants in roots of *P. major* was a response to light and chilling stress. These results conformed to that of Wildi and Lütz (1996). It was notable that altitude might affect antioxidant component and contents of high alpine plant from different altitudes in a different manner. The effect of altitudinal gradients on leaves and roots might differ with seasons. So further research is required to determine the seasonal changes of antioxidant system in leaves and roots at high elevations.

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