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The relationship between seasonal changes in anti-oxidative system and freezing tolerance in the leaves of evergreen woody plants of *Sabina*

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Abstract

Although the relationships between reactive oxygen species (ROS) and membrane damage, between anti-oxidative enzyme activity and chilling tolerance, and between antioxidant concentration and chilling tolerance are well documented, the mechanisms responsible for survival of evergreen woody *Sabina* plants to survive temperatures below −30 °C are not well understood. In this study, seasonal changes in the activities of the anti-oxidative enzymes superoxide dismutase (SOD), catalase (CAT), peroxidase (POD) and ascorbate peroxidase (APX), as well as levels of ascorbic acid (ASA), glutathione (GSH), proline (Pro), carotenoids (Car) and lipid peroxidation were examined in the leaves of *Sabina przewalskii* Kom. and *Sabina chinensis* (Lin.) Ant. Leaves were sampled at monthly intervals from the summer of 2004 to spring of 2005. Anti-oxidative enzyme activities and antioxidant contents were affected by declining temperatures in the autumn (September to October) and winter (November to January) and increasing temperatures in the spring (February to April). Activities of POD, CAT and APX, as well as contents of ASA, GSH, Car and Pro increased with the decrease in temperature. The highest values were found in the winter. In contrast, the leaf content of thiobarbituric acid-reactive substances (TBARS) increased markedly in the autumn and remained relatively constant during the following winter. In the autumn, presumably in anticipation of acclimation during the winter, leaf freezing tolerance was closely correlated with the activities of anti-oxidative enzymes and contents of antioxidants, indicating that anti-oxidative systems in the leaves played an important role in limiting the production of free radicals to protect membrane integrity. Freezing tolerance in evergreen woody plants of *Sabina* is correlated with an increased capacity to scavenge or detoxify ROS by the anti-oxidative system. ROS accumulated as part of the cold acclimation response may induce anti-oxidative defence systems for the acquisition of freezing tolerance in the leaves. The results also suggested that a better capacity for anti-oxidative defences in *S. przewalskii* might account for its greater capacity for freezing tolerance than *S. chinensis*.

Keywords: Sabina; Anti-oxidative system; Freezing tolerance; Seasonal change

1. Introduction

Leaves of evergreen woody plants experience freeze–thaw cycles in late autumn and throughout the winter, and maintain the potential for re-growth during the following spring. However, seasonal variations in the freezing tolerance of many perennial evergreen woody plants are reported (Silim and Lavender, 1994). During the summer months, needles of Scots pine (*Pinus sylvestris* L.) are lethally damaged when exposed to −10 °C, while in mid-winter, they survive exposure to −80 °C (Beck et al., 2004). Frost hardening and dehardening of a plant are extremely slow processes which cannot be studied like metabolic reactions. The maximum freezing tolerance of plants is not constitutively expressed, but it is induced in response to low temperature exposure (Thomashow, 1998). Therefore, knowledge of the physiological events that occur during cold acclimation will allow better understanding of freezing tolerance in evergreen woody plants leaves. At the cellular level, membranes are directly involved in cold acclimation and freezing tolerance. Plasma membranes are the primary sites of freezing injury (Arora and Palta, 1991). Membrane damage from exposure to

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chilling stress may be mediated by reactive oxygen species (ROS) such as superoxide radicals, singlet oxygen, H_2O_2, and hydroxyl radicals (Kendall and Mckersie, 1989). The accumulation of these ROS initiates lipid peroxidation (Halliwell and Gutteridge, 1990). Although ROS are produced as a by-product of normal cell metabolism, their levels are enhanced by diverse environmental stresses (Allen, 1995; Rao et al., 1996). Plants have evolved several mechanisms to prevent damage from ROS. One of them is to use the enzymatic antioxidant system that includes superoxide dismutase (SOD), catalase (CAT), peroxidase (POD) and ascorbate peroxidase (APX). The other is to use a system of physiological antioxidant molecules, which includes ascorbate (ASA), glutathione (GSH), carotenoids (Car) and tocopherols. Usually, both act in tandem (Bowler et al., 1992; Foyer et al., 1994; Allen, 1995; Rao et al., 1996). Superoxide dismutase, the first enzyme in the detoxifying process, catalyses the transformation of superoxide to hydrogen peroxide (Bowler et al., 1992). The resulting hydrogen peroxide is removed peroxidases (Foyer et al., 1994). Kendall and Mckersie (1989) reported that freeze–thaw injury at the membrane level can be simulated by treatment with the superoxide radical, and that acclimation to chilling stress enhanced the concentration of lipid-soluble antioxidants. Borsos and Blake (2001) reported that seed treatment with the antioxidant Ambiol enhances membrane protection of seedlings exposed to drought and low temperatures. Furthermore, free proline (Pro) accumulation was associated with frost resistance in *Nothofagus dombeyi* leaves (Luis et al., 1986). Proline can scavenge hydroxyl radicals to increase cellular freezing tolerance (Nicholas and Quinton, 1989). Tolerance of adverse environmental conditions is correlated with an increased capacity to scavenge or detoxify ROS. It has been shown that tolerant plants respond to low temperatures by increasing levels of these antioxidant compounds and the activities of anti-oxidative enzymes (Walker and Mckersie, 1993).

Evergreen woody plants of *Sabina* are mainly distributed in alpine habitats of northwest China, and play an important role in water conservation in forest ecosystems. *Sabina przewalskii* grows mainly at altitudes ranged from 2600 m to 4000 m on the Qinghai–Tibet Plateau, where the annual average temperature is about 0.5 °C. It is an ideal material to study plant freezing tolerance. In contrast, *Sabina chinensis* distributes mainly at altitudes ranging from 500 m to 1900 m on plains of the China where the annual average temperature is about 8.5 °C (Feng, 1994; Fu, 1990). For that reason, we used *S. przewalskii* and *S. chinensis* as materials to study the relation of seasonal changes in anti-oxidative system to freezing tolerance in the leaves of *Sabina* by monitoring lipid peroxidation (TBARS content), activities of various anti-oxidative enzymes such as SOD, POD, CAT and APX, and contents of various antioxidants such as GSH, ASA, Pro and Car from autumn to early spring. This period of time included both cold acclimation during autumn and cold de-acclimation in early spring. To our knowledge, this is the first report on seasonal changes of the levels of anti-oxidative systems in northwest China evergreen trees of *Sabina* during winter stress.

2. Materials and methods

2.1. Growth of plants

Seedlings of *S. przewalskii* and *S. chinensis* were established at the experimental station of Lanzhou University, which is located in a temperate semi-arid and semi-humid region (Long, 103°E, Lat 35°N, 1900 m elevation, in central Gansu province, China), in mid-May of 2001. Species were arranged in a randomized complete block design with three replications. Plots were hand-weeded and insect controlled. Mean air temperatures between August 2004 and April 2005 were collected from local weather stations near the research site (Fig. 1).

2.2. Sampling and analysis of material contents

There were four sampling blocks randomly chosen and labeled in each replicated tree plot. Each sample was taken from a labeled sampling block at each sampling time. Leaf samples were collected on the 22nd day of every month from August 2004 to April 2005. All samples were collected between 10:00 am and 12:00 am to avoid the effects of rhythmic phenomena and large temperature differences between day and night on the different variables studied. Part of the harvested fresh leaves was used to test electrolyte leakage immediately; the remaining leaves were frozen in liquid N_2 and transported to the laboratory to determine the activities of various anti-oxidative enzymes, contents of various antioxidants and thiobarbituric acid reactive substances (TBARS).

2.3. Assessment of chilling injury

Electrolyte leakage (EL), determined as described by Zwiazek et al. (1990) with some modifications, was used to assess chilling injury. Leaves (0.2 g) were cut into 0.2-cm segments and placed in a test tube with 10 mL of de-ionized water at 25 °C for 2 h. After the incubation, the conductivity in the bathing solution was determined (C_1). The samples were then heated in boiling water for 20 min. After cooling to room temperature, conductivity was read again in the bathing

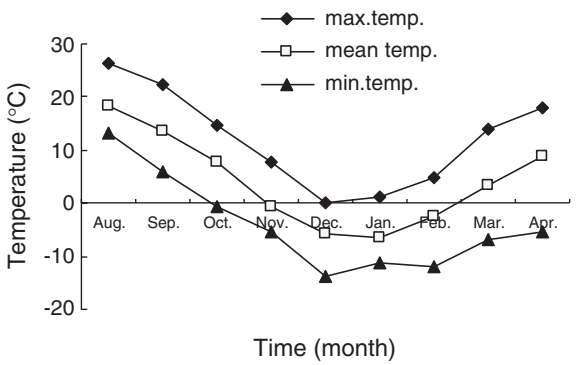

Fig. 1. Seasonal variations of air temperature each month from August 2004 to April 2005 of research site (Long, 103°E, Lat 35°N, 1900 m elevation, in central Gansu province, China).

solution (C_2). Electrolyte leakage was expressed as a percentage of the total conductivity after heating in boiling water ($EL = C_1/C_2 \times 100\%$).

2.4. Determination of TBARS

The extent of lipid peroxidation was estimated by determining the concentration of TBARS in the leaves, according to Cakmak and Horst (1991). Briefly, leaves (1 g) were homogenized in 5 ml 0.1% (w/v) trichloroacetic acid (TCA) and centrifuged at 10,000×*g* for 10 min. An aliquot of 1 ml of supernatant was mixed with 4 ml 0.5% thiobarbituric acid in 20% TCA. The mixture was heated at 95 °C for 30 min, and the reaction was stopped by placing the reaction tubes in an ice-water bath. The samples were then centrifuged for 10 min at 6000×*g*. The non-specific, background absorbance was read at 600 nm and was subtracted from the specific absorbance of the product read at 532 nm. The concentration of TBARS was calculated by using a molar absorptive value of 155 mM^{-1} cm^{-1}.

2.5. Extraction and assay of enzymes

To extract antioxidant enzymes, leaves (0.5 g) were homogenized in 5 ml 50 mM sodium phosphate buffer (pH 7 for catalase and pH7.8 for SOD, POD and APX) containing 1% (w/v) PVP and 0.1 mM Na_2EDTA. The homogenate was filtered through four layers of cheesecloth and centrifuged at 15,000×*g* for 20 min. After centrifugation, aliquots of the supernatant was passed through a Sephadex G-25 (PD-10 column, Amersham Biosciences, Uppsala, Sweden) and used to determine the enzyme activities and protein concentration. All extractions were prepared at 0–4 °C, and enzyme assays were performed at 25 °C.

The SOD activity was measured spectrophotometrically as described by Beyer and Fridovich (1987). In this assay, the activity of SOD was determined, with one unit of SOD being defined as the amount required to inhibit the photoreduction of nitroblue tetrazolium by 50%. The specific activity of SOD was expressed as units mg^{-1} protein.

The CAT activity was assayed by the method of Clairborne (1985). The decomposition of H_2O_2 was followed by a decline in absorbance at 240 nm for 2 min. One unit of catalase converts 1 mmol of H_2O_2 min^{-1}.

The POD activity was determined by the method of Chance and Maehly (1955) using guaiacol as an electron donor.

The APX activity was determined according to the method of Gupta et al. (1993) by measuring the oxidation of ascorbate at 290 nm in 1 ml of solution that contained 50 mM Hepes (pH7.0), 1 mM EDTA, 1 mM H_2O_2 and 25 μl of protein extract. The specific activity of APX was expressed as μmol ascorbate oxidized h^{-1} mg^{-1} protein.

Protein concentration in the enzymatic extraction was measured by the method of Bradford (1976).

2.6. Antioxidant assays

Frozen samples (100 mg FW) were ground in a mortar with liquid nitrogen and soluble metabolites were extracted first with 1 ml of 2.5 N $HClO_4$ and later adjusted with succinate buffer (200 mM, pH 12.7) to reach a pH between 4 and 5 for ascorbate determination, or between 6 and 7 for glutathione determination (Foyer et al., 1995).

An aliquot of 0.2 ml of the extract was then mixed with 0.8 ml of sodium phosphate buffer (pH 5.6). In the same extracts dehydroascorbate was reduced by the addition of DTT (20 mM) in phosphate buffer (100 mM, pH 7.0). Total ascorbate was measured by the change in absorbance at 265 nm after the addition of 10 ml ascorbate oxidase (100 kU l^{-1}), as described by Foyer et al. (1995).

Total glutathione was assayed according to the method of Griffith (1980). This method is based on the sequential oxidation of glutathione by 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) and later reduced again by NADPH in the presence of glutathione reductase (GR). The rate of 2-nitro-5 thiobenzoic acid (TNB) formation was proportional to the glutathione concentration and was monitored at 412 nm and compared with a standard curve. For this assay an aliquot of 0.2 ml of the neutralised extract was mixed with 0.7 ml of NADPH (0.3 mM) in potassium phosphate buffer (20 mM, pH 7.8), 0.1 ml DTNB (6 mM) and 5 ml of GR (200 kU l^{-1}).

Carotenoids were extracted and estimated according to the method described by Arnon (1949).

Table 1
Pearson correlations between anti-oxidative system and mean temperature, between anti-oxidative system and lipid peroxidation, and between anti-oxidative system and EL from August 2004 to January 2005

	Temperature		TBARS		EL	
	S. chinensis	*S. przewalskii*	*S. chinensis*	*S. przewalskii*	*S. chinensis*	*S. przewalskii*
SOD	0.880[a]	0.912[a]	0.579	0.535	0.684	0.432
POD	−0.487	−0.700	−0.388	−0.780	0.415	0.123
CAT	−0.326	−0.242	−0.624	−0.724	−0.486	−0.434
APX	−0.327	−0.774	−0.513	−0.634	0.034	−0.221
ASA	−0.966[b]	−0.940[b]	−0.761	−0.680	−0.449	−0.094
GSH	−0.142	−0.984[b]	−0.220	−0.824[a]	0.416	−0.279
Car	−0.443	−0.894[a]	−0.564	−0.854[a]	0.106	−0.152
Pro	−0.693	−0.954[b]	−0.220	−0.827[a]	0.090	−0.242

[a] Correlation significant at the 0.05 level.
[b] Correlation significant at the 0.01 level.

Table 2
Differences of anti-oxidative system and freezing tolerance between two tree species during early cold acclimation, cold acclimation and freezing stages

		TBARS	SOD	CAT	POD	APX	ASA	GSH	Car	Pro
Early cold acclimation	*S. przewalskii*	4.82±0.55	91.14±6.89	0.12±0.02	20.99±3.25	0.29±0.48	0.63±0.03	0.19±0.02	0.15±0.01	0.17±0.05
(August to September)	*S. chinensis*	5.26±0.76	90.60±1.59	0.09±0.02	14.41±7.42	0.29±0.04	0.52±0.02	0.16±0.02	0.15±0.02	0.16±0.04
	t-test	$p=0.277$	$p=0.857$	$p=0.010$	$p=0.088$	$p=0.996$	$p=0.415$	$p=0.273$	$p=0.713$	$p=0.851$
Cold acclimation	*S. przewalskii*	3.67±0.49	84.79±6.03	0.13±0.01	57. 50±7.21	0.36±0.11	0.83±0.01	0.32±0.04	0.29±0.02	0.65±0.08
(October to November)	*S. chinensis*	4.10±0.33	77.07±8.22	0.09±0.02	52.40±3.17	0.34±0.07	0.77±0.16	0.23±0.04	0.27±0.03	0.25±0.03
	t-test	$p=0.022$	$p=0.096$	$p=0.003$	$p=0.158$	$p=0.654$	$p=0.484$	$p=0.005$	$p=0.432$	$p=0.000$
Freezing	*S. przewalskii*	3.29±0.37	68.51±4.94	0.13±0.02	46.98±12.25	0.37±0.05	0.90±0.02	0.39±0.03	0.31±0.07	0.74±0.14
(December to January)	*S. chinensis*	3.69±0.28	55.95±13.23	0.12±0.03	30.02±4.35	0.30±0.04	0.92±0.02	0.18±0.01	0.22±0.01	0.26±0.05
	t-test	$p=0.002$	$p=0.070$	$p=0.324$	$p=0.018$	$p=0.037$	$p=0.325$	$p=0.000$	$p=0.177$	$p=0.001$

Values are means±SE ($n=18$).

Proline content was determined using a colorimetric method (Zhu et al., 1983). Leaf samples were treated with 5 ml of 3% sulphosalicylic acid and kept at 100 °C for 10 min. The supernatant (1 ml) was added to 2 ml of distilled water, 2 ml of glacial acetic, and 4 ml of 2.5% (w/v) acidic ninhydrin and kept at 100 °C for 60 min. The absorbance of the extract was read at 520 nm.

2.7. Statistical analysis

Each experiment was repeated at least three times. Correlations between variables were analyzed using the SPSS 10.0 analysis of bivariate correlations procedure (Table 1). Mean ($n=18$) comparisons of anti-oxidative system and freezing tolerance between two tree species in the same stage were analyzed using the SPSS analysis of independent-samples *t*-test procedure (Table 2).

3. Results

The study was conducted at the experimental station of Lanzhou University at an altitude of 1900 m. Mean annual temperature was 7.6 °C. The highest mean monthly temperature was about 28.4 °C (July) and the lowest was −13.9 °C (December). There were 107 days with temperatures below 0 °C. Note the occurrence of minimum temperatures below 0 °C from October to the following April (Fig. 1). The evergreen woody plants survived the winter and supported re-growth the subsequent spring. Minimum temperatures above zero were present from May to September. During this period, the evergreen woody plants finished a new growth experience.

3.1. Seasonal changes in TBARS and electrolyte leakage in the leaves

Fig. 2 shows changes in TBARS and EL during the whole experiment. Although some differences were found in the initial TBARS contents of the two species, it is evident that they displayed a similar seasonal pattern of lipid peroxidation (Fig. 2a). At the end of the summer, mean daily temperature decreased from 18.4 °C (August) to 13.6 °C (September) and leaf TBARS contents increased by 30.2% and 23.3% in *S. chinensis* and *S. przewalskii*, respectively. From September to November, a decrease in TBARS contents of the leaves was found. The values of TBARS contents of the leaves observed in October were maintained with only small fluctuations at the next sampling dates and with a slight increase during the following spring. Concerning the differences between the two species, in general the higher values of TBARS were found in the leaves of *S. chinensis* and the lower contents in *S. przewalskii.* The most evident differences occurred at the end of the summer and early autumn, and in the middle of the spring season (Fig. 2a). A similar behavior was found for EL (Fig. 2b) during the whole experiment.

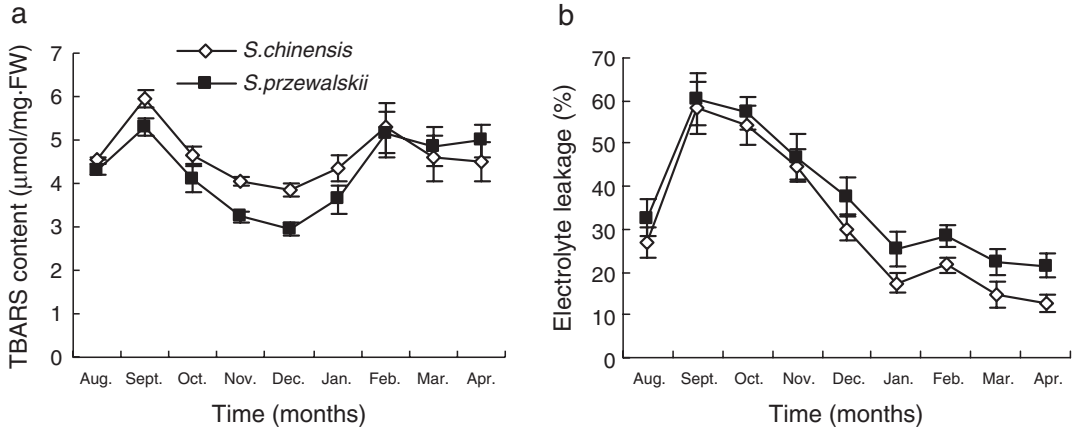

Fig. 2. Seasonal variations in TBARS content (a) and EL (b) in the leaves. Values are mean±SE of three replicates.

3.2. Seasonal changes in SOD, CAT, POD and APX activities

SOD activity in the leaves of the two species did not markedly change from August to October. However, a significant decrease was observed from November to January, and SOD activity in the leaves of the two species reached minimum values in January and February (Fig. 3a). With increasing temperature in the spring, an opposite changing pattern occurred: SOD activity increased and reached a maximum value in March (Fig. 3a), which coincided with shoot sprout development (mean temperature about 3.3 °C).

In contrast with SOD activity, CAT and POD activities in the leaves of the two species increased rapidly from September and reached maximum values in December and November, respectively (Fig. 3b,c). In this case, maximum increases for CAT were 106.1% and 33.5%, and for POD were 156.6% and 170% for *S. chinensis* and *S. przewalskii*, respectively. By January, the enzymes activities declined to lower values, but they were still higher than those observed in September. In spring, the CAT and POD activity increased slightly with rising temperatures. Concerning the differences between the two species, CAT activity in the leaves of *S. przewalskii* increased linearly and reached a maximum in December, however values in the leaves of *S. chinensis* fluctuated according to the season. There were two peaks in October and December (Fig. 3b). There were no substantial differences in the seasonal pattern of POD activity between the two species (Fig. 3c).

In comparison with CAT and POD activity, APX activity in the leaves was relatively constant during temperature decreasing from September (13.6 °C) to October (7.6 °C) (Fig. 3d). Later, APX activity increased gradually and reached a peak in November. By December, the enzyme activity declined to the value of September. In spring, APX activity in the leaves increased rapidly and reached the maximum values found during the year in March. From December to this last date, increases in APX activity were 100.0 and 97.1% for *S. chinensis* and *S. przewalskii*, respectively.

3.3. Seasonal changes in ASA, GSH, Car and pro contents

Ascorbic acid (ASA) contents in the leaves of the two species increased slowly from August to November (Fig. 4a). Later, values were maintained with only small fluctuations in the whole winter from December to January. In spring, ASA contents increased slowly again and reached the maximum values found during the year in March. From December to this last date, the increments in ASA contents observed were 5.38% and 23.4% for *S. chinensis* and *S. przewalskii*, respectively.

Compared with ASA, GSH contents in *S. przewalskii* increased continually and reached the first peak value in December. In contrast, values in *S. chinensis* decreased in November after a rapid increase in October, but still higher than those observed in September (Fig. 4b). In spring, GSH contents in *S. przewalskii* increased rapidly and reached the maximum values found during the year in March. However, the levels of GSH in *S. chinensis* increased slightly in spring. Afterwards, GSH content decreased to values similar to those observed in September.

Like GSH, contents of Car and Pro increased significantly from August to October (Fig. 4c,d). The increases in Car were

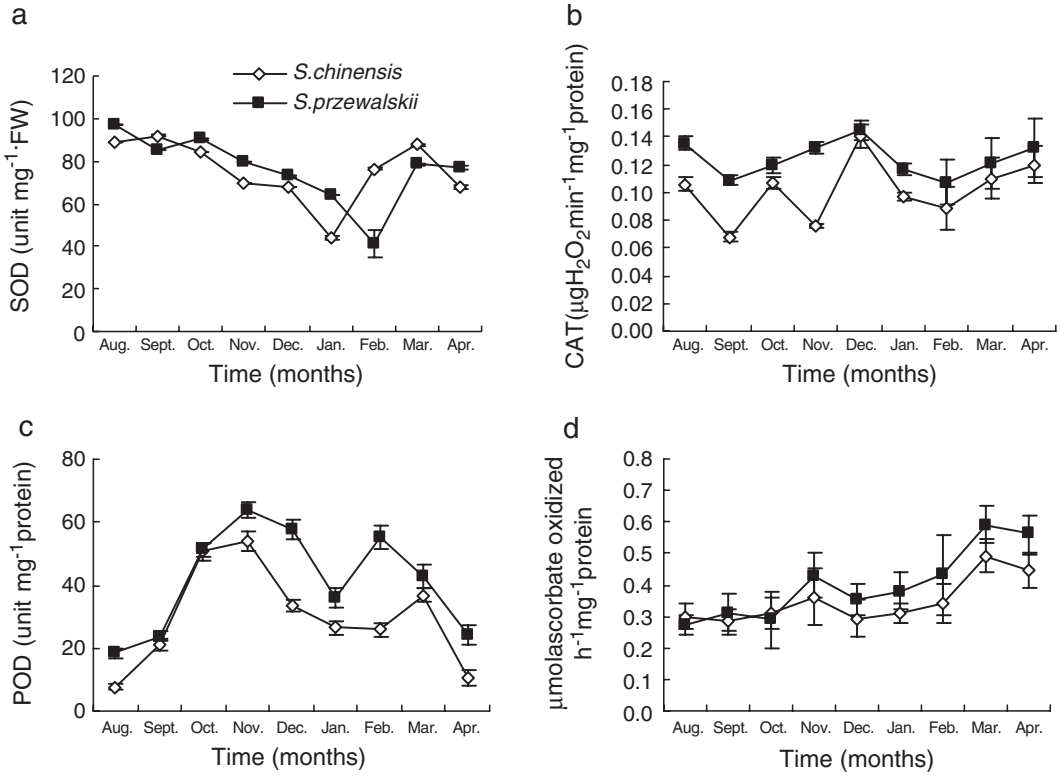

Fig. 3. Seasonal variations in activities of SOD (a), CAT (b), POD (c) and APX (d) in the leaves. Values are mean ± SE of three replicates.

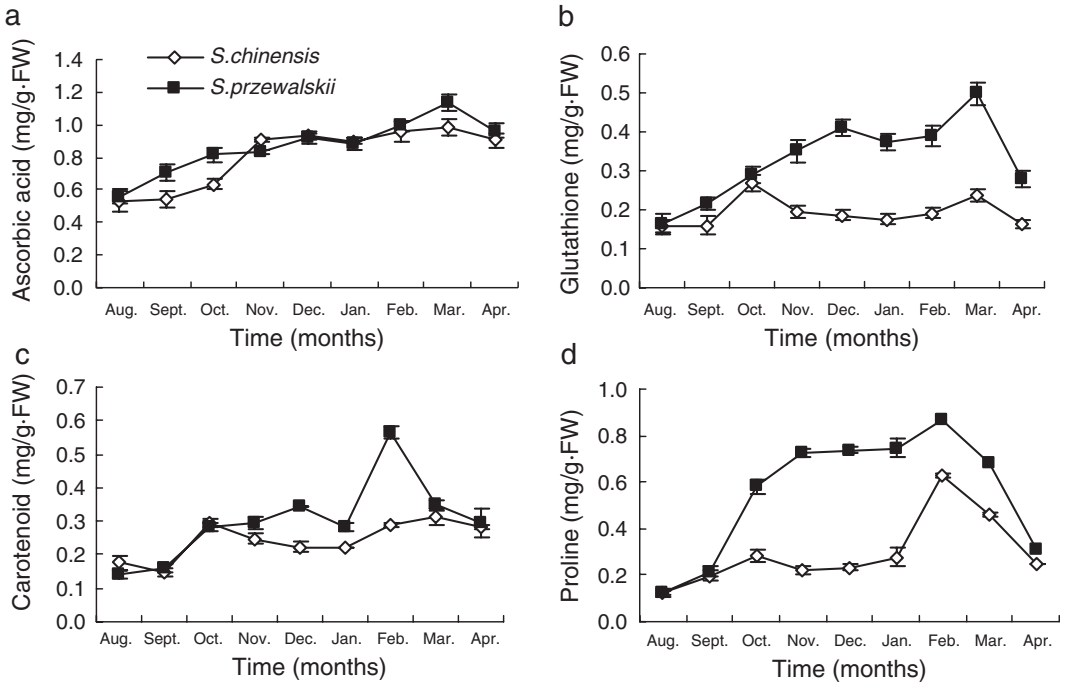

Fig. 4. Seasonal variations in contents of ASA (a), GSH (b), Car (c) and Pro (d) in the leaves. Values are mean ± SE of three replicates.

103.1% and 100.8%, and increases in Pro were 138.3% and 355.2%, for *S. chinensis* and *S. przewalskii*, respectively. Later, there were remarkable differences between the two species throughout the winter. Car and Pro contents in *S. przewalskii* increased continually and reached the maximum values in December, in contrast, values in *S. chinensis* decreased although they were still higher than those observed in September (Fig. 4c,d). In spring, contents of both antioxidants in the leaves increased and reached the maximum values found during the year in February. Afterwards, contents decreased to values similar to those observed in October (Fig. 4c,d).

Comparing the seasonal pattern of anti-oxidative system and mean temperature, lipid peroxidation (TBARS) and EL in the leaves of the two species, correlations between anti-oxidative system and mean temperature, between anti-oxidative system and lipid peroxidation, and between anti-oxidative system and EL were all observed (Table 1). With mean temperature decreasing from August to January, except for SOD, the activities of POD, CAT, APX and contents of ASA, GSH, Car and Pro were negatively correlated with mean temperature and TBARS content in both species. As for EL, in *S. przewalskii*, except for SOD and POD, the activities of CAT, APX and contents of ASA, GSH, Car and Pro were negatively correlated with EL. However, there is no obvious correlation between anti-oxidative defence capacity and EL in *S. chinensis* (Table 1).

3.4. Differences between the two species

Some evident differences between the two species were found in our results. The experimental time can conveniently be categorized into three main stages, that is early cold acclimation (August to September), cold acclimation (October to November) and freezing stage (December to January). Mean values of various parameters over these 2-month intervals are listed in Table 2. In general, higher TBARS contents were found in the leaves of *S. chinensis* than in *S. przewalskii*. During the early stages of cold acclimation, there were not significant differences between the two species ($p > 0.05$), but significant differences ($p < 0.01$) were evident during freezing after cold acclimation (Table 2). Activities of anti-oxidative enzymes and levels of antioxidants were higher in *S. przewalskii* than in *S. chinensis*. Furthermore, anti-oxidative enzymes and antioxidants were also significantly different between the two species in the freezing stage after experiencing cold acclimation ($p < 0.05$ for POD and APX, $p < 0.001$ for GSH and Pro) (Table 2).

4. Discussion

At the first stage (early cold acclimation) from August to September, the mean maximum and minimum temperature were all over 0 °C. The second stage (cold acclimation), also known as the freeze–thaw stage, occurs as a result of changes in day and night temperatures from late October to early November. The third stage (freezing stage) occurs from December to the following January when the temperature was below 0 °C (relatively stable temperature) during both day and night. Under this climate, leaves of evergreen woody plants of *Sabina* are able to resist freezing and to survive the winter. Probably, they are cold acclimated during the freeze–thaw stage and later accomplish frost hardening during the freezing stage in the winter. The behavior of our plants matches very well with this general assumption.

In this study, when temperature decreased in September, we found a loss of membrane integrity (higher electrolyte leakage), which was positively correlated with lipid peroxidation (Fig.

2a,b). On the other hand, this lipid peroxidation could be the result of the decreased activity of the different antioxidant enzymes (CAT, SOD and APX) assayed (Fig. 3a,b,d). Antioxidant contents did not increase remarkably during this period (Fig. 4a–d). The higher electrolyte leakage of leaves in the autumn may arise from ROS accumulation in excess of the capacity of anti-oxidative systems to scavenging ROS. This would cause loss of membrane integrity and subcellular compartmentation (Prasad et al., 1994). In fact, chilling stress is known to result in extensive oxidative stress (Prasad, 1997). In addition, increasing evidence indicates that membrane injury under lower temperature is related to increased production of highly toxic ROS (Kendall and Mckersie, 1989; Hernandez and Almansa, 2002). However, this initial increment of ROS was stopped or reversed and this coincided with an abrupt increase in antioxidant enzyme activities and antioxidant contents (Figs. 3 and 4), possibly as a consequence of cold-acclimation. Initially, low temperatures would result in cellular damage in the leaves but later the leaves are able to develop cold-hardiness.

In the third stage (freezing stage), TBARS content in the leaves remained stable indicating that ROS accumulation did not occur (Fig. 2a). The level of anti-oxidative enzyme activities and antioxidant contents also decreased (Figs. 3 and 4), suggesting that the constitutive levels of these anti-oxidative enzyme activities and antioxidant contents could be enough to regulate and control ROS levels, and enough to maintain freezing tolerance in the leaves. During the spring, when dehardening occurred, a very slight increase in TBARS content occurred and an increase in the anti-oxidative enzyme activities and antioxidant contents was discerned, followed by a decrease. This cycle is similar to that observed during the autumn. In both cases a freeze–thaw cycle occurs, although in April the accumulation of TBARS is prevented. Freeze–thaw cycles may be sensed by the leaves as a signal to develop cold acclimation and dehardening processes (Zhou and Zhao, 2004).

SOD, CAT, POD and APX are usually considered to be the most predominant ROS-scavenging in plants systems (Bowler et al., 1992; Foyer et al., 1994; Allen, 1995; Rao et al., 1996; Saruyama and Tanida, 1995). In our study, CAT, POD and APX were important antioxidant enzymes (Fig. 3). Consistent with previous studies (Prasad, 1997; Prasad et al., 1994), tolerance of both species to freezing was linked closely with the activities of antioxidant enzymes (Table 1). Our results indicate that, unlike POD, CAT and APX (Fig. 3a), SOD decreased during the time course of cold acclimation (Fig. 3a). It is possible that SOD plays a more important role as an anti-oxidative enzyme in organs other than leaves. ASA, GSH, Car and Pro constitute non-enzymatic defences against oxidative stress (Long and Humphrie, 1994; Kendall and Mckersie, 1989), and have been reported to play very important roles in scavenging of H_2O_2,$\cdot O_2^-$ and OH (Nicholas and Quinton, 1989). Our study indicates that the tolerance of *Sabina* trees grown in natural conditions was also closely linked to the accumulation of ASA, GSH, Car and Pro (Fig. 4, Table 1), which is consistent with previous studies (Rada and Rcia, 2001).

Some differences between the two species were observed. The higher enzymatic activities and antioxidant contents were linked with the lower TBARS content in leaves of *S. przewalskii*. In contrast, the lower enzymatic activities and antioxidant contents were linked with the higher TBARS content in the leave of *S. chinensis* (Table 2). Furthermore, at early cold acclimation, there were not significant differences between the two trees, but significant differences occurred in the freezing stage after experiencing cold acclimation (Table 2). These suggested that freezing tolerance of *S. przewalskii* is higher than *S. chinensis*, which is consistent with their natural environment. *S. przewalskii* with higher freezing tolerance grows mainly at altitudes ranging from 2600 m to 4000 m on Qinghai–Tibet Plateau (including Qilian Mountains), where the average temperature is about 0.5 °C. Instead, *S. chinensis* with lower freezing tolerance distributes mainly at altitudes ranging from 500 m to 1900 m on Plains of China where the average temperature is about 8.5 °C (Feng, 1994; Fu, 1990).

5. Conclusion

This study demonstrated that freezing tolerance in the leaves of evergreen woody plants of *Sabina* is correlated with an increased capacity to scavenge or detoxify ROS by anti-oxidative enzyme systems and antioxidant systems. POD, CAT and APX enzymatic activities are especially active during the hardening and de-hardening processes. SOD is only active during de-hardening. The antioxidant system includes at least four antioxidants (ASA, GSH, Car and Pro) that all accumulate especially during the hardening and de-hardening processes. Compared with *S. chinensis*, *S. przewalskii* showed a prevailing strategy in anti-oxidative defence systems in order to develop the tolerance to freezing.

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