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RELATIONSHIP BETWEEN NITRIC OXIDE ACCUMULATION, ANTI-OXIDATIVE SYSTEM AND FREEZING TOLERANCE IN THE LEAVES OF *SABINA* DURING COLD ADAPTATION

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ABSTRACT

Signal function of endogenous nitric oxide (NO) and freezing-resistant traits in the leaves of *Sabina przewalskii* Kom. (SP) and *Sabina chinensis* (Lin.) Ant. (SC) during cold acclimation and freezing tolerance were investigated. The results indicated that with the decrease in temperature electrolyte leakage (EL) and thiobarbituric acid-reactive substances (TBARS) content increased markedly and reached two peaks in September and February, ratio of free water content (FWC) to bound water content (BWC) decreased and the lowest value was found in the winter (during November to January). Both NO and the anti-oxidative systems played an important role in potentiating freezing-resistance and limiting the production of free radicals to protect membrane integrity. Activities of catalase (CAT), perixidease (POD) and ascorbate peroxidase (APX), contents of ascorbic acid (ASA), glutathione (GSH), proline (Pro) and carotenoid (Car) increased with temperature decrease, and highest in the winter, the rate of NO release, NO synthase (NOS) and nitrate reductase (NR) activity increased markedly and reached two peaks respectively in September and February. Meanwhile, the increment of NO generation is always found before decreasing of the ratio of FWC to BWC and increasing of anti-oxidative enzyme activity and antioxidant content. It indicated that NO as an early signal molecule is able to induce the freezing tolerance by increasing the content of bound-water in cell and activating the anti-oxidative system.

Key words: *Sabina*, anti-oxidative system, nitric oxide, freezing tolerance.

INTRODUCTION

The tissues present in the leaves of evergreen woody plants are exposed to freeze-thaw cycles in late autumn and throughout the winter, and they maintain a regrowth potential in the following spring. It was reported that the freezing tolerance of these plants changes with seasonal variations. Needles of the Central European Scots pine (*Pinus sylvestris* L.) are lethally damaged when exposed to –10°C during the summer months, while in mid winter they survive in 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 (cold acclimation). Therefore, knowledge of the physiological events that occur during cold acclimation would allow a better understanding of freezing tolerance in evergreen woody plants leaves. At the cellular level, it has been demonstrated that cell membranes are directly involved in cold acclimation and freezing tolerance and the primary sites of freezing injury (Lu *et al*., 2004). Membrane damage from exposure to chilling stress may be mediated by reactive oxygen species (ROS) such as superoxide radicals, singlet oxygen, H_2O_2, and hydroxyl radicals. The accumulation of these ROS initiates lipid peroxidation. ROS are produced as a by-product of normal cell metabolism and their levels are enhanced by diverse environmental stresses (Sharma and Dietz 2009).

However, plants have evolved several mechanisms to develop a hardening process. This process would include the expression of anti-oxidative system and changes in the different pools of water present in the cells. Anti-oxidative system includes such anti-oxidative enzymes as SOD, CAT, POD, and APX, and such physiological antioxidant molecules as ASA, GSH, Car and Pro. Usually both act in tandem (Sharma and Dietz 2009). It has been shown that tolerant plants respond to low temperatures by an increase of the levels of these antioxidant compounds and also by an increment in the activity of some anti-oxidative enzymes (Gabriela *et al.*, 2011).The water content of a tissue is inversely related to freezing tolerance (Yoshida *et al*., 1997). Acclimation promotes water loss from the tissue. The loss of water has obvious adaptive advantages because there would be less water to freeze, less ice to accommodate, and physically less expansion in the intercellular spaces (Chang *et al*., 2001). Yoshida *et al.* (1997) reported that a decrease in very weakly bound water and an increase in the amount

of tightly or moderately bound water would produce a markedly increased freezing tolerance in winter wheat.

Nitric oxide (NO) is synthesized by NO synthase (NOS) or nitrate/nitrite reductase (NR/NiR) (Foresi *et a1*., 2010). As a bioactive molecule, NO has also been suggested to act as a signal molecular mediating responses to biotic and abiotic stresses in the plant kingdom. It could induce germination instead of red right (Zhang *et al*. 2003), affect on growth and development of plant tissue (Xiong *et a1*., 2009). Also, NO was suggested to be involved in the responses to drought stress (Arasimowicz *et al*., 2009), heat stress (Song *et al*., 2008), salt stress (Zheng *et al*., 2009), UV-B radiation (An *et al*., 2005) and heavy metal (Wang and Yang 2005). However, the experimental evidence for NO as a signal molecular in plants during cold acclimation remains elusive, especially in plants which grown in natural condition.

Evergreen woody plants of *Sabina* are mainly distributed in alpine habitat of northwest China, and play an important role in ecosystem function as water-conservation forest, such as *SP* which grows mainly at altitudes ranging between 2600m to 4000m on Qinghai-Tibet Plateau (including Qilian Mountains), where the annual average temperature is about 0.5°C. In contrast, *SC* distributes mainly at altitudes ranging between 500m to 1900m on Plains of the China where the annual average temperature is about 8.5°C (Feng 1994; Fu 1990). For that reason, we use *SP* and *SC* as materials to study the role of NO as a signal molecule and the several freezing-resistant traits including the relation of seasonal pattern in anti-oxidative system and modification of water pattern in the tissue to freezing tolerance of *Sabina*, and investigated them to induce adaptive responses from autumn (during September to October) to early spring (during February to April), involving in the cold acclimation that occurred during autumn, as well as de-acclimation in early spring. To our knowledge, this is the first report on NO functions as a signal and seasonal changes of anti-oxidative systems in freezing tolerance as well as modification of water pattern caused by environmental temperature in plant tissue in northwest China evergreen trees of *Sabina* during winter stress.

MATERIALS AND METHODS

Three years old seedlings of two evergreen woody plants of *Sabina*, *SP* and *SC* were planted at the experimental station of Lanzhou University, which is located in a temperate semi-arid and semi-humid region (103°E, 35°N, altitude 1900 m, in central Gansu province, China), in mid-May of 2001. Plots were hand-weeded and insects controlled as necessary each year. Mean air temperature data were collected from August 2004 to April 2005 and it is summarized in Fig. 1 (the weather data was collected from local weather stations near the research site). Mean annual temperature was 7.6°C. The highest temperature was about 28.4°C (July) and the lowest 6.4°C (January). Note the occurrence of temperatures below 0°C from October to the following year, in April (Fig. 1). The evergreen woody plants survived the winter and they supported the regrowth of the next spring. Minimum temperatures above zero were present from May to September. During this period, the evergreen woody plants finished a new growth experience.

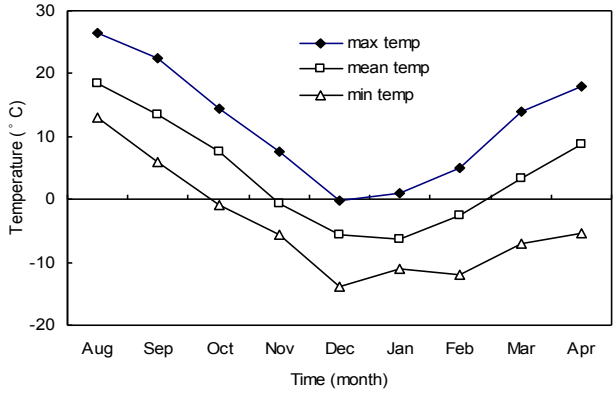

Fig.1. Seasonal variations of mean air temperature and mean maximum and minimum temperature each month from 2004 to 2005 of research site (103°E, 35°N, altitude 1900 m, in central Gansu province, China)

There were four sampling blocks randomly chosen and labelled in each replicated tree plot. Each sample was taken from a labelled sampling block at each sampling time. Leaf samples were collected at 22 of every month from August 2004 to April 2005. All samples were taken at 10:00 to 12:00 am. (cst) 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 NO content, water content and EL immediately, the remaining leaves were frozen in liquid N_2 and transported to the laboratory to determine the activities of various antioxidant enzymes, the activities of NOS and NR, the content of various antioxidants and TBARS.

NOS activity and NO content were determined as described by Murphy and Noack (1994). NR assays were performed essentially as described by Wray and Fido (1990). Dry material of leaves was obtained after being revised and heated at 80°C for 48 h. Relative Water Content (RWC) was measured as the formula: RWC = (fresh weight – dry weight)/fresh weight. FWC and BWC were determined by the method of Li (2000). Measurement of EL was used to assess chilling injury. EL of the leaves was determined as described by Zwiazek *et al.* (1990). The extent of lipid peroxidation was estimated by determining the concentration of TBARS.

The TBARS contents in the leaves were measured according to Cakmak and Horst (1991).

The SOD activity was measured spectrophotometrically as described by Beyer and Fridovich (1987). The CAT activity was assayed by the method of Clairborne (1985). 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 (1993). Protein concentration in the enzymatic extraction was measured by the method of Bradford (1976).

Total ascorbate was measured as described by Foyer *et al.* (1995). Total glutathione was assayed according to the method of Griffith (1980). Carotenoid were extracted and estimated according to the method described by Arnon (1949). Proline content was determined using a colorimetric method (Zhu *et al*., 1983).

Each experiment was repeated at least three times. Correlations between variables were analyzed using the SPSS 13.0 analysis of bivariate correlations procedure (Table 1). Mean (n=18) comparisons between two tree species in the same stage were analyzed using the SPSS analysis of independent-samples t-test procedure (Table 2).

RESULTS AND DISCUSSION

The present study was performed in a semi-arid area, where the annual mean temperature was 7.6°C. The mean minimum air temperature was below 0°C from later October to April (Fig. 1). The climate in the area is divided into two main stages. The first stage, known as the freeze-thaw stage, occurs as a result of the day and night temperature changes from later October to early November. The second stage, known as the freezing stage, occurs from December to the following January when the temperature was below 0°C (relatively stable temperature) during both day and night. In this climate, leaves of evergreen woody plants of *Sabina* are able to resist freezing and survive the winter. Probably, they are cold acclimation during the freeze-thaw stage and later they reach frost hardening during the freezing stage in the winter.

When temperature decreased in September, we found a loss of membrane integrity (higher electrolyte leakage), which was positively correlated with lipid peroxidation ($p<0.05$) (Fig. 2). On the other hand, this lipid peroxidation could be the result of the decreased activity of the different anti-oxidative enzymes (SOD, CAT and APX) assayed ($p>0.05$) (Fig. 3a, b, d), furthermore, antioxidant contents did not increased remarkably during this period ($p>0.05$) (Fig. 4a, b, c, d). Higher electrolyte leakage of leaves in the autumn may be due to the rate of ROS accumulation (TBARS content increased) being greater than the rate of scavenging ROS by anti-oxidative system, which led to damage of the membrane integrity and loss of subcellular compartmentation. In fact, chilling stress is known to result in extensive oxidative stress (Lu *et al*., 2004). In addition, more and more evidence indicate that membrane injury under lower temperature was related to an increased production of highly toxic ROS (Hernandez and Almonsa 2002). However, this initial increment of ROS was stopped or reversed and this coincided with an abrupt increase in anti-oxidative enzymes activities and antioxidants contents ($p<0.05$) (Fig. 3, 4). This may be an implication as a result of the cold-acclimation process. Initially, low temperatures would result in cellular damage in the leaves but later the leaves are able to develop a hardening process.

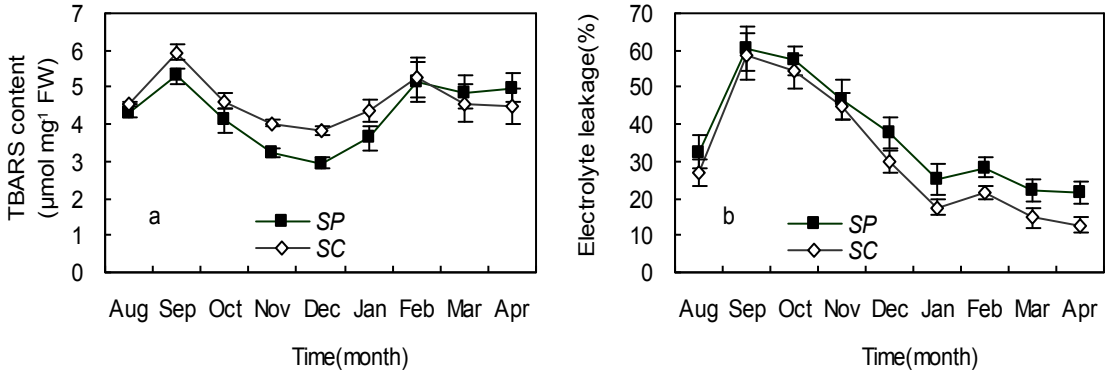

Fig.2. Seasonal variations in TBARS content (a) and EL (b) in the leaves. Values are mean ± S.E. of three replicates.

In the second stage (freezing period from December to the following January), TBARS content in the leaves remained stable indicating that ROS accumulation did not occur (Fig. 2a). Anti-oxidative enzymes activities and antioxidants contents also decreased a little or remained steadily (Fig. 3, 4), which

could be enough to regulate and control ROS levels, and enough to maintain the active operation of the freezing tolerance mechanism in the leaves. During the spring when dehardening took place, a very slight increase in TBARS content occurred ($p<0.05$) and an increase in the anti-oxidative enzymes activities and antioxidants contents was assayed followed later ($p<0.05$). This cycle was similar to that observed during the autumn, in both cases a freeze-thaw cycle occurred, although in April the accumulation of TBARS got controlled. Freeze-thaw cycles may be sensed by the leaves as a signal to develop cold acclimation and dehardening processes (Zhou and Zhao 2004).

Tolerance of two species to freezing was linked closely with activity of anti-oxidative enzymes (Table 1), which is consistent with previous studies (Lu *et al*., 2004; Sharma and Dietz, 2009). But activity of SOD decreased during the time course of cold acclimation unlike POD, CAT and APX (Fig. 3a). It is possible that SOD plays a more important role as an anti-oxidative enzyme in other organs rather than in the leaves. ASA, GSH, Car and Pro constituted the unenzymatic defence against oxidation stress, and they play a very important role in scavenging of H_2O_2, O_2^- and ·OH. Tolerance of trees of *Sabina* 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).

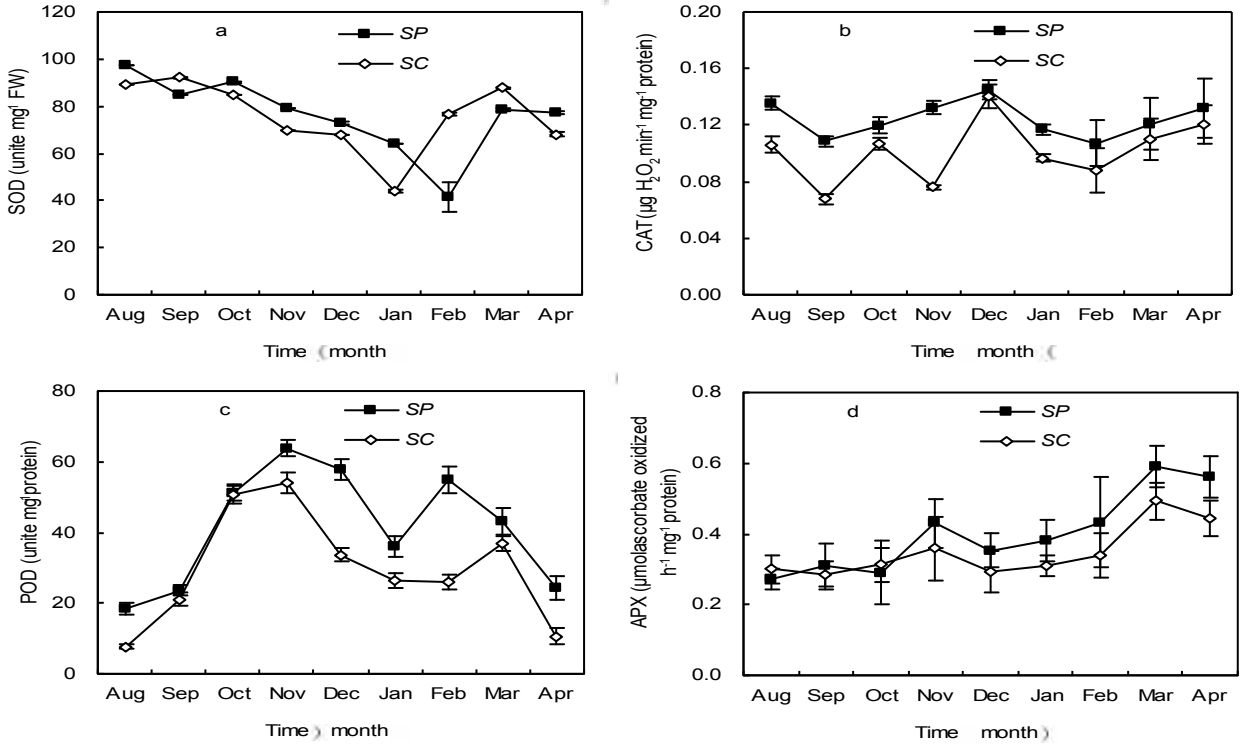

Fig.3. Seasonal variations in activities of SOD (a), CAT (b), POD (c) and APX (d) in the leaves. Values are mean ± S.E. of three replicates.

Comparing the seasonal pattern of anti-oxidative system and mean temperature, lipid peroxidation (TBARS), EL in the leaves of the two species, correlation between anti-oxidative system and mean temperature, between anti-oxidative system and lipid peroxidation, between anti-oxidative system and EL was observed. With mean temperature decreasing from September (13.6°C) to November (–0.6°C), the activities of POD, CAT, APX and contents of ASA, GSH, Car and Pro were negatively correlated with temperature, TBARS content and EL (Table 1).

When Freeze-thaw cycles take place, plants always develop cold acclimation and dehardening processes (Zhou and Zhao 2004), this process would also include changes in the different pools of water present in the cells. Yoshida *et al.* (1997) reported in winter wheat a decrease in very weakly bound water and an increase in the amount of tightly or moderately bound water that produced an increased freezing tolerance. Our study showed that two stages of hardening are associated with marked changes both in content and physical state of tissue water, with the temperature decreasing in autumn,

RWC, FWC and ratio of FWC to BWC decreased (p<0.05), whereas an opposite pattern occurred when temperature increased in spring (p<0.05) and the lowest values were found in the freezing stage (Fig. 5a, b, d). In contrast, BWC decreased in spite of decreasing temperature or increasing temperature in the freeze-thaw stage, but it increased in the freezing stage when mean temperatures were just below 0°C (p<0.05) and reaching a maximum in late December (Fig. 5c). The decrease in RWC is perhaps due to a decrease in water absorption by roots at low soil temperatures. A decrease in the FWC in leaves to avoid mechanical damage by ice formation, and an increase in BWC which prevents withdrawal of water for ice formation are, therefore, necessary for plant survival in freezing temperatures.

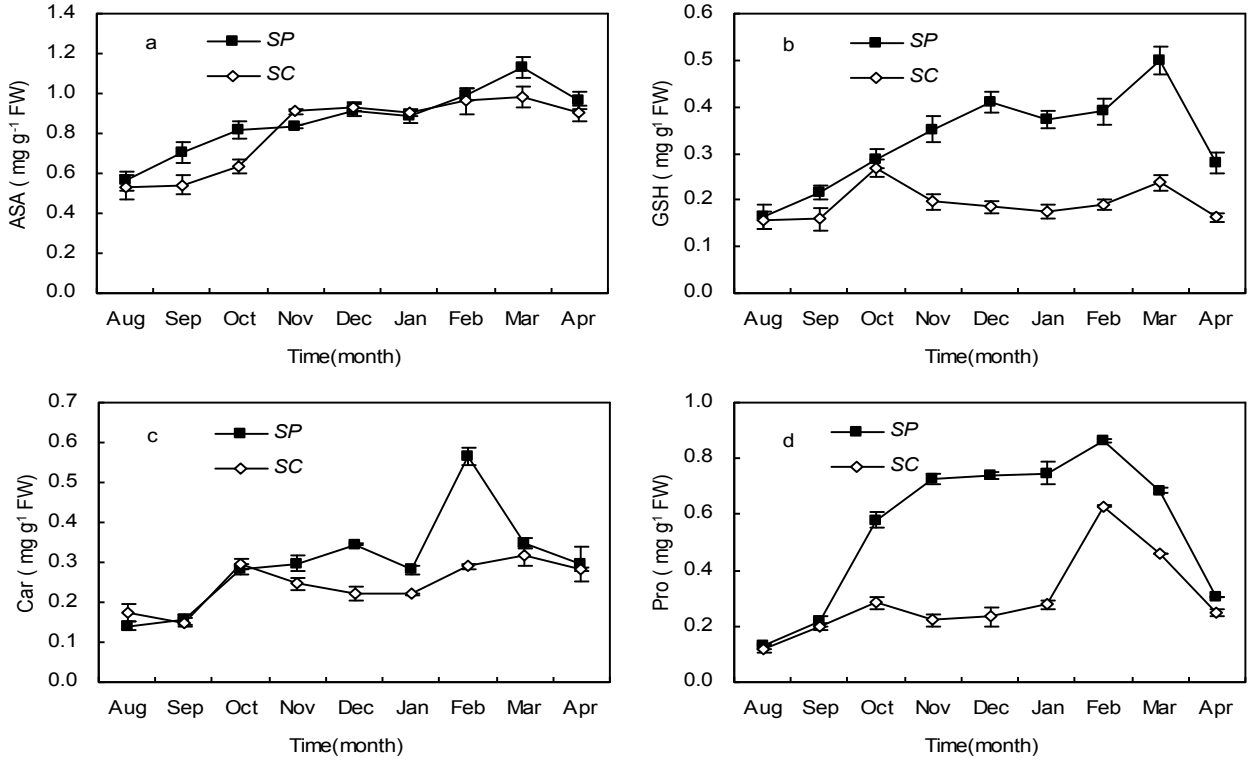

Fig.4. Seasonal variations in contents of ASA (a), GSH (b), Car (c) and Pro (d) in the leaves. Values are mean ± S.E. of three replicates.

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	
	SC	***SP***	***SC***	***SP***	***SC***	***SP***
SOD	0.880*	0.912*	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**	-0.940**	-0.761	-0.680	-0.449	-0.094
GSH	-0.142	-0.984**	-0.220	-0.824*	0.416	-0.279
Car	-0.443	-0.894*	-0.564	-0.854*	0.106	-0.152
Pro	-0.693	-0.954**	-0.220	-0.827*	0.090	-0.242

** Correlation significant at the 0.01 level,
* Correlation significant at the 0.05 level

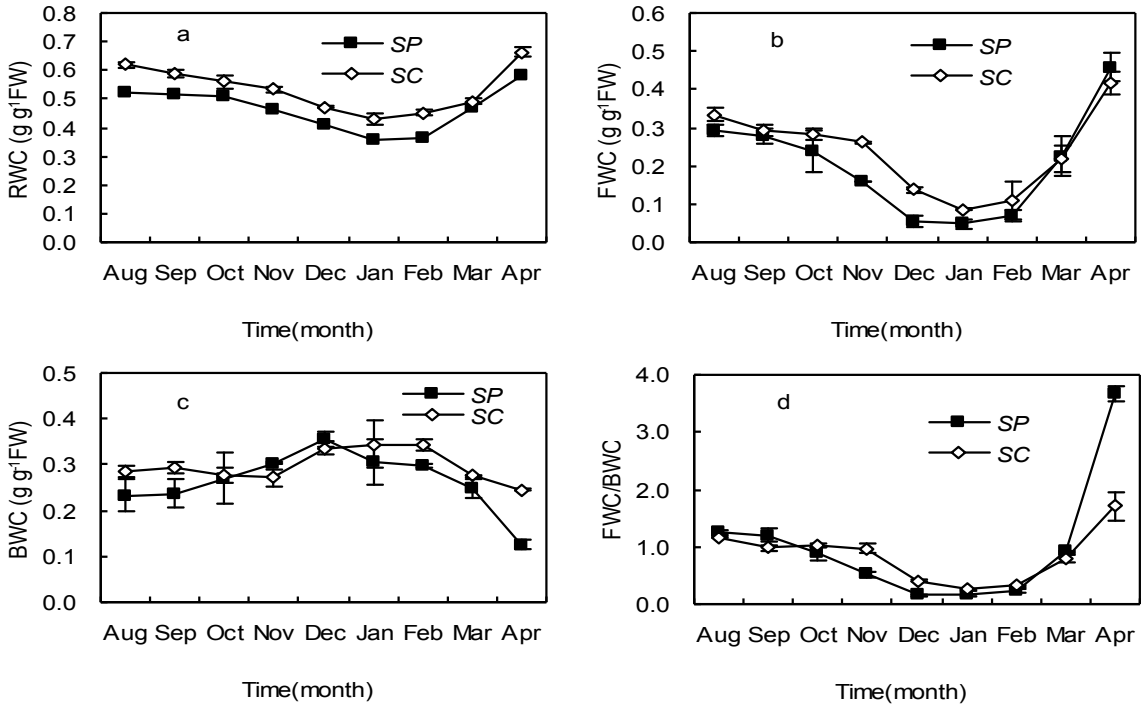

Fig.5. Seasonal variations in Water content (a), FWC (b), BWC (c) and the ratio of FWC to BWC (d) in the leaves. Values are mean ± S.E. of three replicates.

NO, as a signaling molecular, is involved in multiple plant-resistant reactions against environmental stresses (Arasimowicz and Floryszak 2007). NO accumulated in environmental stresses can scavenge $O_2^{\cdot-}$ and lipid free radical($R^{\cdot}$), prevent oxidative stress induced by ROS (Tewari *et al*., 2008). Also, it can induce expression of antioxidant enzymes (Laspina *et al*., 2004). The higher NO accumulation, antioxidant enzymatic activities and antioxidant contents were linked with the lower ratio of FWC to BWC and TBARS content in the leaves of *SP*. In contrast, the lower NO accumulation, antioxidant enzymatic activities and antioxidant contents were linked with the higher ratio of FWC to BWC and TBARS content in the leaves of *SC* (Fig. 6, Table 2). Compared with in the leaves of *SC,* more NO accumulation was found in the leaves of *SP* in the freeze-thaw stage. Therefore, NO as an early signal molecule is able to induce the freezing tolerance. On one hand, NO activated the anti-oxidative system involved in both anti-oxidative enzymes and antioxidants, on the other hand, NO may induce the accumulation of hydrophilic substances to increase the content of bound-water in cell. As a result, in the freezing stage, activities of SOD, CAT, POD, APX, and contents of ASA, GSH, Car and Pro in *SP* was higher than values in *SC*. In contrast, the ratio of FWC to BWC and TBARS content in *SP* is lower than values in *SC* (Table 2). Furthermore, at early cold acclimation, there were not significant differences between the two species, but significant differences occurred in the freezing stage after experiencing cold acclimation (Table 2). These suggested that freezing tolerance of *SP* is higher than *SC*, which is consistent with their natural environment. To be noticeable, ROS and NO accumulated simultaneously in September and February (Fig. 2, 6), subsequently the anti-oxidative system was triggered, increments of antioxidant enzymes activities and antioxidants contents occurred (Fig. 3, 4), which were just coupled with the freeze temperature to increase freezing tolerance (Fig. 2). This suggested that freeze-thaw cycles taking place in both autumn and spring may be sensed by leaves, ROS accumulated (TBARS content increased) due to oxidative stress, and the membrane integrity was damaged. Afterward, decrease of FWC and increase of BWC took place after largely releasing of NO during cold acclimation (Fig. 5, 6). Plants may develop the capacity not only to retain water in tissues but also to protect cell structures against desiccation by accumulating hydrophilic substances such as Metabolic Enzymes (Desaturases), Osmoprotectant producing enzymes, ROS scavenging enzymes and Structural Proteins (COR/LEA, Dehydrins, AFPs) that bind water molecules, which leads to a reduction in free water and an increase in bound water (Yoshida *et al*., 1997; Breton *et al*., 2000). Consequently, in our experiments, it is possible that NO as an early signal molecule can not only induce the expression of anti-oxidative enzymes and increment of antioxidants contents so as to adapt to cold stress, but also induce the

accumulation of hydrophilic substances to decrease free water, increase bound water and freezing tolerance.

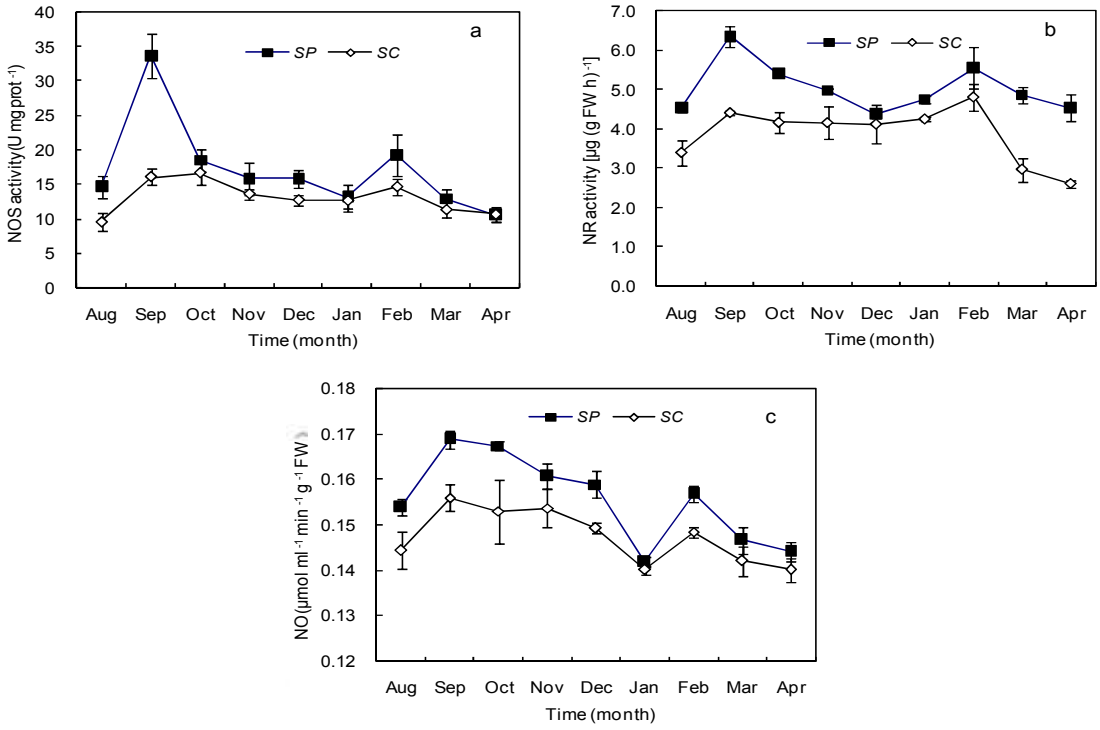

Fig.6. Seasonal variations in activities of NOS (a), NR (b) and the rate of NO release (c) in the leaves. Values are mean ± S.E. of three replicates.

Table 2. Differences of freezing-resistant traits between two tree species during early cold acclimation, cold acclimation and freezing tolerance. Values are means ± S.E. (n=18)

Item	Early cold acclimation (Aug. to Sept.)			Cold acclimation (Oct. to Nov.)			Freezing tolerance (Dec. to Jan.)		
	SP	*SC*	*T- test*	*SP*	*SC*	*T- test*	*SP*	*SC*	*T- test*
FWC /BWC	0.98±0.17	1.02±0.14	p=0.482	0.97±0.16	1.05±0.09	p=0.456	0.16±0.01	0.33±0.09	p=0.016
TBARS	4.82±0.55	5.26±0.76	p=0.277	3.67±0.49	4.10±0.33	p=0.022	3.29±0.37	3.69±0.28	p=0.002
SOD	91.14±6.89	90.60±1.59	p=0.857	84.79±6.03	77.07±8.22	p=0.096	68.51±4.94	55.95±13.2	p=0.070
CAT	0.12±0.02	0.09±0.02	p=0.010	0.13±0.01	0.09±0.02	p=0.003	0.13±0.02	0.12±0.03	p=0.324
POD	20.99±3.25	14.41±7.42	p=0.088	57. 50±7.21	52.40±3.17	p=0.158	46.98±12.25	30.02±4.35	p=0.018
APX	0.29±0.48	0.29±0.04	p=0.996	0.36±0.11	0.34±0.07	p=0.654	0.37±0.05	0.30±0.04	p=0.037
ASA	0.63±0.03	0.52±0.02	p=0.415	0.83±0.01	0.77±0.16	p=0.484	0.90±0.02	0.92±0.02	p=0.325
GSH	0.19±0.02	0.16±0.02	p=0.273	0.32±0.04	0.23±0.04	p=0.005	0.39±0.03	0.18±0.01	p=0.000
Car	0.15±0.01	0.15±0.02	p=0.713	0.29±0.02	0.27±0.03	p=0.432	0.31±0.07	0.22±0.01	p=0.177
Pro	0.17±0.05	0.16±0.04	p=0.851	0.65±0.08	0.25±0.03	p=0.000	0.74±0.14	0.26±0.05	p=0.001
NOS	24.2±10.72	12.84±3.72	p=0.048	17.17±2.39	15.13±2.04	p=0.144	14.52±1.98	12.71±1.07	p=0.078
NR	5.46±0.49	3.91±0.70	p=0.000	5.20±0.24	4.17±0.48	p=0.001	4.88±0.15	4.15±0.65	p=0.053
NO	0.16±0.01	0.15±0.01	p=0.004	0.16±0.01	0.15±0.01	p=0.003	0.15±0.01	0.14±0.01	p=0.241

Conclusion: In conclusion, the present study has demonstrated that freezing tolerance in the leaves of evergreen woody plants of *Sabina* is correlated with signaling cascade transduction activating, priming and preparing in plant cell to scavenge or detoxify ROS by anti-oxidative enzyme systems and antioxidant systems. Enzyme system includes at least three enzymatic activities, POD, CAT and APX, is especially active during the hardening and dehardening processes. SOD is only active during the dehardening processes. Antioxidant system includes at least four antioxidants, ASA, GSH, Car and Pro, accumulated especially during the hardening and dehardening processes. Our study also indicated that evergreen woody plants of *Sabina* can increase freezing tolerance by decreasing free water and increasing bound water during cold acclimation.

Furthermore, our results gave the first indication, to our knowledge, that NO functions as an early messenger inducing freezing tolerance in the leaves of evergreen woody plants of *Sabina* by activating the anti-oxidative system, and probably by inducing the accumulation of hydrophilic substances to increase bound water. Compared with *SC*, *SP* showed a prevailing strategy in signal function of NO and traits of freezing-tolerance in order to develop the tolerance to freezing.

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