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ORIGINAL ARTICLE

Differential antioxidation activities in two alfalfa cultivars under chilling stress

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Abstract To understand the adaptability of alfalfa (*Medicago sativa* L.) to chilling stress, we analyzed the antioxidative mechanism during seed germination. The germination rates of six alfalfa cultivars were studied comparatively at 10°C. Xinmu No. 1 and Northstar were selected as chilling stress-tolerant and stress-sensitive cultivars for further characterization. After chilling treatment, Xinmu No. 1 showed higher seedling growth than Northstar. Xinmu No. 1 exhibited low levels of hydrogen peroxide and lipid peroxidation compared with Northstar. In addition, shoots in Xinmu No. 1 treated with chilling showed higher activities of the superoxide dismutase, ascorbate peroxidase (APX), and catalase than those of Northstar, whereas Xinmu No. 1 showed higher APX activity in roots that Northstar. These results indicated that high antioxidation activity in Xinmu No. 1 under chilling stress is well associated with tolerance to chilling condition during germination.

Keywords Alfalfa · Antioxidant enzymes · Chilling stress · Germination · Lipid peroxidation

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Introduction

Extreme temperature represents one of the principal limitations affecting distribution and plant growth of diverse plant species (Thomashow 1998). Many of the world's most important annual crops have their origins in the tropics/sub-tropics or as a summer species in temperate environments. Thus, low temperature condition by wind and water erosion is a serious problem in such crops (Clarke and Siddique 2004). It can also result in poor germination and weak growth, and further reduce crop production. Thus, understanding the mechanisms of plants tolerance to chilling stress during germination and early growth stage is a crucial environmental research topic.

Chilling stress can induce the overproduction of reactive oxygen species (ROS) in plants, which then in turn negatively affects cellular structures and metabolism by oxidative stress. Higher plants have developed several strategies to cope with oxidative stress. One of the defense mechanisms is the antioxidation defense system, including antioxidant enzymes and low molecular weight antioxidants (Asada 1999; Foyer and Noctor 2005). The superoxide radical (O_2^-) is dismutated to hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD). Ascorbate peroxidase (APX), peroxidase (POD), and catalase (CAT) metabolize H_2O_2 into water. In the presence of O_2^- and H_2O_2, trace amounts of transition metals can give rise to the highly toxic hydroxyl radical (OH^-). Rapid detoxification of both O_2^- and H_2O_2 is essential for preventing oxidative damage. Activities of antioxidant enzymes under chilling stress have been correlated with tolerance to the stress. Chilling-tolerant maize (Hodges et al. 1997; Taka 2004), cucumber (Kang and Saltveit 2002), and rice (Huang and Guo 2005) cultivars had higher antioxidation activities.

Alfalfa (*Medicago sativa* L.) is one of the most important perennial forage crops in the world. It was originally used for ruminant livestock feed but recently a number of nontraditional uses of alfalfa are being investigated, including as a bioreactor for the production of industrial enzymes or other products via transgenic plants and as a bioenergy crop. Moreover, it has high yield and good quality with high protein content and can be cultivated in marginal lands (Croughan et al. 1978; Ehsanpour and Fatahian 2003). Previous studies on alfalfa have reported on its antioxidant mechanism under heavy metals (Zhou et al. 2007, 2008) and drought stresses (Naya et al. 2007). Recently, we studied the enzyme activity during germination of alfalfa under salt and drought stresses (Wang et al. 2009). However, comparative analyses of antioxidant modulation in the tissues of alfalfa roots and shoots between chilling stress-tolerant and stress-sensitive cultivars during germination under chilling stress are sparse.

In the present study, to better understand the adaptability of alfalfa plants to chilling stress during germination, we evaluated the stress tolerance of alfalfa cultivars under 10°C treatment by assessing seedling growth, content of H_2O_2, lipid peroxidation, and antioxidant enzyme activity in shoots and roots.

Materials and methods

Plant materials and culture conditions

Seeds (cv. Xinjiang Daye and Xinmu No. 1) of alfalfa (*Medicago sativa* L.) were provided by Prof. Zhang Bo from Xinjiang Agriculture University in China. The other cultivars (cv. Algonquin, Golden Empress, Victor, and Northstar) were stocked in our laboratory (State Key Laboratory of Soil Erosion and Dryland Farming on Loess Plateau). Seeds were surface-sterilized with 0.5% sodium hypochloride solution for 5 min, thoroughly rinsed 7–8 times with distilled water, and germinated on half-strength MS medium (pH 5.7) (Murashige and Skoog 1962). These cultures were kept under aseptic conditions for 3 days in the dark and 4 days in a 12-h light/dark cycle, with a light intensity of 350 μmol $m^{-2}s^{-1}$ and a relative humidity of 65% at 25°C.

Analysis of germination rate and chilling treatment

Germination tests were conducted using six alfalfa cultivars germinated on half-strength MS media. Three replicates (50 seeds per cultivar) were germinated on similar medium conditions, with chilling treatment. Germination rate was determined by counting the number of germinated seeds after 7 days, at 24-h intervals. Alfalfa seeds were considered to be germinated when the radical visibly protruded from the seed coat by at least 2 mm (Kim et al. 2006). To mimic chilling stress conditions, seeds were incubated on half-strength MS medium including 3% sucrose and 7% Phyto agar at 25 (control) or 10°C.

Analysis of seedling weight and length

The length of shoots and roots was measured in 120 seven-day-old seedlings from each cultivar grown under control or chilling treatment conditions. Length was assessed by image analysis using the Image J software available for free download from the National Institutes of Health (http://rsb.info.nih.gov/ij/index.html) (Verslues et al. 2006). The seedlings were photographed, separated into shoots or roots for measuring of fresh weight (Turk et al. 2003), and then immediately frozen in liquid nitrogen and stored at −70°C for biochemical analysis.

Analysis of H_2O_2 content

The H_2O_2 content in alfalfa shoots and roots was assessed using the xylenol orange method, in which H_2O_2 is reduced by ferrous ions in an acidic solution to form a ferric product: the xylenol orange complex. Detection of these complexes was performed in our samples at 560 nm (Bindschedler et al. 2001). H_2O_2 content was expressed as μmol of H_2O_2 per gram of fresh weight of plant tissue.

Analysis of lipid peroxidation

Lipid peroxidation was measured using a modified thiobarbituric acid (TBA) method (Puckette et al. 2007). The specific absorbance of extracts was recorded at 532 nm. Non-specific absorbance at 600 nm was measured and subtracted from the 532 nm readings. The concentration of malondialdehyde (MDA) was calculated as a measure of lipid peroxidation.

Determination of antioxidant enzyme activity

Shoots and roots of alfalfa seedlings were homogenized on ice with a mortar and pestle in a 0.1 M potassium phosphate buffer (pH 7.0). The homogenate was centrifuged at 12,000*g* for 15 min at 4°C. The supernatant was used immediately for enzyme assays. Total protein concentration was determined according to the Bradford method (1976) using the Bio-Rad protein assay reagent. The activity of SOD was measured according to a method using xanthine, xanthine oxidase, and cytochrome *c* (McCord and Fridovich 1969). One unit of SOD was defined as the amount of enzyme that inhibits the rate of ferricytochrome *c* reduction by 50%. The activity of APX was assayed

according to the method described by Nakano and Asada (1981), using ascorbic acid as a substrate. The oxidation of ascorbate was initiated by H_2O_2, and the decrease at 290 nm was monitored for 1.5 min. One unit of APX was defined as the amount of enzyme required to oxidize 1 μmol of ascorbate. The CAT activity was assayed according to the method described by Aebi (1984), by measuring the decrease at 240 nm for 1 min, due to H_2O_2 consumption. One unit of CAT activity was defined as the amount of enzyme required for oxidize 1 μmol of H_2O_2 per min. The activity of POD was assayed according to the method described by Kwak et al. (1995) using pyrogallol as a substrate. One unit of POD activity was defined as the amount of enzyme necessary to obtain 1 mg of purpurogallin from pyrogallol in 20 s, at 420 nm.

Statistical analysis

The experimental assays used to obtain all results were repeated at least three times, under the same conditions, and yielded essentially the same results. Data were statistically analyzed with Statistical Package for the Social Sciences (SPSS 12). Means were separated using Duncan's multiple range test at $P = 0.05$.

Results

Germination analysis of six alfalfa cultivars

To evaluate the differential responses of six alfalfa cultivars to chilling stress during germination, we analyzed the germination rate at 10°C chilling stress. The germination rate in six alfalfa cultivars was differently affected by treatments with 10°C. The germination rate of the six cultivars was delayed or inhibited under chilling stress (Fig. 1a). Among the alfalfa varieties, three cultivars, Xinjang Daye, Xinmu No. 1, and Victor, germinated faster than the other cultivars and showed the highest germination rate. In contrast, Golden Empress and Northstar cultivars showed the lowest germination rate under the same conditions. At 7 days after imbibition, the germination rate of Xinjang Daye, Xinmu No. 1, and Victor were approximately 97–99%, whereas those of Northstar and Golden Empress were 80 and 79% under 10°C treatment, respectively (Fig. 1b). From these results, Xinmu No. 1 and Northstar were selected as representative chilling stress-tolerant and stress-sensitive cultivars for further characterization.

Changes in fresh weight and growth of two alfalfa seedlings

To investigate the physiological changes in seedling growth of two alfalfa cultivars (Xinmu No. 1 and Northstar) under chilling stress, we analyzed the fresh weight and length in shoots and roots of alfalfa seedlings at 7 days after imbibition. Under normal conditions, Xinmu No. 1 showed lower levels of fresh weight and length in shoots and roots of seedlings than Northstar (Fig. 2). The fresh weight and length of shoots and roots of both cultivars were significantly inhibited by 10°C treatments. Interestingly, Xinmu No. 1 exhibited a slightly higher biomass and longer length of seedlings under treatment with chilling compared with Northstar.

Changes in H_2O_2 content and lipid peroxidation of two alfalfa seedlings

To investigate the differential oxidative damage derived from chilling stress in chilling-tolerant and chilling-sensitive alfalfa cultivars, we measured the contents of H_2O_2 and lipid peroxidation in alfalfa shoots and roots. Under normal conditions, H_2O_2 contents of Xinmu No. 1 and Northstar were similar (Fig. 3a). The level of H_2O_2 was significantly increased in the shoots and roots of both cultivars under chilling stress condition. However, the level of H_2O_2 in

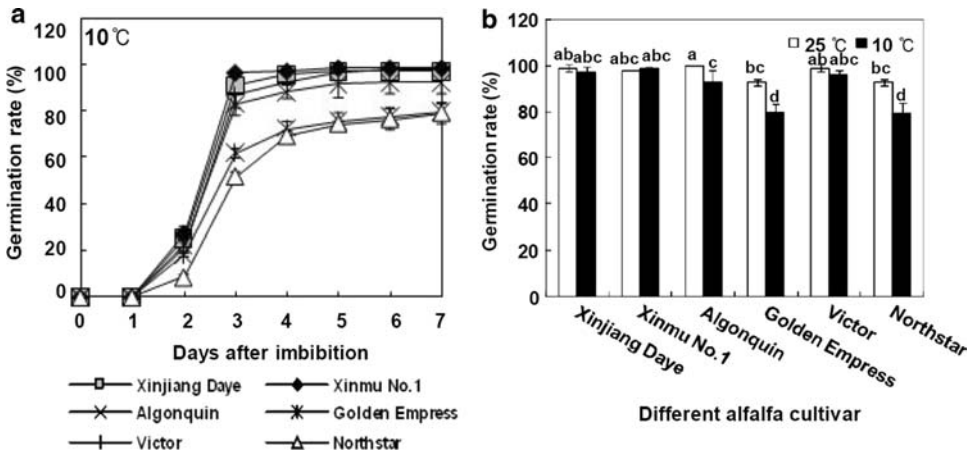

Fig. 1 Seed germination of six alfalfa cultivars under 10°C treatment. **a** Seeds were germinated for 3 days in the dark and 4 days in a 12-h light/dark cycle on half-strength MS medium at 10°C. **b** Germination rate of six alfalfa cultivars under 10°C at day 7. Data are shown as mean ± SD of nine independent measurements. *Bars* carrying the same *letter* are not significantly different ($P = 0.05$) according to Duncan's multiple range test

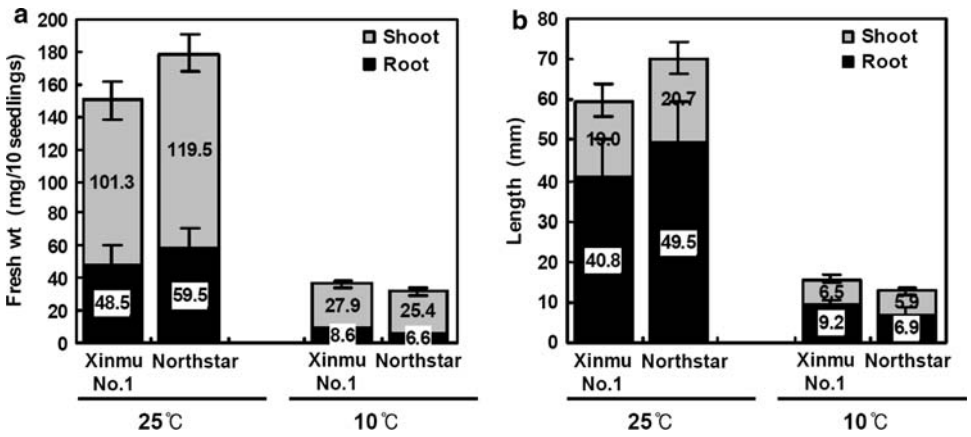

Fig. 2 Changes in plant growth in chilling stress-tolerant and -sensitive alfalfa cultivars under 10°C treatment. **a** Fresh weight of shoots and roots of alfalfa seedlings at 7 days under 10°C treatment. **b** Length of shoots and roots of alfalfa seedlings at 7 days under 10°C treatment. Data are shown as mean ± SD of nine independent measurements

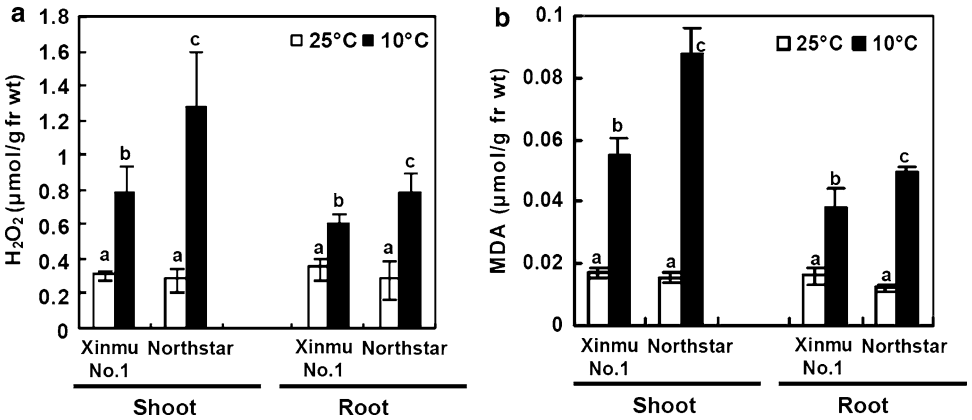

Fig. 3 Changes in H_2O_2 content and MDA levels of chilling stress-tolerant and stress-sensitive alfalfa cultivars under 10°C treatment. **a** H_2O_2 content of shoots and roots of alfalfa seedlings at 7 days under 10°C treatment. **b** MDA levels of shoots and roots of alfalfa seedlings at 7 days under 10°C treatment. Data are shown as mean ± SD of three independent measurements. *Bars* carrying the *same letter* are not significantly different ($P = 0.05$) according to Duncan's multiple range test

shoots and roots subjected to chilling stress was higher in Northstar than in Xinmu No. 1. Similarly, lipid peroxidation assessed by MDA content in alfalfa shoots and roots significantly increased after 7 days under chilling treatment (Fig. 3b). The level of lipid peroxidation was lower in Xinmu No. 1 than in Northstar, under chilling stress condition.

Changes in activities of antioxidant enzymes of two alfalfa seedlings

In order to determine the nature of the antioxidant responses of alfalfa to chilling stress during germination, we measured the enzymatic activity of SOD, APX, CAT, and POD in shoots and roots of seedlings of two cultivars treated with 10°C. As shown in Fig. 4, 10°C treatment increased the activity of SOD, APX, and CAT in only shoots of both cultivars. More precisely, the SOD activity of Xinmu No. 1 shoots was higher than that of Northstar (activity increase was 1.86-fold) at 10°C (Fig. 4a). The two cultivars exhibited a similar SOD activity in control conditions. Similarly, the activity of APX in shoots of both cultivars sharply increased upon treatment with 10°C. Furthermore, Xinmu No. 1 also showed higher APX activity in its shoots, when compared with Northstar (increases of 1.54-fold) (Fig. 4b). The activity of CAT in Xinmu No. 1 shoot tissues was 1.53-fold higher than that in the shoots of Northstar under 10°C treatment (Fig. 4c). However, the activity of CAT in root tissues of both cultivars was not significantly different under chilling stress. In normal conditions, POD activity in the two cultivars was higher in roots than in shoots (Fig. 4d). Treatment with 10°C brought about a significant decrease (~10-fold) in the activity of POD in roots of both cultivars, when compared to control conditions. However, the activities of POD in shoot and root tissues of both cultivars showed similar level under chilling stress.

Discussion

Seed germination is normally limited by increasing strength of abiotic stresses, such as chilling stress. Chilling

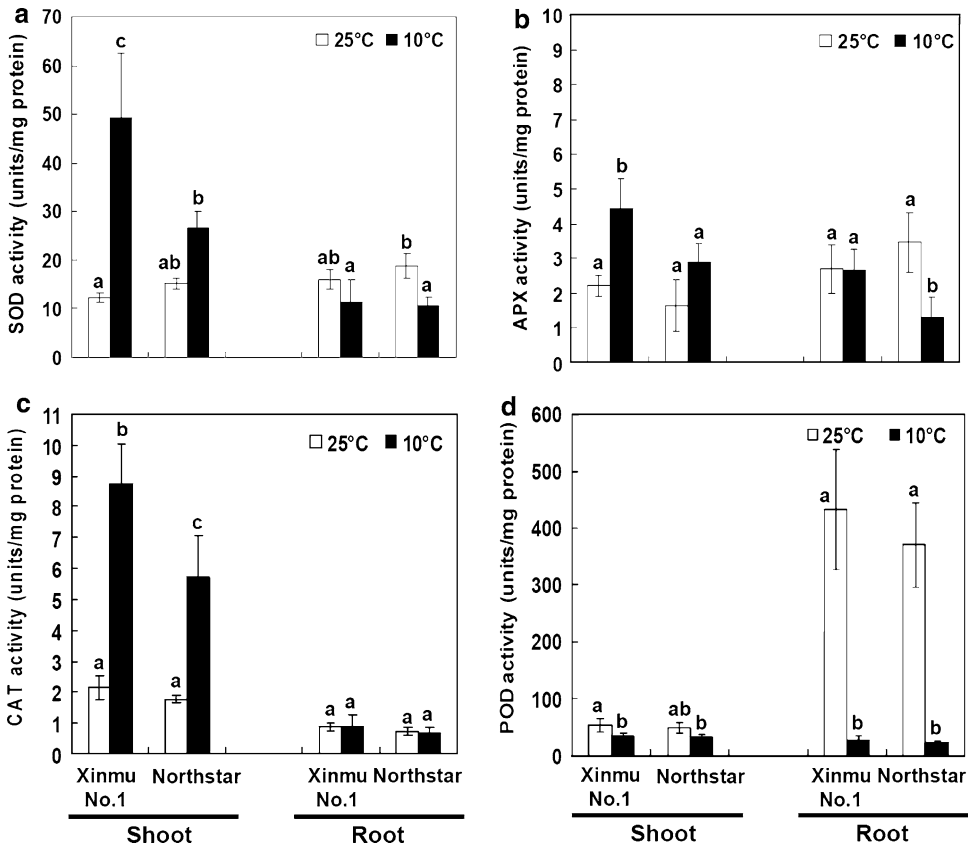

Fig. 4 Changes in antioxidant enzyme activity in chilling stress-tolerant and stress-sensitive alfalfa cultivars after treatment with 10°C. **a** Specific SOD activity, **b** APX activity, **c** CAT activity, and **d** POD activity. Data are shown as mean ± SD of three independent measurements. *Bars* carrying the *same letter* are not significantly different ($P = 0.05$) according to Duncan's multiple range test

tolerance or sensitivity in plants is well correlated with inherent antioxidant responses. Tolerant plant species generally have a better capacity to protect themselves from chilling-induced oxidative stress, via the enhancement of antioxidant enzyme activity (Hodges et al. 1997; Kang and Saltveit 2002; Taka 2004; Huang and Guo 2005). In this study, we observed a significant difference in the germination rate of six alfalfa cultivars under 10°C conditions. To investigate the correlation between the activity of antioxidant enzymes and chilling stress tolerance, we subsequently selected the Xinmu No. 1 variety as a chilling stress-tolerant cultivar and the Northstar variety as a chilling stress-sensitive cultivar. Interestingly, tolerance to chilling stress in Xinmu No. 1 during germination was associated with increased activity of antioxidant enzymes, such as SOD, APX, and CAT in shoot tissue, and the sensitivity of alfalfa to chilling condition was linearly correlated to lower levels of activity of antioxidant enzymes.

H_2O_2 is one type of ROS. Its level during germination of alfalfa seedlings subjected to chilling stress was increased, indicating that oxidative stress occurred. MDA is an indicator of lipid peroxidation and oxidative damages on membranes. Our results showed that lower levels of H_2O_2 and lipid peroxidation increases were observed in Xinmu No. 1 under 10°C, suggesting a better protection from oxidative damage by chilling stress (Fig. 3). Numerous investigations have demonstrated that the cellular injury to plants by chilling stress is the consequence of oxidative damage. It was similarly observed in rice (Huang and Guo 2005; Guo et al. 2006; Morsy et al. 2007) and maize (Hodges et al. 1997; Taka 2004) that chilling-tolerant cultivars showed higher tolerance to oxidative damage.

SOD dismutates O_2^- into H_2O_2 and plays a key role in cellular defense against ROS. In roots, the SOD activity in the two cultivars decreased after the plants were subjected to chilling stress whereas the SOD activity in the two cultivars increased in shoots (Fig. 4a). Higher SOD activity in the chilling-tolerant cultivar indicated higher O_2^- scavenging activity under stress. Our results are consistent with the reports that higher SOD activity has been shown in chilling-tolerant cultivars, such as that of maize (Taka 2004), cucumber (Kang and Saltveit 2002), and rice (Huang and Guo 2005), than in chilling-susceptive cultivars when plants were subjected to chilling stress. H_2O_2 is a toxic ROS to cells. Therefore, it is important that H_2O_2 is scavenged rapidly by the antioxidative defense system. APX, CAT, and POD are primary H_2O_2 scavenging enzymes in various cellular organs such as cytosol, chloroplast, mitochondria, peroxisome, and cell wall. These enzymes have important roles in protecting plants from oxidative damage. Transgenic plants elevating APX

improved the recovery of cotton after chilling treatment (Payton et al. 2001). Matsumura et al. (2002) also reported that transgenic rice expressing CAT also showed a tolerance against chilling injury by the effective detoxification of H_2O_2. In this work, the antioxidant enzymes involved in H_2O_2 scavenging, APX and CAT activities had similar responses to chilling stress (Fig. 4b, c). They increased in both cultivars, but greatly increased in shoots of tolerant cultivars under chilling stress. This result was confirmed by previous observation that APX and CAT activities were higher in chilling stress-tolerant rice than those in sensitive rice under chilling stress (Huang and Guo 2005; Guo et al. 2006). The enhanced scavenging ability for H_2O_2 in tolerant cultivars inhibited the accumulation of ROS and thus protected the plants from lipid peroxidation of membrane systems and oxidative damage under chilling stress. However, POD activity in shoots and roots of the two cultivars decreased when subjected to chilling stress, and both cultivars showed similar levels of POD activity (Fig. 4d). PODs are generally involved not only in scavenging of H_2O_2 but also in diverse plant physiological processes, such as plant growth, development, lignification, and suberization (Passardi et al. 2005). In a previous study, Morsy et al. (2007) reported that a chilling-tolerant rice cultivar showed lower H_2O_2 and MDA level than those of a chilling-sensitive cultivar under chilling condition. Like our study, chilling-tolerant rice exhibited higher CAT activity than that of chilling-sensitive rice, but both cultivars showed a similar POD activity level under chilling stress. These results indicate that APX and CAT have more effective H_2O_2 detoxification capacity than POD during alfalfa germination under chilling stress.

Our data demonstrated that the two alfalfa cultivars showed higher levels of H_2O_2 and lipid peroxidation in shoots than in roots, thereby suggesting that APX and CAT are important for the removal of high concentrations of H_2O_2, particularly in the shoots of alfalfa under chilling stress. Therefore, the enhanced H_2O_2 scavenging ability in the tolerant alfalfa cultivar inhibited the accumulation of ROS and thus protected the plants from lipid peroxidation of membrane systems and oxidative damage under chilling stress during germination. The evidence in this study also suggests an important role for antioxidant enzymes in alfalfa seedling establishment under typical chilling conditions. The levels of various low molecular antioxidants remains to be determined to further elucidate the understanding of the difference in chilling stress tolerance in the two cultivars.

We are currently in the process of generating transgenic alfalfa (cv. Xinmu No. 1) using choline oxidase (*codA*) gene under the control of an oxidative stress-inducible *SWPA2* promoter (Kim et al. 2003; Ahmad et al. 2008). We expect that enhanced stress tolerance of transgenic alfalfa using the Xinmu No. 1 cultivar might be useful for sustainable agriculture in marginal soils, including low temperature and desertification areas.

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