Plant Physiology and Biochemistry 47 (2009) 570–577

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Research article

Analysis of antioxidant enzyme activity during germination of alfalfa under salt and drought stresses

Wen-Bin Wang [a,b], Yun-Hee Kim [b], Haeng-Soon Lee [b], Ki-Yong Kim [c], Xi-Ping Deng [a,*], Sang-Soo Kwak [b,**]

[a] State Key Laboratory of Soil Erosion and Dryland Farming on Loess Plateau, Northwest A&F University, Xinong Road No. 26, Yangling, Shaanxi 712100, P.R.China
[b] Environmental Biotechnology Research Center, Korea Research Institute of Bioscience and Biotechnology (KRIBB), Oun-dong 52, Yuseong-gu, Daejeon, Republic of Korea
[c] National Institute of Animal Science, Rural Development Administration, Republic of Korea

ARTICLE INFO

Article history:
Received 12 September 2008
Accepted 17 February 2009
Available online 28 February 2009

Keywords:
Alfalfa
Antioxidant enzymes
Drought
Germination
Salt

ABSTRACT

To understand the adaptability of alfalfa (*Medicago sativa* L.) to environmental stresses, we analyzed the activity of several antioxidant enzymes, including superoxide dismutase (SOD), peroxidase (POD), ascorbate peroxidase (APX), and catalase (CAT), in alfalfa shoots and roots subjected to salt and drought stresses during germination. The germination rate of six alfalfa cultivars was comparatively studied under 200 mM NaCl or 35% PEG treatment. Alfalfa Xinmu No. 1 and Northstar varieties were selected as stress-tolerant and -sensitive cultivars, respectively, and were used for further characterization. After NaCl or PEG treatment, Xinmu No. 1 showed enhanced seedling growth, compared with Northstar. Xinmu No. 1 also exhibited low levels of hydrogen peroxide (H_2O_2) production and lipid peroxidation, compared with Northstar. In addition, Xinmu No. 1 showed higher enzymatic activity of SOD, APX, CAT, and POD in its shoots and roots than Northstar. These results seem to indicate that Xinmu No. 1 cultivar's tolerance to salt or drought stresses during germination is associated with enhanced activity of antioxidant enzymes. This study highlights the importance of antioxidant enzymes in the establishment of alfalfa seedlings under drought and salinity conditions typical of desertification.

1. Introduction

Drought and high salinity are two major environmental determinants of plant growth and productivity. Desertification and salinization are rapidly increasing on a global scale and currently affect more than 10% of arable land, which results in a decline of the average yields of major crops greater than 50% [6,8]. Therefore, understanding the mechanisms of plant tolerance to drought stress and high salinity is a crucial environmental research topic [3]. Generally, exposure to drought or salt stresses triggers many common reactions in plants that lead to cellular dehydration with concomitant osmotic changes; removal of water from the cytoplasm into the extracellular space results in a decrease of the cytosolic and vacuolar volumes. Another consequence of exposure to these stresses is the generation of reactive oxygen species (ROS), which in turn have a negative oxidative stress effect on cellular structures and metabolism [3].

As water and salt stresses occur frequently and can affect most habitats, plants have developed several strategies to cope with these challenges. One of the stress defense mechanisms is the antioxidant defense system, which includes antioxidant enzymes and low-molecular antioxidants [12,23]. SOD converts superoxide radicals (O_2^-) into hydrogen peroxide (H_2O_2), POD reduces H_2O_2 to water using various substrates as electron donors, APX uses ascorbate as an electron donor to reduce H_2O_2 to water, and CAT dismutates H_2O_2 into water and oxygen. 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 therefore essential to prevent oxidative damage. Numerous studies indicate that the activity of antioxidant enzymes is correlated with plant tolerance to abiotic stresses, including drought and high salt [5,10,34].

As a perennial forage crop, alfalfa (*Medicago sativa* L.) can be cultivated in marginal lands and has a high yield and good quality high-protein content [9,11]. In addition, Alfalfa's deep-root system can help prevent from soil and water loss in semi-dry lands [24]. Previous studies on alfalfa report on antioxidant mechanisms triggered by stress conditions, such as heavy metals [38,39],

Abbreviations: APX, ascorbate peroxidase; CAT, catalase; MDA, malondialdehyde; POD, peroxidase; ROS, reactive oxygen species; SOD, superoxide dismutase.

* Corresponding author. Tel.: +86 29 87012437; fax: +86 29 87012210.
** Corresponding author. Tel.: +82 42 860 4432; fax: +82 42 860 4608.
E-mail addresses: dengxiping@ms.iswc.ac (X.-P. Deng), sskwak@kribb.re.kr (S.-S. Kwak).

0981-9428/$ – see front matter © 2009 Elsevier Masson SAS. All rights reserved.
doi:10.1016/j.plaphy.2009.02.009

drought [27], and pathogen infection [14]. However, comparative analyses of antioxidant modulation in the tissues of alfalfa roots and shoots between stress-tolerant and -sensitive cultivars under various abiotic stress conditions, including high salt and drought, are sparse.

In the present study, to better understand the adaptability of alfalfa plants to salt and drought stresses during germination, we evaluated the stress tolerance of alfalfa cultivars under 200 mM NaCl and 35% PEG treatment by assessing seedling growth, content of H_2O_2, lipid peroxidation, and antioxidant enzyme activity conditions in alfalfa shoots and roots.

2. Materials and methods

2.1. Plant material and culture conditions

Seeds (cv. Xinmu No. 1 and Xinjiang Daye) of alfalfa (*M. 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 Murashige and Skoog (MS) medium (pH 5.7) [25]. These cultures were kept under aseptic conditions for 3 days in the dark and 4 days in 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.

2.2. NaCl and PEG treatment

To mimic salt stress conditions, seeds were incubated on half-strength MS medium including 3% sucrose and 7% Phyto agar with 0 (control) or 200 mM NaCl. To reproduce drought stress conditions, PEG 6000 (polyethylene glycol, Sigma, USA)-infused plates were used, according to a modified version of the method reported in Verslues et al. [35]. PEG-infused plates were prepared by dissolving solid PEG in a sterilized solution of half-strength MS medium with 2 mM MES buffer (pH 5.7), followed by overlaying of the PEG solution onto agar-solidified half-strength MS medium plates containing 7% Phyto agar. The agar medium and PEG solution were equilibrated for at least 12 h before the excess PEG solution was removed. Drought stress strength was expressed as the concentration of the overlayed PEG solution: 0% (control) or 35% (drought stress). The addition of high concentration of sucrose can in itself induce an osmotic response; we thus used half-strength MS medium without sucrose. For salt treatment, 3% sucrose is typically used to stimulate root elongation and allow easier detection of differences between cultivars of different genotypes [35].

2.3. Analysis of germination rate and seedling weight and length

A germination test was conducted using six alfalfa cultivars germinated on half-strength MS media. Three replicates (50 seeds per cultivar) were germinated on similar medium conditions, with NaCl or PEG treatment. Germination rate was determined by counting the number of germinated seeds for 7 days, at 24 h

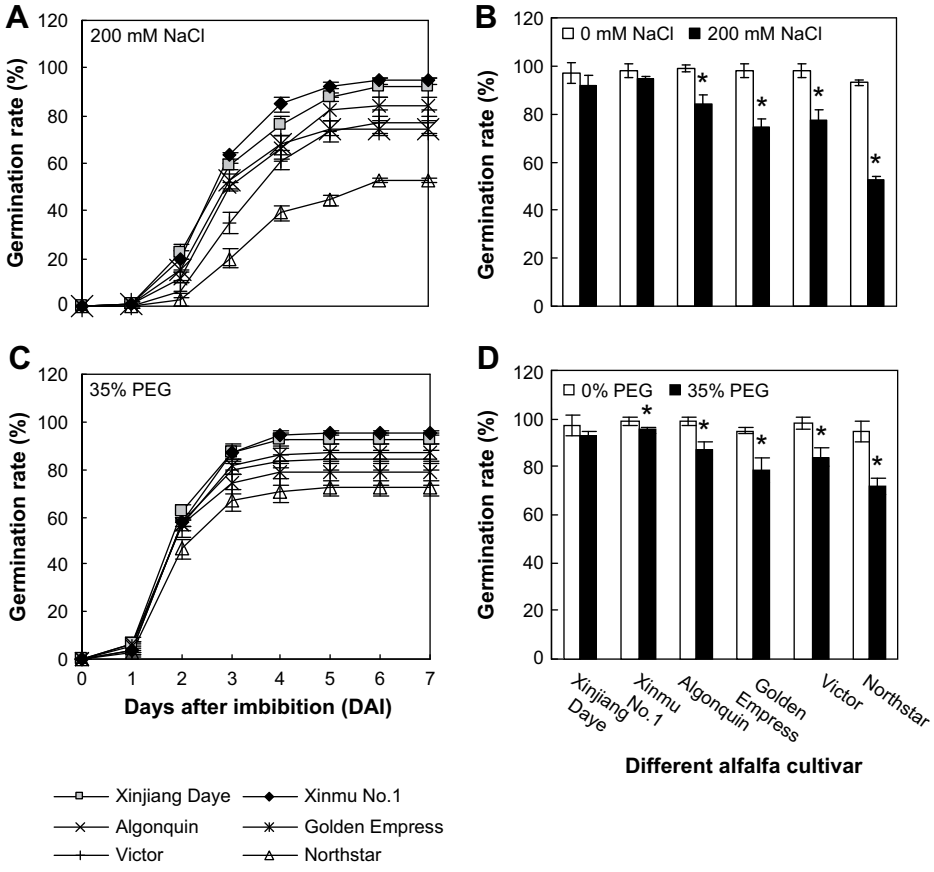

Fig. 1. Seed germination of six alfalfa cultivars under 200 mM NaCl or 35% PEG treatment. Seeds were *germinated* for 3 days in the dark and 4 days in 12-h light/dark cycle on half-strength MS medium containing 200 mM NaCl (A). Germination rate of six alfalfa cultivars under 200 mM NaCl stress at day 7 (B). Seeds were *germinated* on half-strength MS medium equilibrated with 35% PEG for 7 days (C). Germination rate of six alfalfa varieties under 35% PEG stress at day 7 (D). Data are shown as mean ± SD of nine independent measurements. Asterisk indicates that mean values were significantly different between the treatments and the control ($P < 0.05$).

intervals. Alfalfa seeds were considered to have germinated when the radical visibly protruded from the seed coat by at least 2 mm [16].

The length of shoots and roots was measured in 120 7-day-old seedlings from each cultivar grown under control, 200 mM NaCl, or 35% PEG 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) [35]. The seedlings were photographed, separated into shoots or roots for measuring of fresh weight [33], and then immediately frozen in liquid nitrogen and stored at −70 °C for biochemical analysis.

2.4. Analysis of H_2O_2 content

The H_2O_2 content in alfalfa roots and shoots 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 [4]. H_2O_2 content was expressed as μmol of H_2O_2 per gram of fresh weight of plant tissue.

2.5. Analysis of lipid peroxidation

Lipid peroxidation was measured using a modified thiobarbituric acid (TBA) method [29]. 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.

2.6. Determination of total protein content and 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 [7] using the Bio-Rad protein assay reagent. The activity of SOD was measured according to a method using xanthine, xanthine oxidase, and cytochrome *c* [22]. 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 [26], 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 μM of ascorbate. The activity of POD was assayed according to the method described by Kwak et al. [19], 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. CAT activity was assayed according to the method described by Aebi [1], by measuring the decrease at 240 nm for 1 min, due to H_2O_2 consumption.

2.7. 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. All measurements were subjected to analysis

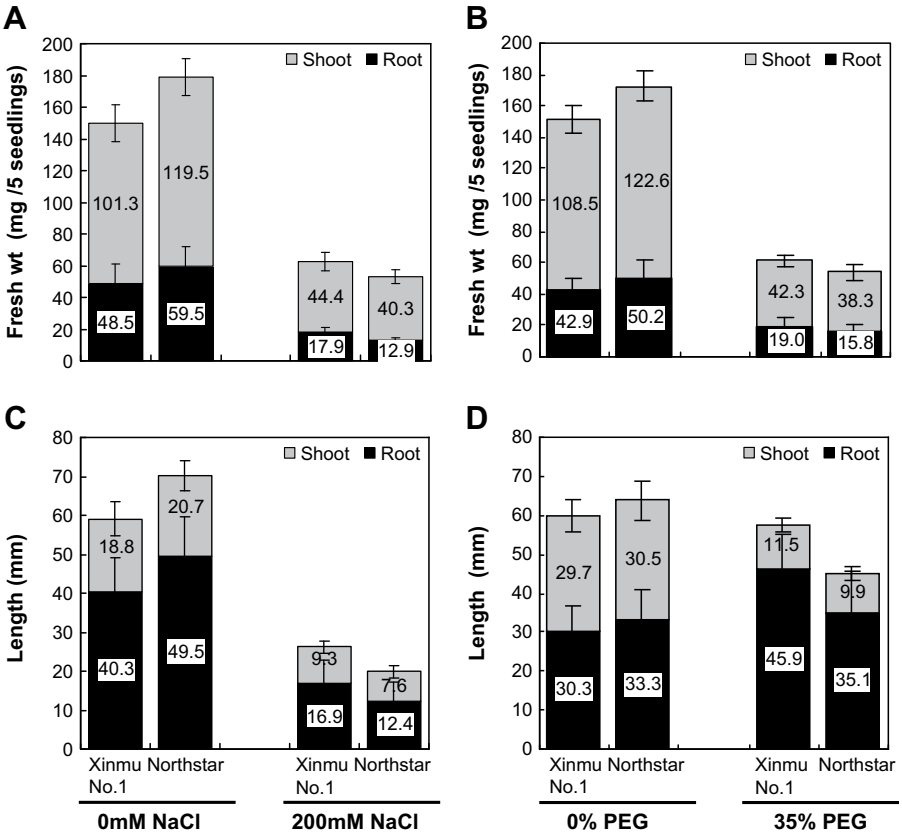

Fig. 2. Changes in fresh weight and length of stress-tolerant and -sensitive alfalfa cultivars under 200 mM NaCl and 35% PEG treatment. Fresh weight of shoots and roots of alfalfa seedlings at 7 days under 200 mM NaCl (A) or 35% PEG (B) treatment. Length of shoots and roots of alfalfa seedlings at 7 days under 200 mM NaCl (C) or 35% PEG (D) treatment. Data are shown as mean ± SD of nine independent measurements.

of variance (ANOVA) to discriminate significant differences (defined as $P \leq 0.05$). Data are shown as the mean ± standard deviation (SD).

3. Results

3.1. Germination analysis of six alfalfa cultivars

To evaluate the differential responses of six alfalfa cultivars to salt and drought stresses during germination, we analyzed the germination rate under stresses corresponding to different concentrations of NaCl (0, 100, 200, 300, and 400 mM) or PEG (0, 10, 20, 30, 35, and 40%) (data not shown). For further experiments, we adopted 200 mM NaCl and 35% PEG for salt stress and drought stress, respectively.

The germination rate in six alfalfa cultivars was differently affected by treatments with 200 mM NaCl and 35% PEG (Fig. 1). Under stress treatment conditions, 200 mM NaCl treatment had a faster effect than 35% PEG treatment (Fig. 1A and C). The germination rate of the six cultivars was delayed or inhibited after 7 days of germination under salt or drought stress. Among the alfalfa varieties, two cultivars, Xinmu No. 1 and Xinjang Daye, germinated faster than the other cultivars and showed the highest germination rate. In contrast, the Northstar cultivar showed the lowest germination rate, under the same conditions. At 7 days after imbibition, the germination rate of Xinjang Daye and Xinmu No. 1 was approximately 90–95%, whereas that of Northstar was only 52.7% under 200 mM NaCl treatment and 72% under 35% PEG treatment (Fig. 1B and D).

These results indicate that Xinmu No. 1 is a cultivar tolerant to high salt and drought stresses, while Northstar is sensitive to the same stresses, during the germination stage. Therefore, among the six alfalfa varieties examined, these two stress-tolerant and -sensitive cultivars were used for further characterization.

3.2. Changes in fresh weight and growth of two alfalfa seedlings

To investigate the physiological changes in two alfalfa seedlings under salt and drought stresses, 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). However, the fresh weight and length of shoots and roots of both cultivars were significantly inhibited by 200 mM NaCl or 35% PEG treatments. Interestingly, Xinmu No. 1 exhibited a slightly higher biomass and longer length of seedlings under treatment with NaCl or PEG, when compared with Northstar.

3.3. Changes in H_2O_2 content and lipid peroxidation of two alfalfa seedlings

To investigate the differential oxidative damage brought about salt and drought stresses in stress-tolerant and -sensitive alfalfa cultivars, we measured the contents of H_2O_2 and lipid peroxidation in alfalfa roots and shoots (Fig. 3). Under normal conditions, H_2O_2 contents of Xinmu No. 1 and Northstar were similar ($P > 0.05$). However, the level of H_2O_2 was significantly increased in the roots and shoots of both cultivars under all experimental stress conditions, with the exception of the roots of Xinmu No. 1 treated with 200 mM NaCl (Fig. 3A and B). In addition, the level of H_2O_2 in roots and shoots subjected to salt and drought stresses was higher in Northstar than in Xinmu No. 1.

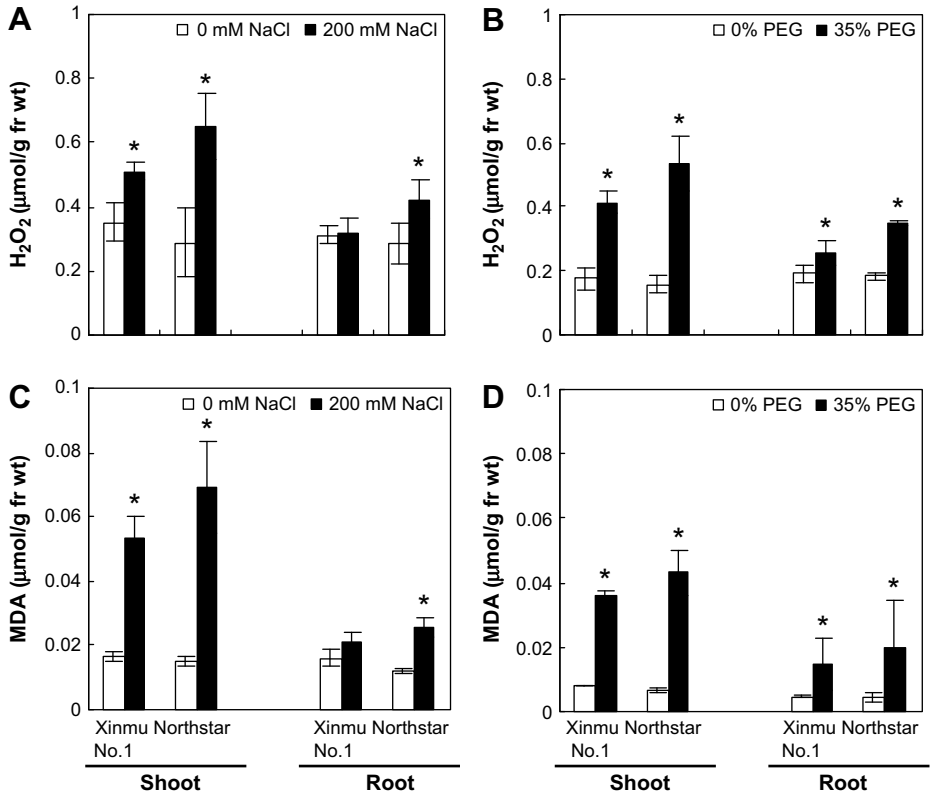

Fig. 3. Changes in H_2O_2 content and MDA levels of stress-tolerant and -sensitive alfalfa cultivars under 200 mM NaCl and 35% PEG treatment. H_2O_2 content of shoots and roots of alfalfa seedlings at 7 days under 200 mM NaCl (A) or 35% PEG (B) treatment. MDA levels of shoots and roots of alfalfa seedlings at 7 days under 200 mM NaCl (C) or 35% PEG (D) treatment. Data are shown as mean ± SD of three independent measurements. Asterisk indicates that mean values were significantly different between the treatments and the control ($P < 0.05$).

Similarly, lipid peroxidation assessed by MDA content in alfalfa roots and shoots significantly increased after 7 days under salt and drought treatment, with the exception of the roots of Xinmu No. 1 subjected to 200 mM NaCl stress (Fig. 3C and D). The level of lipid peroxidation was lower in Xinmu No. 1 than in Northstar under all stress conditions.

3.4. Changes in activities of antioxidant enzymes of two alfalfa seedlings

In order to determine the nature of the antioxidant responses of alfalfa to salt and drought stresses during germination, we measured the enzymatic activity of SOD, APX, POD, and CAT in shoots and roots of seedlings of two cultivars treated with 200 mM NaCl or 35% PEG.

As shown in Fig. 4A, 200 mM NaCl treatment increased the activity of SOD in shoots and roots of both cultivars. More precisely, the SOD activity of Xinmu No. 1 shoots and roots was higher than that of Northstar (activity increases were 2.19-fold and 1.25-fold, respectively). However, the two cultivars exhibited a similar SOD activity in control conditions. Similarly, the activity of APX in shoots and roots of both cultivars sharply increased upon treatment with 200 mM NaCl. Furthermore, Xinmu No. 1 also showed higher APX activity in its shoots and roots, when compared with Northstar (increases of 1.59-fold and 1.48-fold, respectively) (Fig. 4B). In normal conditions, POD activity in the two cultivars was higher in roots than in shoots (Fig. 4C). In contrast, treatment with 200 mM NaCl brought about a significant decrease (~50%) in the activity of POD in Northstar roots, when compared to control conditions, whereas that of Xinmu No. 1 was only slightly decreased, displaying an activity 1.96-fold higher than that of Northstar. The activity of CAT in Xinmu No. 1 shoot tissues was 1.59-fold higher than the CAT activity found in the shoots of Northstar under 200 mM NaCl treatment (Fig. 4D). However, the activity of CAT in root tissues of both cultivars was not significantly different under salt stress.

The 35% PEG stress also significantly increased SOD activity in Xinmu No. 1 and Northstar cultivars, with the exception of shoots of Northstar (Fig. 5A). The SOD activity of shoots and roots in Xinmu No. 1 was 1.75-fold and 1.66-fold higher, respectively, than in Northstar. The APX activity in both tissues of Xinmu No. 1 was slightly higher than that of Northstar, after PEG treatment (Fig. 5B). In addition, 35% PEG treatment decreased the POD activity in the roots of both cultivars, but the POD activity in Xinmu No. 1 was 1.68-fold higher than that of Northstar (Fig. 5C). Similarly to what was observed for the salt treatment, the CAT activity in shoots of Xinmu No. 1 was 2.67-fold higher than that of Northstar, under PEG treatment (Fig. 5D).

4. Discussion

Seed germination is normally limited by increasing strength of abiotic stresses, such as high salinity and drought. Salinity and drought tolerance or sensitivity in plants is well-correlated with inherent antioxidant responses. Tolerant plant species generally have a better capacity to protect themselves from salt- or drought-induced oxidative stress, via the enhancement of antioxidant enzyme activity [30,34]. In this study, we observed a significant difference in the germination rate of six alfalfa cultivars under 200 mM NaCl or 35% PEG stress conditions. To investigate the correlation between the activity of antioxidant enzymes and

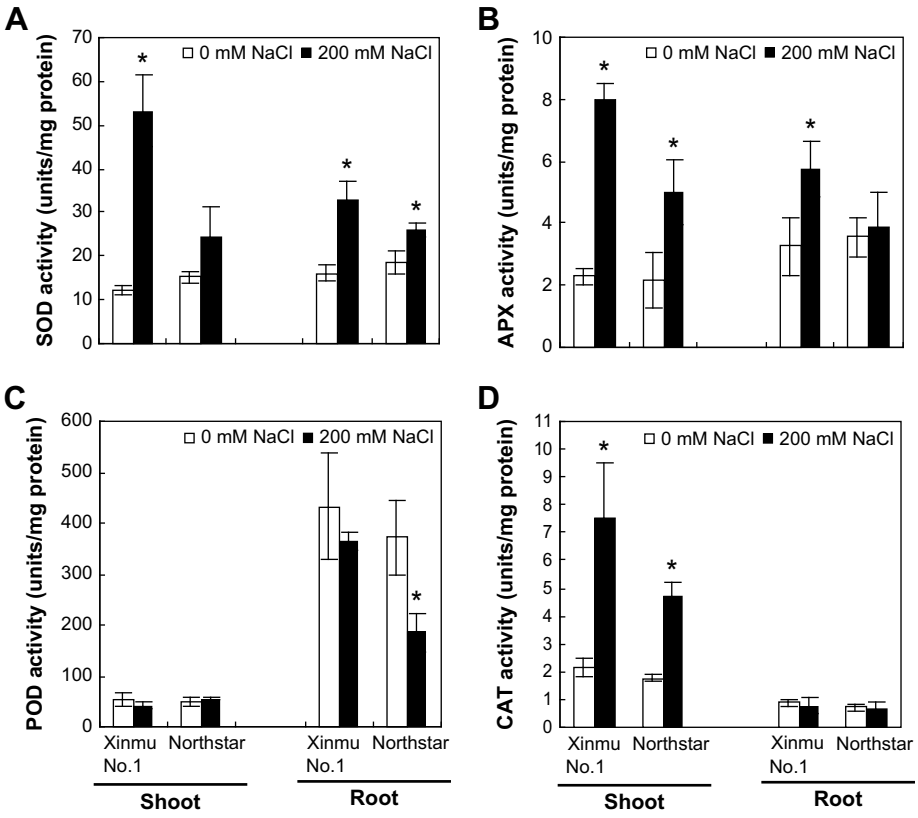

Fig. 4. Changes in antioxidant enzyme activity in stress-tolerant and -sensitive alfalfa cultivars after treatment with 200 mM NaCl. Specific SOD activity (A), APX activity (B), POD activity (C), and CAT activity (D). Data are shown as mean ± SD of three independent measurements. Asterisk indicates that mean values were significantly different between the treatments and the control ($P < 0.05$).

stress tolerance, we subsequently selected the Xinmu No. 1 variety as a stress-tolerant cultivar and the Northstar variety as a stress-sensitive cultivar. Interestingly, tolerance to salt or drought stresses in Xinmu No. 1 during germination was associated with increase activity of antioxidant enzymes, and the sensitivity of alfalfa to salt and drought was linearly correlated to lower levels of activity of antioxidant enzymes.

Environmental stresses, including high salinity and drought, cause oxidative stress via the production and accumulation of ROS. H_2O_2 is a ROS that can be adequately controlled by antioxidants and antioxidant enzymes, under normal conditions. However, a risk for serious cellular damage may arise when ROS are overproduced under stress conditions [39]. In addition, free radical-induced peroxidation in lipid membrane is both a reflection and a measure of stress-induced damage at the cellular level [10,34]. Lower levels of H_2O_2 and lipid peroxidation were observed in Xinmu No. 1 tissues treated with NaCl or PEG, suggesting an enhanced capacity for protection from oxidative damage by salt or drought stresses in this alfalfa variety (Fig. 3). Similar results were reported in *M. sativa* L. and *Medicago truncatula* L., since drought [27], heavy metal [38,39], or O_3 [29] stress-tolerant cultivars of these species exhibited a higher tolerance to oxidative damage under stress.

To cope with oxidative damage under extremely adverse conditions, plants have developed an antioxidant defense system that includes the antioxidant enzymes SOD, APX, POD, and CAT [12,23]. The levels of antioxidant enzymes are higher in tolerant than in sensitive species under various environmental stresses [5,10,34]. Accordingly, we also found higher SOD activity in our stress experimental conditions (Figs. 4A and 5A). In the present study, a higher SOD activity was observed in Xinmu No. 1, compared with Northstar under salt or drought stress, which suggests that the salt- and drought-tolerant alfalfa cultivar possesses a better O_2^- scavenging ability. These results are also in agreement with reports of increased SOD activity in salt-tolerant cultivars of pea [13], sugar beet [5], and tomato [18] under salt stress, and drought-tolerant cultivars of maize [15] and common bean [34].

H_2O_2, a toxic species, is a byproduct of the activity of SOD to prevent cellular damage, and must be eliminated by conversion to H_2O in subsequent reactions involving APX, POD, and CAT, which regulate H_2O_2 levels in plants. We found a significant increase of APX activity in Xinmu No. 1 under NaCl treatment, and a slight increase in its activity under PEG treatment (Figs. 4B and 5B). APX is a component of the ascorbate-glutathione pathway, which plays a key role in scavenging H_2O_2 [12,23]. Lower levels of lipid peroxidation are associated with higher APX activity in drought- or salt-tolerant tomato [31], sugar beet [5], and rice [10] plants.

Interestingly, our data showed that POD activity in roots (Figs. 4C and 5C) and CAT activity in shoots (Figs. 4D and 5D) of Xinmu No. 1 were higher than Northstar, under salt and drought stresses. Furthermore, the elevated POD activity in Xinmu No. 1 roots treated with NaCl or PEG may reflect an increased ROS scavenging capacity and decreased damage to lipids of the plasma membrane under stress conditions. PODs are involved not only in scavenging H_2O_2 but also in plant growth, development, lignification, suberization, and cross-linking of cell wall compounds [28]. Salt- or drought-tolerant plants often have higher POD activity than sensitive plants under stress conditions; this is true for salt-tolerant tomato [31] and *Plantago martima* [30], and drought-tolerant common bean

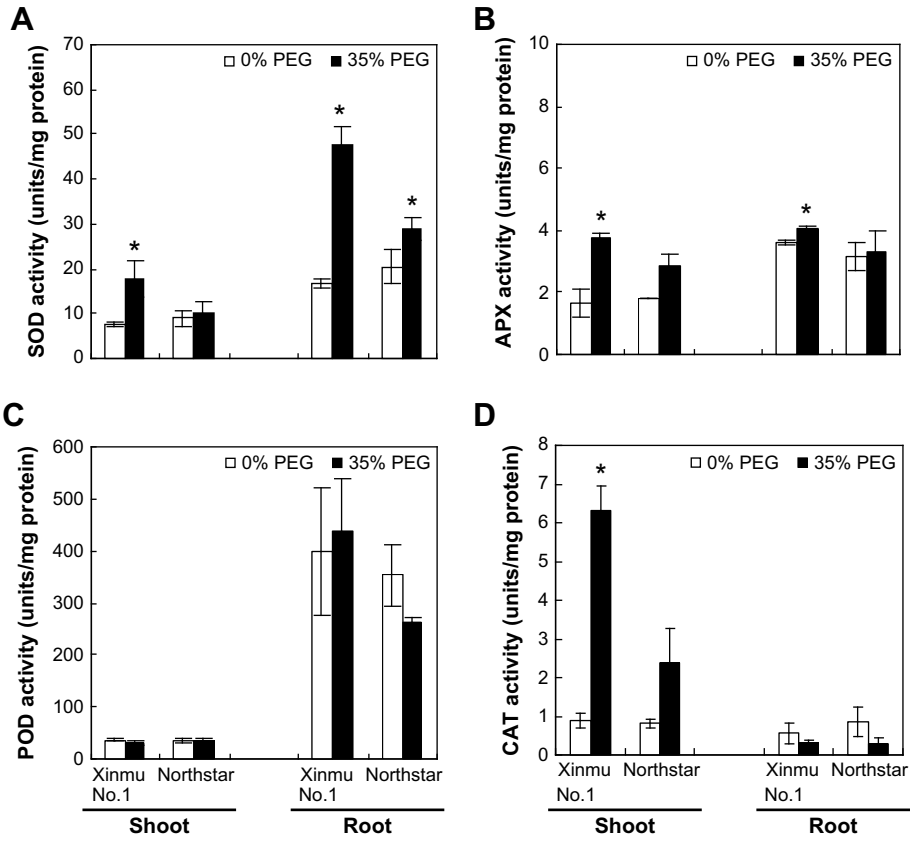

Fig. 5. Changes in antioxidant enzyme activity in stress-tolerant and -sensitive alfalfa cultivars after treatment with 35% PEG. Specific SOD activity (A), APX activity (B), POD activity (C), and CAT activity (D). Data are shown as mean ± SD of three independent measurements. Asterisk indicates that mean values were significantly different between the treatments and the control ($P < 0.05$).

[34], sunflower [37], and sorghum [37]. CAT eliminates H_2O_2 by breaking it down directly to form water and oxygen. Thus, this enzyme does not require a reducing power and has a high reaction rate but a low affinity for H_2O_2, thereby only removing the high concentration of H_2O_2 [36]. 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 CAT is important for the removal of high concentrations of H_2O_2 particularly in the shoots of alfalfa under NaCl and PEG stresses. 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 damages under salt or drought stress during germination. The evidences in this study also suggest an important role for antioxidant enzymes in alfalfa seedling establishment under the drought and salinity conditions typical of desertification. The levels of various low-molecular antioxidants remain to be determined to further elucidate the understanding of the difference of stress tolerance in two cultivars.

In a previous study, we isolated a strong oxidative stress-inducible POD (*SWPA2*) promoter from cultured cells of sweet potato and characterized its function in transgenic tobacco plants and cultured cells, under environmental stress conditions [17]. Accumulative evidence suggests that a stress-inducible *SWPA2* promoter is applicable to the development of stress-tolerant transgenic varieties in other plant systems, such as sweet potato, potato, and tall fescue plants [2,20,21,32]. Recently, transgenic potato plants overexpressing the *NDPK2* [32] or the *codA* [2] genes under the control of the *SWPA2* promoter resulted in an acquired higher tolerance to methyl viologen (MV)-mediated oxidative stress, as well as to salt, drought, and extreme temperature stresses. Therefore, we are currently in the process of generating transgenic alfalfa (cv. Xinmu No. 1) overexpressing *NDPK2* and *codA* under the control of the *SWPA2* promoter. We expect that transgenic alfalfa with enhanced stress tolerance might be useful for sustainable agriculture in marginal soils, including desertified areas and alkalinized soils.

Acknowledgments

This study was supported by the Project of Knowledge Innovation Engineering of the Chinese Academy of Sciences (KZCX3-SW-444), the Plan for Outstanding Personnel of Northwest A&F University, National Basic Research Program of China (2009CB118604), the Korea Foundation for International Cooperation of Science & Technology (KICOS), MEST, and KRIBB Initiative Program.

References

[1] H. Aebi, Catalase in vitro, Meth. Enzymol. 105 (1984) 121–126.
[2] R. Ahmad, M.D. Kim, K.H. Back, H.S. Kim, H.S. Lee, S.Y. Kwon, N. Murata, W.I. Chung, S.S. Kwak, Stress-induced expression of choline oxidase in potato plant chloroplasts confers enhanced tolerance to oxidative, salt, and drought stresses, Plant Cell Rep. 27 (2008) 687–698.
[3] D. Bartels, R. Sunkar, Drought and salt tolerance in plants, Crit. Rev. Plant Sci. 24 (2005) 23–58.
[4] L.V. Bindschedler, F. Minibayeva, S.L. Gardner, C. Gerrish, D.R. Davies, P.G. Bolwell, Early signalling events in the apoplastic oxidative burst in suspension cultured French bean cells involve cAMP and Ca^{2+}_2, New Phytol. 151 (2001) 185–194.
[5] M. Bor, F. Ozdemir, I. Turkan, The effect of salt stress on lipid peroxidation and antioxidants in leaves of sugar beet *Beta vulgaris* L. and wild beet *Beta maritima* L. Plant Sci. 164 (2003) 77–84.
[6] J.S. Boyer, Plant productivity and environment, Science 218 (1982) 443–448.
[7] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding, Anal. Biochem. 72 (1976) 248–254.
[8] E.A. Bray, Plant responses to water deficit, Trends Plant Sci. 2 (1997) 48–54.
[9] T.P. Croughan, S.J. Stavarek, D.W. Rains, Selection of NaCl tolerant of cultured alfalfa cells, Crop Sci. 18 (1978) 959–963.
[10] T. Demiral, I. Turkan, Comparative lipid peroxidation, antioxidant systems and proline content in roots of two rice cultivars differing in salt tolerance, Environ. Exp. Bot. 53 (2005) 247–257.
[11] A.A. Ehsanpour, N. Fatahian, Effects of salt and proline on *Medicago sativa* callus, Plant Cell Tiss. Org. 73 (2003) 53–56.
[12] C.H. Foyer, G. Noctor, Oxidant and antioxidant signalling in plants: a re-evalu ation of the concept of oxidative stress in a physiological context, Plant Cell Environ. 28 (2005) 1056–1071.
[13] J.A. Hernandez, M.S. Almansa, Short-term effects of salt stress on antioxidant systems and leaf water relations of pea leaves, Physiol. Plant. 115 (2002) 251–257.
[14] W. Huang, J. Zhikuan, Q.F. Han, Effects of herbivore stress by *Aphis medicaginis* Koch on the Malondialdehyde contents and the activities of protective enzymes in different alfalfa varieties, Acta Ecol. Sin. 27 (2007) 2177–2183.
[15] V. Jagtap, S. Bhargava, Variation in the antioxidant metabolism of drought tolerant and drought susceptible varieties of *Sorghum bicolor* (L.) Moench. Exposed to high light, low water and high temperature stress, J. Plant Physiol. 145 (1995) 195–197.
[16] H.J. Kim, H. Feng, M.M. Kushad, X. Fan, Effect of ultrasound, irradiation, and acidic electrolyzed water on germination of alfalfa and broccoli seeds and *Escherichia coli* O157:H7, J. Food Sci. 71 (2006) 168–173.
[17] K.Y. Kim, S.Y. Kwon, H.S. Lee, Y. Hur, J.W. Bang, S.S. Kwak, A novel oxidative stress-inducible peroxidase promoter from sweetpotato: molecular cloning and characterization in transgenic tobacco plants and cultured cells, Plant Mol. Biol. 51 (2003) 831–838.
[18] H. Koca, F. Ozdemir, I. Turkan, Effect of salt stress on lipid peroxidation and superoxide dismutase and peroxidase activities of *Lycopersicon esculentum* and *L. pennellii*, Biol. Plant. 50 (2006) 745–748.
[19] S.S. Kwak, S.K. Kim, M.S. Lee, K.H. Jung, I.H. Park, J.R. Liu, Acidic peroxidases from suspension-cultures of sweet potato, Phytochemistry 39 (1995) 981–984.
[20] S.H. Lee, N. Ahsan, K.W. Lee, D.H. Kim, D.G. Lee, S.S. Kwak, S.Y. Kwon, T.H. Kim, B.H. Lee, Simultaneous overexpression of both CuZn superoxide dismutase and ascorbate peroxidase in transgenic tall fescue plants confers increased tolerance to a wide range of abiotic stresses, J. Plant Physiol. 164 (2007) 1626–1638.
[21] S. Lim, Y.H. Kim, S.H. Kim, S.Y. Kwon, H.S. Lee, J.S. Kim, K.Y. Cho, K.Y. Paek, S.S. Kwak, Enhanced tolerance of transgenic sweetpotato plants that express both CuZnSOD and APX in chloroplasts to methyl viologen-mediated oxidative stress and chilling, Mol. Breed. 19 (2007) 227–239.
[22] J.M. McCord, I. Fridovich, Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein), J. Biol. Chem. 244 (1969) 6049–6055.
[23] R. Mittler, Oxidative stress, antioxidants and stress tolerance, Trends Plant Sci. 7 (2002) 405–410.
[24] J.F. Moran, M. Becana, I. Iturbe-Ormaetxe, S. Frechilla, R.V. Klucas, P. Aparicio-Tejo, Drought induces oxidative stress in pea plants, Planta 194 (1994) 346–352.
[25] T. Murashige, F. Skoog, A revised medium for rapid growth and bioassays with tobacco cultures, Physiol. Plant. 15 (1962) 473–479.
[26] Y. Nakano, K. Asada, Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts, Plant Cell Physiol. 22 (1981) 867–880.
[27] L. Naya, R. Ladrera, J. Ramos, E.M. Gonzalez, C. Arrese-Igor, F.R. Minchin, M. Becana, The response of carbon metabolism and antioxidant defenses of alfalfa nodules to drought stress and to the subsequent recovery of plants, Plant Physiol. 144 (2007) 1104–1114.
[28] F. Passardi, C. Cosio, C. Penel, C. Dunand, Peroxidases have more functions than a Swiss army knife, Plant Cell Rep. 24 (2005) 255–265.
[29] M.C. Puckette, H. Weng, R. Mahalingam, Physiological and biochemical responses to acute ozone-induced oxidative stress in *Medicago truncatula*, Plant Physiol. Biochem. 45 (2007) 70–79.
[30] A.H. Sekmen, I. Turkan, S. Takio, Differential responses of antioxidative enzymes and lipid peroxidation to salt stress in salt-tolerant *Plantago maritima* and salt-sensitive *Plantago media*, Physiol. Plant. 131 (2007) 399–411.
[31] A. Shalata, M. Tal, The effect of salt stress on lipid peroxidation and antioxidants in the leaf of the cultivated tomato and its wild salt-tolerant relative *Lycopersicon pennellii*, Physiol. Plant. 104 (1998) 169–174.
[32] L. Tang, M.D. Kim, K.S. Yang, S.Y. Kwon, S.H. Kim, J.S. Kim, D.J. Yun, S.S. Kwak, H.S. Lee, Enhanced tolerance of transgenic potato plants overexpressing nucleoside diphosphate kinase 2 against multiple environmental stresses, Transgenic Res. 17 (2008) 705–715.
[33] M.A. Turk, M.K. Shatnawi, A.M. Tawaha, Inhibitory effects of aqueous extracts of black mustard on germination and growth of alfalfa, Weed Biol. Manage. 3 (2003) 37–40.
[34] I. Turkan, M. Bor, F. Ozdemir, H. Koca, Differential responses of lipid peroxidation and antioxidants in the leaves of drought-tolerant *P. acutifolius* Gray and drought-sensitive *P. vulgaris* L. subjected to polyethylene glycol mediated water stress, Plant Sci. 168 (2005) 223–231.
[35] P.E. Verslues, M. Agarwal, S. Katiyar-Agarwal, J. Zhu, J.K. Zhu, Methods and concepts in quantifying resistance to drought, salt and freezing, abiotic stresses that affect plant water status, Plant J. 45 (2006) 523–539.

[36] H. Willekens, S. Chamnongpol, M. Davey, M. Schraudner, C. Langebartels, M. Van Montagu, D. Inze, W. Van Camp, Catalase is a sink for H_2O_2 and is indispensable for stress defence in C3 plants, EMBO J. 16 (1997) 4806–4816.
[37] J. Zhang, M.B. Kirkham, Antioxidant responses to drought in sunflower and sorghum seedlings, New Phytol. 132 (1996) 361–373.
[38] Z.S. Zhou, S.Q. Huang, K. Guo, S. Kant Mehta, P.C. Zhang, Z.M. Yang, Metabolic adaptations to mercury-induced oxidative stress in roots of *Medicago sativa* L. J. Inorg. Biochem. 101 (2007) 1–9.
[39] Z.S. Zhou, S.J. Wang, Z.M. Yang, Biological detection and analysis of mercury toxicity to alfalfa (*Medicago sativa*) plants, Chemosphere 70 (2008) 1500–1509.