

## Exogenous spermidine improves water stress tolerance of white clover (*Trifolium repens* L.) involved in antioxidant defence, gene expression and proline metabolism

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### Abstract

Spermidine (Spd) may be involved in the regulation of plant adaptation to drought stress. The objectives of the study were to identify the physiological effect and elucidate the possible mechanism caused by exogenous Spd (0.05 mM) in white clover under water stress induced by 20% polyethylene glycol 6000 for 12 days. Water stress elevated significantly the accumulation of reactive oxygen species and malonaldehyde, and resulted in the decrease of cell membrane stability, relative water content and relative growth rate. Spd effectively alleviated the damage effect from water stress. Spd-treated plants showed a promoted the ascorbate–glutathione cycle and maintained greater antioxidant enzyme activities (superoxide dismutase, peroxidase and catalase), as well as higher transcript level of genes encoding antioxidant enzymes than untreated plants. Additionally, the plants treated with Spd under water stress exhibited more accumulated organic solutes including soluble sugar, reducing sugar, betaine and free proline. Spd also accelerated proline catabolism and biosynthesis proceeding from glutamate pathway during water stress, but had no effect on the ornithine pathway of proline biosynthesis. These results suggest that exogenous Spd under water stress may directly or indirectly regulate antioxidant defense system, organic solutes accumulation and proline metabolism.

**Keywords:** Antioxidant system; Gene expression; Drought stress; Organic solute; Reactive oxygen species; White clover (*Trifolium repens* L.).

**Abbreviation:** APX\_Ascorbate peroxidase; CAT\_Catalase; DR\_Dehydroascorbate reductase; EL\_Electrolyte leakage; GR\_Glutathione reductase; H<sub>2</sub>O<sub>2</sub>\_Hydrogen peroxide; LSD\_The least significant difference; MDA\_Malondialdehyde; MR\_Monodehydroascorbate reductase; O<sub>2</sub><sup>-</sup>\_Superoxide anion radical; OAT\_Ornithine aminotransferase; P5CS\_Δ<sup>1</sup>-pyrroline-5-carboxylate synthetase; PEG 6000\_Polyethylene glycol 6000; POD\_Peroxidase; ProDH\_Proline dehydrogenase; RGR\_Relative growth rate; ROS\_Reactive oxygen species; RWC\_Relative water content; SOD\_Superoxide dismutase; Spd\_Spermidine.

### Introduction

Drought is one of the most detrimental abiotic stress factors for plant growth in water-limiting environments. Under drought stress, Growth and function suppression in plants involves many morphological, physiological and molecular changes including oxidative stress, metabolic disturbance and breakage of DNA (Khanna-Chopra and Selote, 2007; Xu et al., 2011; Li et al., 2013). Reactive oxygen species (ROS) including superoxide radicals (O<sub>2</sub><sup>-</sup>), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and hydroxyl radicals (·OH) can seriously damage plants as a result of lipid peroxidation and cell death under drought stress (Hendry, 1993; Tambussi et al., 2000), and therefore, the most important bases responsible for stress tolerance in plants should be antioxidant including enzymatic and nonenzymatic constituents (Ma et al., 2013). Furthermore, osmotic adjustments were regarded in generally as a mechanism for long-term acclimation in plants under drought stress (Hasegawa et al., 2000). The accumulation of these compatible solutes, such as soluble sugar, betaine and free proline, is one of the most important responses on water deficit (Morgan, 1984). Many previous studies have implied the importance of maintaining a favorable water status and antioxidative level for adaptation to drought stress in plants (Karsten and Macadam, 2001; Chen and Murata, 2002; Sharma and Dubey, 2005; DaCosta and Huang, 2007; Li and Peng, 2012).

Polyamines (PAs), such as putrescine (Put), spermidine (Spd) and spermine (Spm), are ubiquitous low-molecular-weight aliphatic amines and involved in various physiological changes associated with the regulation of plant growth and development (Martin-Tannguy, 2001; Roychoudhury et al., 2011). More and more evidences proved that spermidine was participating in the regulation of plant responses to diverse abiotic stresses like drought or osmotic stress, salinity, heat and chilling through directly binding to membrane phospholipids, osmotic adjustment, scavenging of free radicals and maintaining a cation-anion balance (Bors et al., 1989; Kakkar and Sawhney, 2002; Tang and Newton, 2005). It has been shown that stress-tolerant plants had significantly higher endogenous PAs levels as compared to sensitive ones under stress. (Lee, 1997). Several biochemical and physiological effects were elicited by exogenously applied spermidine under environmental stress. The study of Yiu et al. (2009) showed that exogenous spermidine helped to maintain antioxidant enzyme activities of welsh onion (*Allium fistulosum* L.) which are able to moderate radical scavenging system to lessen oxidative stress in this way. A similar result was reported that exogenous spermidine was effective in enhancing the activity of peroxidase under salinity stress and the salt-induced increase in reducing sugar and free proline level was further promoted by spermidine in indica rice

(Roychoudhury et al., 2011). Moreover, it has been demonstrated that overexpression of spermidine synthase gene in transgenic *Arabidopsis thaliana* maintained higher levels of spermidine content and enhanced tolerance to chilling, salinity, hyperosmosis and drought relative to the wild-type plants, which suggests that spermidine plays an important role in stress signaling pathway as a signaling regulator, leading to build a stress tolerance mechanisms for plants (Kasukabe et al., 2004). These studies further highlight the importance of spermidine for stress tolerance in plants. Previous studies have indicated that spermidine was involved in a number of environmental stress, not only exogenous application but also transgene (such as spermidine synthase gene) could alleviate damage from these stresses. However, most of studies centered around antioxidant enzyme activities and organic solutes accumulation.

Limited research has focused on the gene expression patterns in conjunction with the underlying enzymes and proline metabolism under water stress. The role of spermidine in regulation of water stress tolerance is still unclear and not fully understood. Further understanding the association of antioxidant enzyme activity and gene expression under water stress condition is important for studying molecular factors controlling antioxidant defense. Additionally, effects of exogenous spermidine on changes of different osmolytes and ascorbate-glutathione cycle (AsA-GSH cycle) have not been well revealed during water stress. The objectives of this study are (i) to determine whether the acquired water stress tolerance induced by exogenous spermidine is associated with the changes in antioxidant defense system and osmoregulatory solutes accumulation; (ii) to test the effects of exogenous spermidine on differential antioxidant enzymes gene expression patterns during water deficit; and (iii) to assess the possible influence of exogenous spermidine under water stress conditions on proline metabolism in an essential cool-season perennial forage legume, white clover, widely used because of its contributions to nitrogen fixation, feed quality and complementary growth patterns. Such information will help further understand the effects of plant tolerance to water stress and gain more insights on the possible mechanisms of the enhanced water stress tolerance induced by exogenous spermidine.

## Results

### *Relative water content and relative growth rate of leaves*

RWC of the leaves was not significantly different at the start of treatments with PEG and PEG+Spd (Fig. 1A). Leaf RWC of both treatments decreased in response to water stress, but RWC maintained at a significantly higher level in PEG+Spd treatment than that in PEG treatment after 6 and 9 days of water stress (Fig. 1A). At the end of the water stress, although RWC of both treatments almost declined to 30%, plants RGR was significantly higher in PEG+Spd treatment than that in PEG treatment (Fig. 1B).

### *Reactive oxygen species production and membrane damage*

Water stress caused an increase in  $O_2^{\cdot-}$ ,  $H_2O_2$ , MDA content and EL of leaves in both treatments (Fig. 2).  $O_2^{\cdot-}$  generation rate in both treatments increased similarly in response to stress; however, PEG+Spd treatment had a significantly lower  $O_2^{\cdot-}$  generation rate than PEG treatment at 3 and 6 d of water stress (Fig. 2A). Exogenous Spd was effective in decreasing  $H_2O_2$  and MDA content under water stress (Fig. 2B, C). At 6 and 9 d of stress, PEG+Spd treatment maintained significantly lower  $H_2O_2$  and MDA content than PEG treatment; at the last day of

stress, MDA content was also significantly different in PEG+Spd plants from the PEG plants (Fig. 2C). EL increased with decreasing RWC in both treatments, but PEG treatment had a significantly higher EL level relative to PEG+Spd treatment (Fig. 2D). These results showed that exogenous Spd could effectively alleviate membrane damage induced by water stress.

### *Activities of antioxidant enzymes*

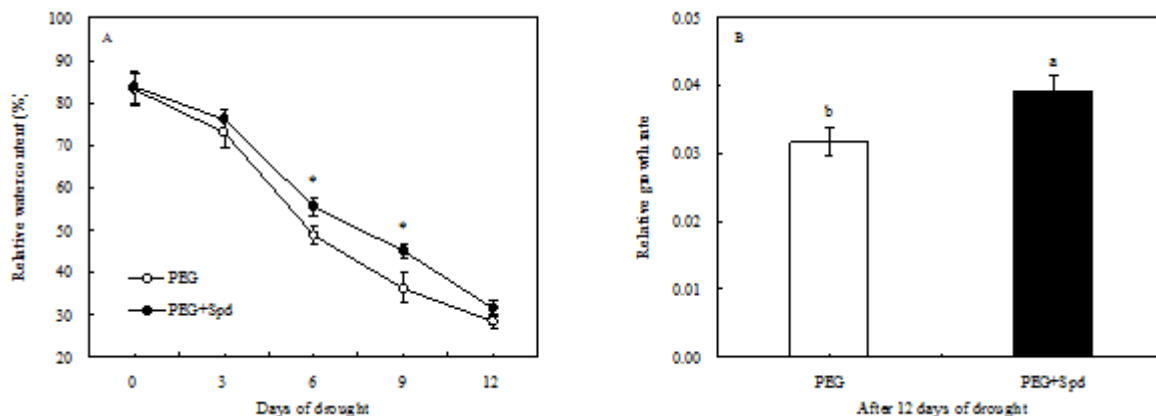
Exogenous Spd had stimulative effects on SOD, POD and CAT activities under water stress (Fig. 3). In response to water stress, SOD activities gradually increased and reached a peak value at 9 d of stress and then started to decrease in both treatment plants. However, SOD activity of PEG+Spd treatment was significantly higher than that of PEG treatment at 3 d and 9 d of stress (Fig. 3A). In the beginning of water stress, there was a slight increase in POD activity for PEG+Spd treatment and then started to decline following aggravating stress, but POD activity of PEG treatment gradually declined from beginning of water stress (Fig. 3B). The POD activity of PEG+Spd treatment was significantly higher than that of the PEG treatment at 3 d of stress. The change of CAT activities showed similar trend in both treatment plants, which was increased gradually, with a maximum activity at the third day of water stress and then started to decline with sustained stress, but CAT activity of PEG treatment exhibited a greater reduction as compared to PEG+Spd treatment, which maintained a significantly higher CAT activity at 3 and 9 d of stress (Fig. 3C). The exogenous Spd-treated plants showed higher APX, MR, DR and GR activities compared with untreated plants during 12 d of water stress (Fig. 4). Under well-watered conditions (0 d), APX activities of both treatments kept the same level without statistically significant differences, and APX activities had increased by a wide margin in both treatments before 6 d of stress (Fig. 4A). A significantly higher APX activity was observed in PEG+Spd plants than that in PEG plants at 9 d of water stress (Fig. 4A). MR activity in PEG treatment was relatively unchanged or decline during water stress, but increased by 14% in PEG+Spd treatment exposed to 3 d of stress compared with at 0 d and significant difference between two treatments was also observed at the moment (Fig. 4B). DR activities in both treatments decreased relative to 0 d in response to water stress (Fig. 4C). The reduction in DR activity was greater for the PEG treatment, since the average rate of DR activity was reduced to 41% of the control level (0 d) in PEG treatment and by approximately 34% in PEG+Spd treatment at 12 d of stress. DR activities significantly differ between two treatment under 0 and 9 d of water stress. During the stress period, GR activity in PEG+Spd treatment was higher than that in PEG treatment, but without significant differences (Fig. 4D).

### *Expression of genes encoding antioxidant enzymes*

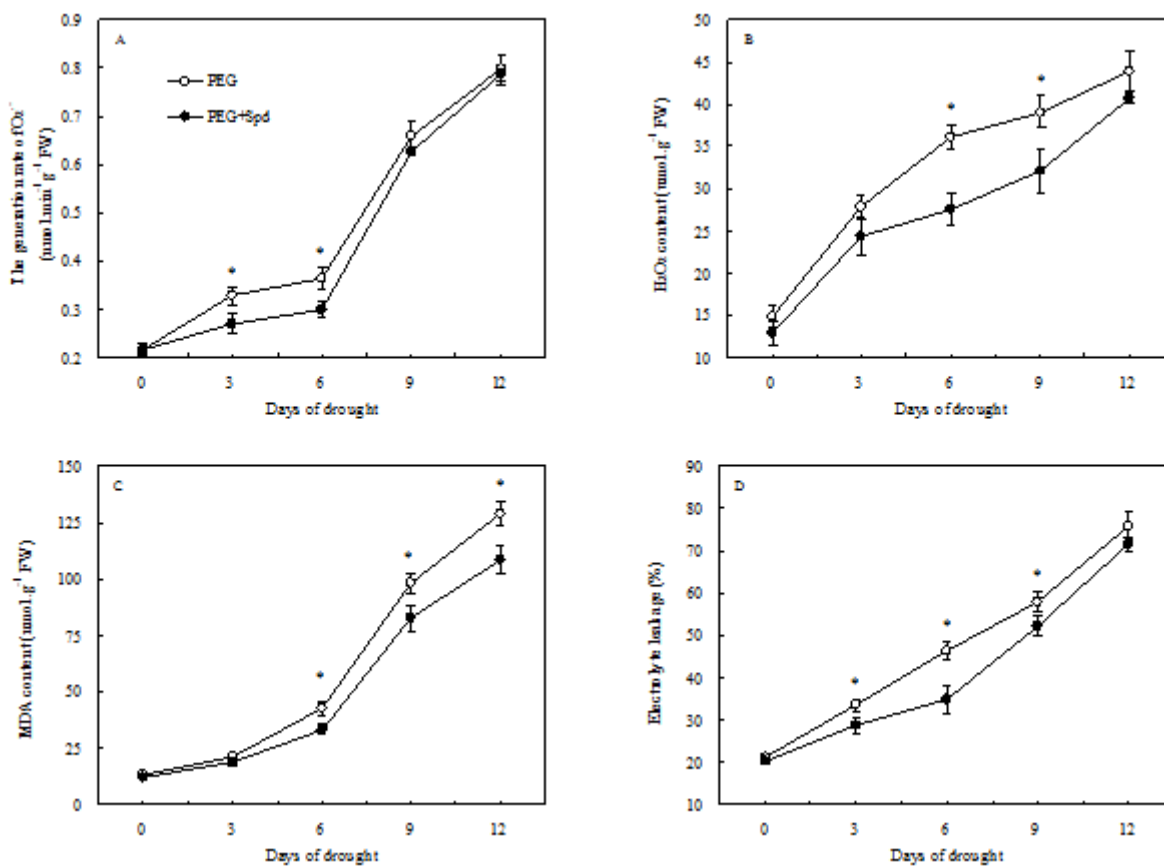
Water stress or exogenous Spd strongly affected the expression of genes encoding antioxidant enzymes in white clover leaves (Fig. 5). Relative expression of *Cu/ZnSOD* in both treatments increased rapidly during the water stress period, and a similar pattern was observed in Fig. 5A. Both treatments displayed a ~4-fold increase in the expression of *Cu/ZnSOD* at 12 d of water stress and PEG+Spd treatment maintained significantly higher *Cu/ZnSOD* expression rate than PEG treatment at the same time (Fig. 5A). The transcript levels of *POD* gene were not different between two treatments under well-watered condition (0 d), but a significantly higher expression rate of *POD* in Spd-treated plants was detected compared with untreated plants

**Table 1.** Primer sequences and their corresponding GeneBank accession numbers of the analyzed genes.

Target gene	Accession no.	Forward primer (5'-3')	Reverse primer (5'-3')
<i>Cu/ZnSOD</i>	FY461274	TCACCTTCCACTTTCAAACCTCTC	TGTTGGACCTTCGTCTTCTTGAGT
<i>CAT</i>	FY464988	GTCTTCTTTGTTACAGATGGGATG	GAAAGTGGGAGAAGAAGTCAAGGAT
<i>POD</i>	AJ011939	TCTAGGGCAACGGTTAATTCATTC	GGTACGGATTTTCCCATTTCTTG
<i>APX</i>	FY460674	GCAGCATCAGTTGGCAAGACC	GGCAAACCTGAGACTAAATACACGA
<i>β-Actin</i>	JF968419	TTACAATGAATTGCGTGTTG	AGAGGACAGCCTGAATGG



**Fig 1.** The effect of exogenous spermidine on (A) relative water content (RWC) and (B) relative growth rate (RGR) in wheat clover under water stress. Vertical bars indicate  $\pm$  SE (n=4). The asterisk “\*” or different letters above columns indicate LSD values where significant differences were detected ( $P \leq 0.05$ ) for comparison between different treatments at a given day.



**Fig 2.** The effect of exogenous spermidine on (A) the generation rate of superoxide anion ( $O_2^-$ ), (B)  $H_2O_2$  content, (C) MDA content and (D) electrolyte leakage (EL) in wheat clover under water stress. Vertical bars indicate  $\pm$  SE (n=4). The asterisk “\*” indicates LSD values where significant differences were detected ( $P \leq 0.05$ ) for comparison between different treatments at a given day.

under water stress (Fig. 5B). Relative expression of *CAT* gene was relatively unchanged during water stress and maintained a significantly higher level in PEG+Spd treatment plants, but decreased by 42% in PEG treatment plants exposed to 12 d of stress compared with that at 0 d (Fig. 5C). The transcript levels of *APX* were significantly inhibited by water stress in both treatments (Fig. 5D). However, there was a significantly higher *APX* transcript level in PEG+Spd treatment than that in PEG treatment under well-watered conditions (0 d). Although without statistically significant difference, PEG+Spd treatment plants also displayed higher *APX* transcript level under the same level of water deficit compared to PEG treatment (Fig. 5D).

#### **Organic solutes accumulation**

Four organic osmoregulatory solutes were detected in the leaves of both treatments. Under well-watered conditions (0 d), the contents of total soluble sugar, reducing sugar, betain and proline were not significantly different between two treatments. However, total soluble sugar, reducing sugar and betain content exhibited a similar variation trend in both treatments, which significantly increased in the beginning of water stress and then declined with the development of stress (Fig. 6A-C). Compared to PEG treatment, exogenous Spd significantly enhanced soluble sugar accumulation, and soluble sugar content reached to its peak value after 9 days of stress in PEG+Spd treatment (Fig. 6A). Furthermore, PEG+Spd treatment had significantly greater reducing sugar content than PEG treatment at 3 and 6 d of water stress and increased by 12% and 7% than PEG treatment at the same time, respectively (Fig. 6B). PEG+Spd treatment also maintained significantly higher betain content in the whole process of water stress as compared with PEG treatment (Fig. 6C). The free proline content generally increased in response to water stress in both treatments and PEG+Spd treatment showed significantly greater free proline content than PEG treatment at 3 d of stress (Fig. 6D).

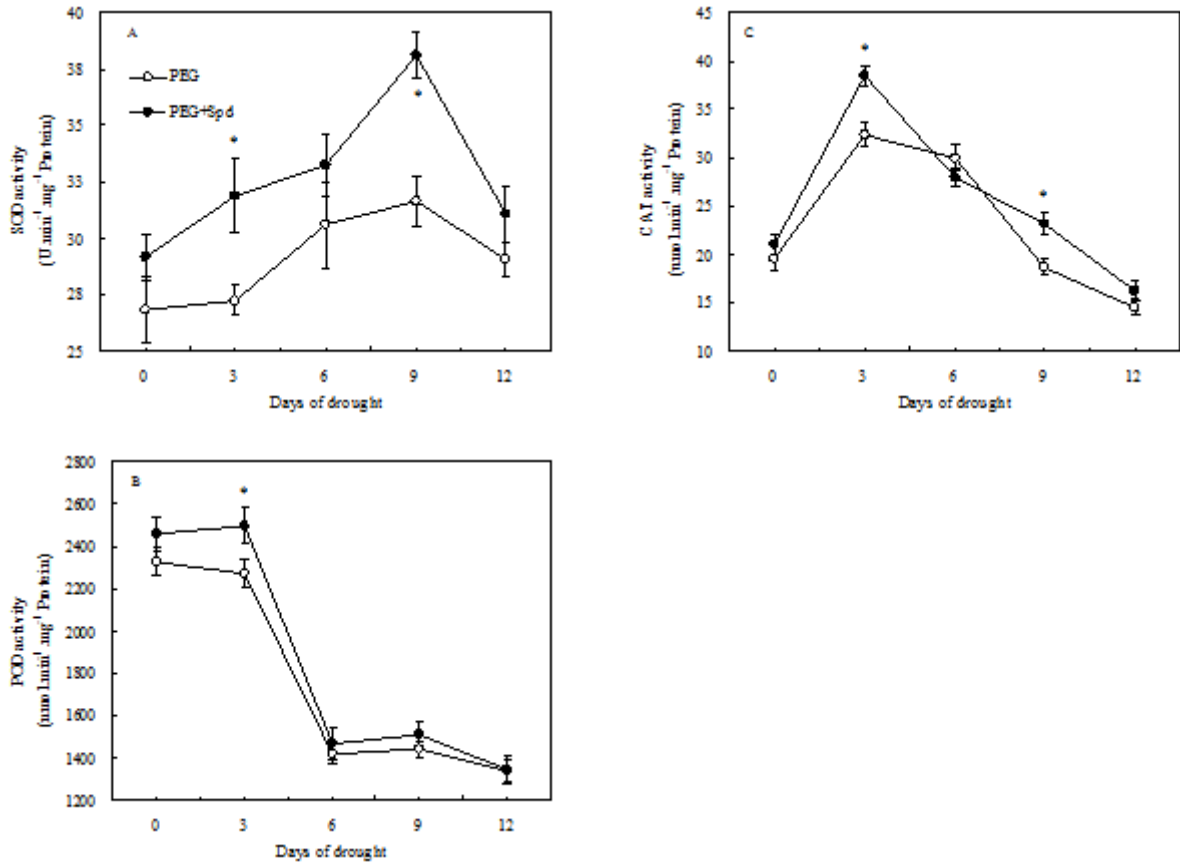
#### **Activities of proline biosynthetic and degradative enzymes**

Progressive water deficit induced significant increase of OAT activities, so that OAT activities of both treatments in leaves reached their maximum at 12 d of water stress, whereas there was no significant difference between two treatments in OAT activities (Fig. 7A). P5CS activities were significantly impacted by water stress or exogenous Spd; P5CS activity exhibiting an increase due to 3 days of water stress in PEG+Spd treatment, whereas there was no significant change in PEG treatment. At the same level of water stress, P5CS activity was increased or maintained better in PEG+Spd treatment compared with PEG treatment (Fig. 7B). ProDH activities gradually changed with the increased stress intensity, but the extent of increase was different for two treatments at the same level of water stress. Accordingly, significantly higher ProDH activity was observed in PEG+Spd treatment compared to that in PEG treatment at 9 d and 12 d of water stress (Fig. 7C).

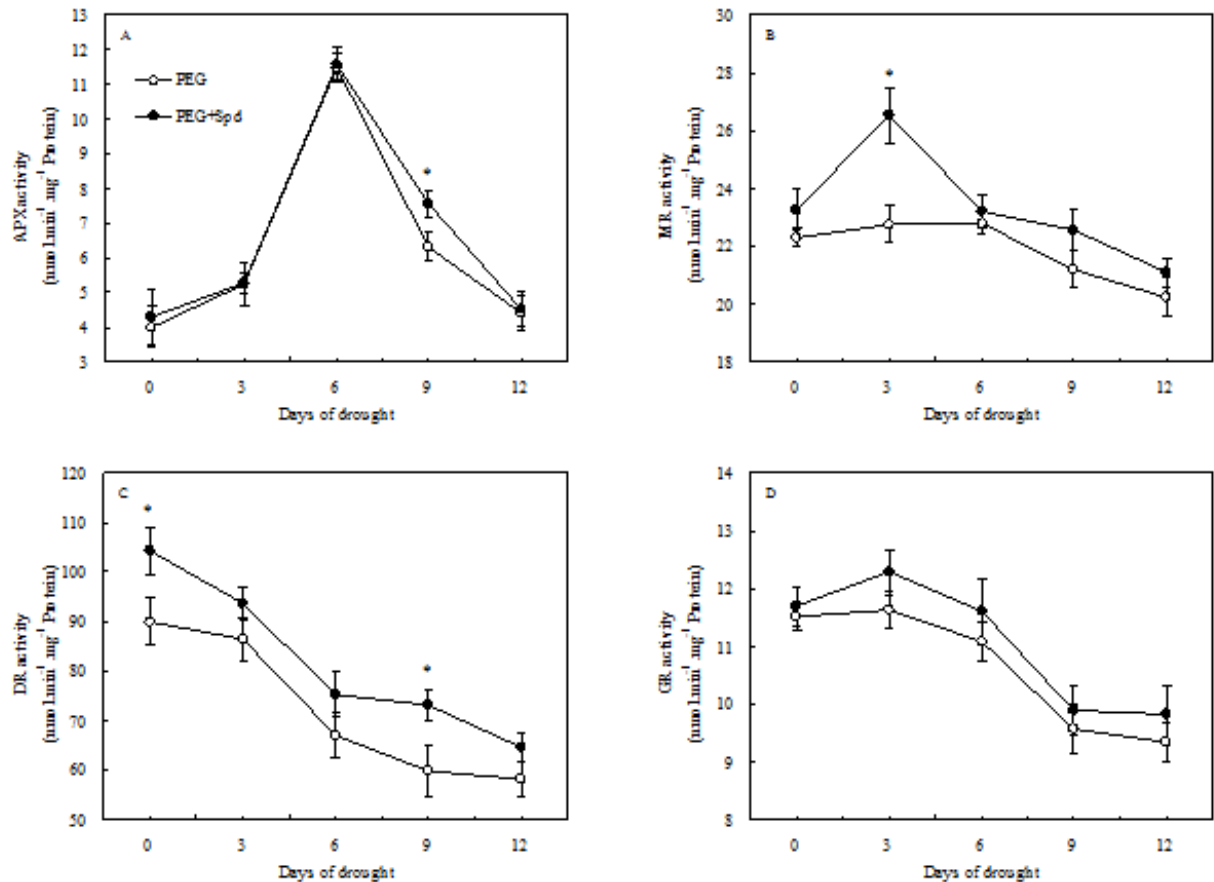
#### **Discussion**

Tolerant plants often have better ability to eliminate ROS by producing higher levels of antioxidants. Thus, the variation in pattern of antioxidants indicates differential resistance mechanism (Roychoudhury et al., 2011). It has been found that alterations in antioxidant enzyme activities under drought stress are dependent on plant species, cultivar, stress intensity and duration (DaCosta and Huang, 2007; Xu et al., 2011). PAs are able to alter some scavenging system enzymes activities (Chattopadhyay et al., 2002; Nayyar and Chander, 2004). Spd was effective in reverting copper-reduced GR activity, but SOD

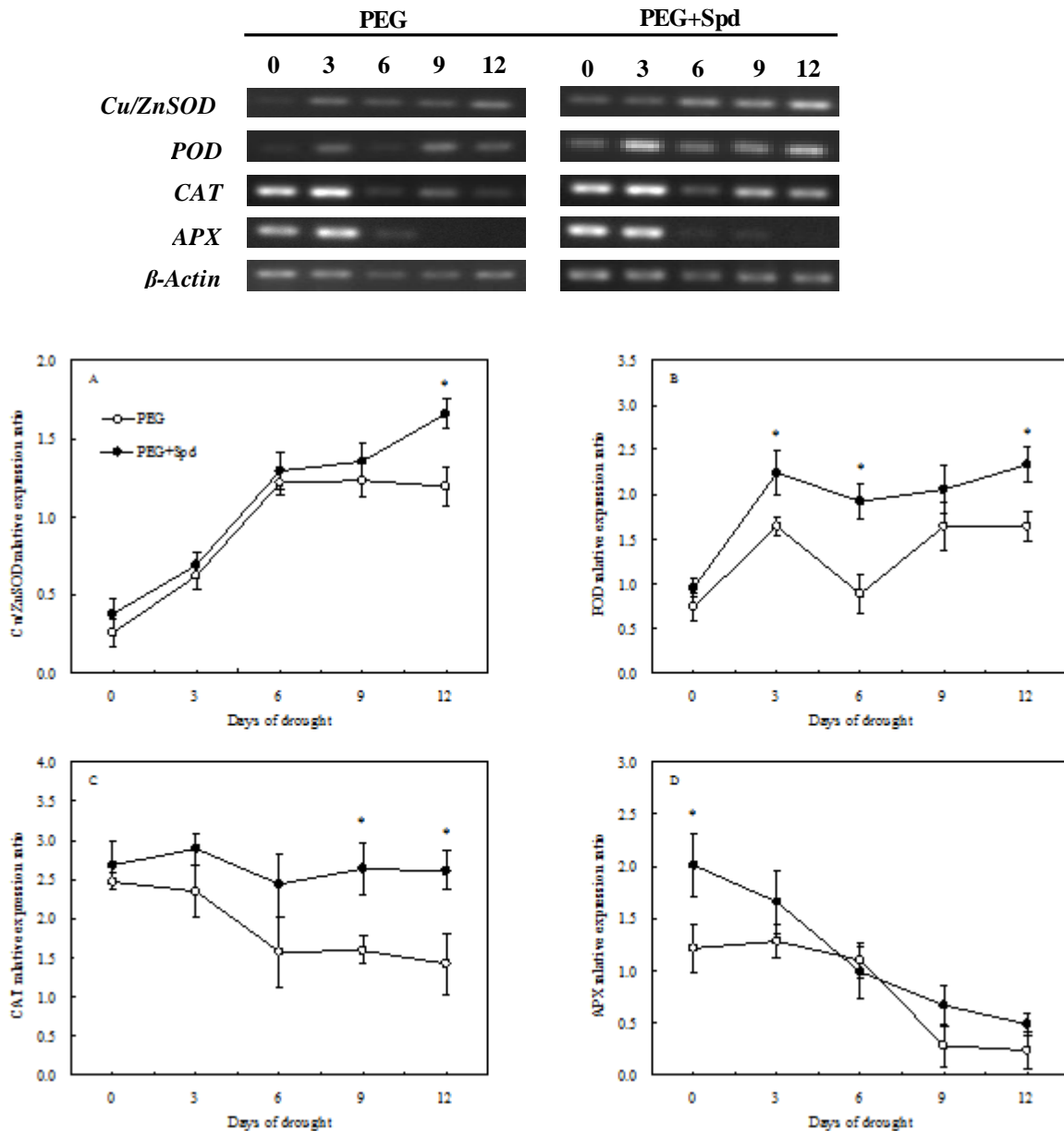
activity was not restored by Spd (Groppa et al., 2001). Kubis (2008) reported that exogenous Spd differentially influenced some antioxidant enzymes, and an increase of POD activity was observed in water-stress cucumber, but to one degree or another, a decline of SOD and CAT activities in Spd-treated plants in comparison to untreated stressed plants. In current study, exogenous Spd significantly promoted activities of SOD, POD, CAT, APX, MR and DR relative to untreated plants at different stress time, but was not effective on altering GR activity level. The result is consistent with early study of Li (2004) about effect of exogenous Spd on wheat seedlings under osmotic stress. Enhanced antioxidant defense system in Spd-treated plants resulted in lower the generation rate of  $O_2^-$ ,  $H_2O_2$  and MDA content and improving cell membrane stability, as demonstrated by lower electrolyte leakage (EL). It suggests that Spd is able to influence oxidative stress intensity through activating some activities of scavenging system enzymes under water stress. PAs were implicated in direct scavenging of free radicals as a stress-protecting compound, thereby reducing oxidative stress (Roberts et al., 1986; Tiburcio et al., 1994). Apart from such direct stress protective roles, Spd may also play an important role in plants as a signaling molecule in response to stress (Kasukabe et al., 2004). Spd and Spm in particular increase the DNA-binding activity of transcription factors and promote gene transcript level (Sudha and Ravishankar, 2002; Childs et al., 2003). Sung et al. (2011) observed that Spd affords fasciata protection against hypersalinity through the up-regulation of *FeSOD* gene, thereby alleviating oxidative damage. The results from RT-PCR analysis in the present study showed that water stress or exogenous Spd strongly affected genes transcript levels encoding antioxidant enzymes in leaves of white clover and four antioxidant enzymes exhibited different expression patterns. Exogenous Spd significantly enhanced expressions of *Cu/ZnSOD*, *POD*, *CAT* and *APX* genes under the same water stress condition. These data suggested that the better drought tolerance of Spd-treated plants could be associated with increased expression of specific genes encoding antioxidant enzymes, which partly influenced or improved antioxidant enzyme activities under stress. Previous studies also indicated that Spd activated antioxidant gene expression with improving tolerance to a variety of environmental stresses (Hiraga et al., 2000; Aronova et al., 2005). For four antioxidant enzymes, the gene expression pattern did not always go along with their activities changes. The discrepancy between gene expression and enzyme activity indicates enzyme activity changes were not only caused by mRNA levels but also regulated at the posttranscriptional level and influenced by cellular metabolism. The function of osmotic adjustment in plants could be involved in two ways in response to water deficit: the one is to improve absorbing water under moderate water deficit; another is to maintain structural and function of cell components and enhance osmoprotection when water stress is severe (Hasegawa et al., 2000; Lambers et al., 2006). Our data clearly showed that exogenous Spd was directly correlated with organic solutes accumulation in response to water stress. More soluble sugar, reducing sugar, betain and free proline were accumulated in Spd-treated plants than that in untreated plants. Formation of such complexes was probably involved in the protection caused by Spd. It has been reported that exogenous Spd strongly promoted reducing sugar and proline content in different plants during salinity stress, which proved that osmolyte levels are regulated by PAs synthesis or absorption in alleviating stress injury (Duan et al., 2008; Roychoudhry et al., 2011). Proline function was considered as an osmoprotectant for the detoxification of ROS, where it can accumulate a huge concentration without detrimental ionic strength effects (Yancey et al., 1982), and as compatible solute for osmotic



**Fig 3.** The effect of exogenous spermidine on (A) SOD activity, (B) POD activity and (C) CAT activity in wheat clover under water stress. Vertical bars indicate  $\pm$  SE (n=4). The asterisk “\*” indicates LSD values where significant differences were detected ( $P \leq 0.05$ ) for comparison between different treatments at a given day.



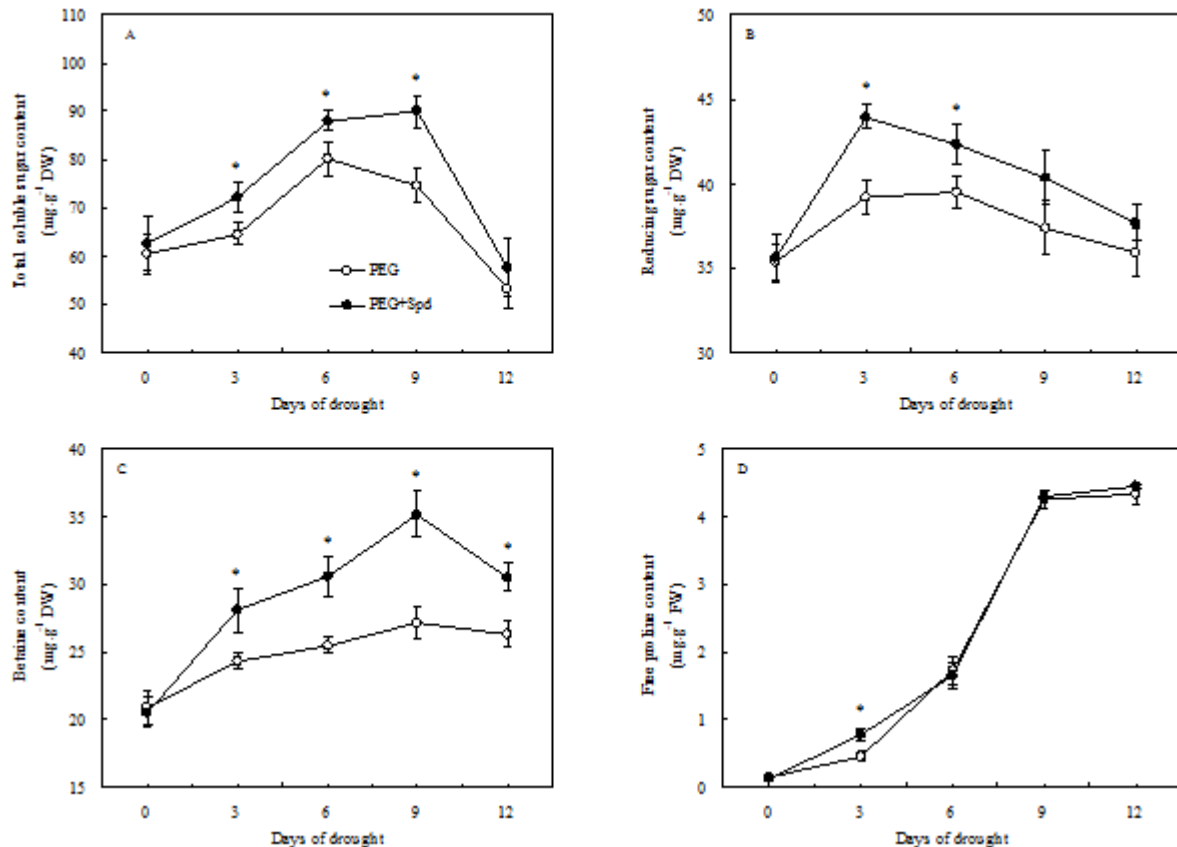
**Fig 4.** The effect of exogenous spermidine on (A) APX activity, (B) MR activity, (C) DR activity and (D) GR activity in wheat clover under water stress. Vertical bars indicate  $\pm$  SE (n=4). The asterisk “\*” indicates LSD values where significant differences were detected ( $P \leq 0.05$ ) for comparison between different treatments at a given day.



**Fig 5.** The effect of exogenous spermidine on (A) *Cu/ZnSOD* gene, (B) *POD* gene, (C) *CAT* gene and (D) *APX* gene relative expression ratio in wheat clover under water stress. Vertical bars indicate  $\pm$  SE (n=4). The asterisk “\*” indicates LSD values where significant differences were detected ( $P \leq 0.05$ ) for comparison between different treatments at a given day.

adjustment (Kocsy et al., 2005; Ashraf and Foolad, 2007) and also as a source of nitrogen or energy (Verslues and Sharp, 1999). Hence, its metabolism is very important for plants to survive the drought. Proline biosynthesis in higher plants may proceed not only from glutamate but also from ornithine pathway.  $\Delta^1$ -pyrroline-5-carboxylate synthetase (P5CS) and ornithine aminotransferase (OAT) are key enzymes involved in glutamate pathway and ornithine pathway, respectively (Sanchez et al., 2001; Szabados and Savoure, 2009). In this study, P5CS and OAT activities increased in both treatment in response to water stress, but exogenous Spd only promoted P5CS activity and failed in OAT activity, which demonstrated that the significantly elevated proline accumulation in Spd-treated plants was dependent upon the up-regulated P5CS activity, while OAT was not essential for this change. The catabolism of proline takes place in mitochondria with the

conversion of proline into pyrroline-5-carboxylate catalyzed by the enzyme proline dehydrogenase (ProDH); contrary to proline biosynthesis, the catabolic pathway involving ProDH seems to be the only way for plants to degrade excess proline (Trovalo et al., 2008). This is very important for cells since oxidation of proline generates NADP/NADPH cycling or redox balance (Hare et al., 1998). According to the study of Cramer et al. (2007), drought stress up-regulated the expression of *ProDH* gene in grapes and proline may be being used as an energy source in young tissues. Exogenous Spd not only improved the P5CS activity, but also ProDH activity in white clover under water stress. It meant that keeping balance between proline biosynthesis and catabolism was as important as proline accumulation for plants to deal with drought stress. More accumulated organic solutes and enhanced proline metabolism caused by exogenous Spd may be involved in osmotic adjustment



**Fig 6.** The effect of exogenous spermidine on (A) total soluble sugar, (B) reducing sugar, (C) betaine and (D) free proline content in wheat clover under water stress. Vertical bars indicate  $\pm$  SE (n=4). The asterisk “\*” indicates LSD values where significant differences were detected ( $P \leq 0.05$ ) for comparison between different treatments at a given day.

and osmoprotection under water stress, which explains the better membrane stability and higher RWC in Spd-treated plants regarding the response of water stress.

## Materials and methods

### Plant materials and treatments

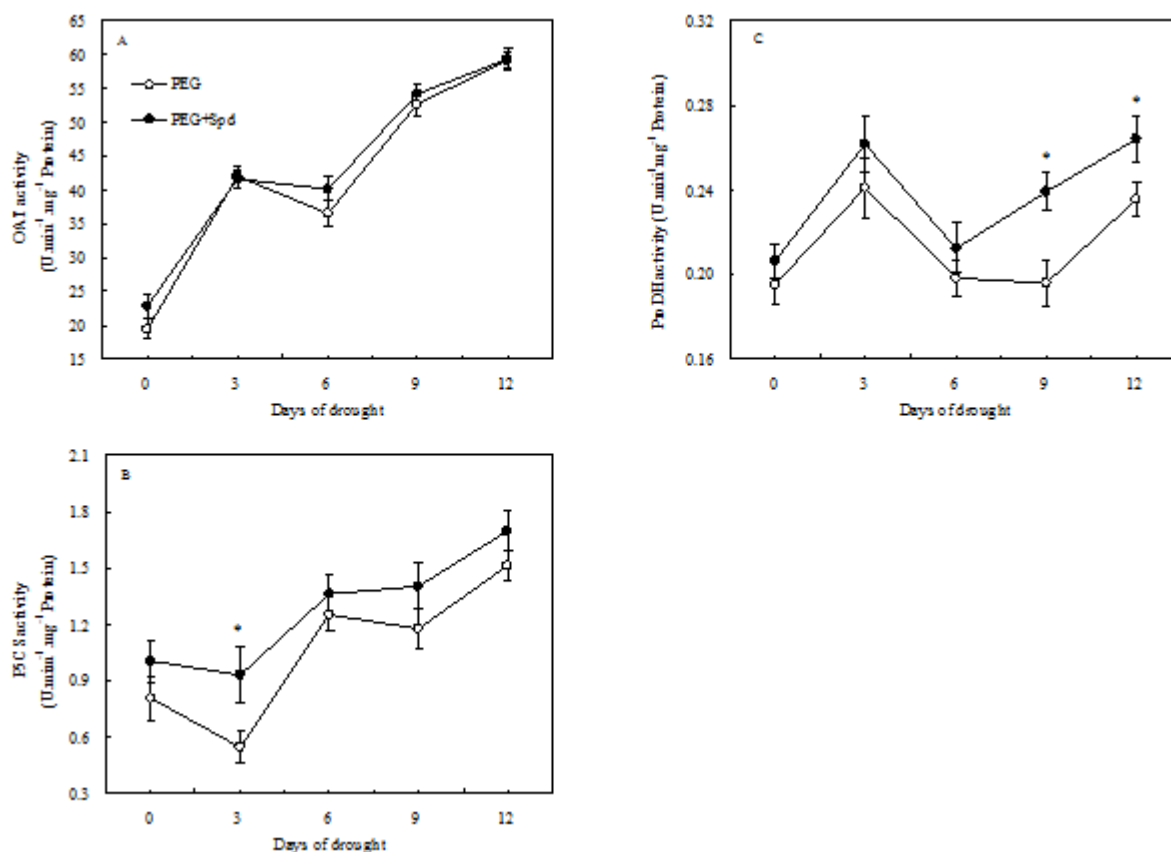
Seeds of white clover cultivar ‘Ladino’ (drought sensitive) were surface-sterilized for 6 min in 0.1 % mercuric chloride, then rinsed four times with ddH<sub>2</sub>O. Afterwards, they were sown in plates (24 cm length, 15 cm width and 8 cm deep) filled with sterilized quartz sand and ddH<sub>2</sub>O in the growth chamber with 12 h photoperiod at average day/night temperature of 23/19 °C and 75 % relative humidity. 7 days later, the ddH<sub>2</sub>O was replaced by Hoagland’s solution (Hoagland and Arnon, 1950) and plants were grown for 23 days in the growth chamber under the same growth conditions. Then plants were exposed to two water stress treatments: 1) PEG: Hoagland’s solution plus 20% PEG 6000 (W/V); 2) PEG+Spd: Hoagland’s solution plus 20% PEG 6000 (W/V) plus 0.5 mmol/L spermidine. Before water stress, the treatment of PEG+Spd was pretreated by 0.5 mmol/L spermidine solution for 2 days in order to make white clover plants absorb enough spermidine. The leaves were sampled at 0, 3, 6, 9 and 12 days after the beginning of water stress. Each treatment had four replicates (four plates) and was arranged in a completely randomized design. Tissue samples were immediately frozen in liquid nitrogen until analyses.

### Determination of relative water content (RWC) and relative growth rate (RGR)

Leaf RWC was determined from fresh weight (FW), dry weight (DW), and turgid weight (TW) using the formula  $RWC (\%) = [(FW - DW)/(TW - DW)] \times 100$ . (Barrs and Weatherley, 1962). Mean relative growth rates (RGR) of plants were calculated as:  $RGR = (\ln W_f - \ln W_i) / \Delta t$ , where  $W_f$  and  $W_i$  are final and initial dry weights of plants, respectively, and  $\Delta t$  is the time elapsed (d) between the two measurements.

### Determination of reactive oxygen species and electrolyte leakage (EL)

The formation rate of O<sub>2</sub><sup>-</sup> was measured by using sulfanilamide method (Elstner and Heupel, 1976) and the absorbance was measured at 530 nm. H<sub>2</sub>O<sub>2</sub> was assayed by potassium iodide method. The oxidation product was measured at 390 nm. The amount of H<sub>2</sub>O<sub>2</sub> formed was computed from the standard curve made earlier with known concentrations of H<sub>2</sub>O<sub>2</sub> (Velikova et al., 2000). For EL, samples of 0.1 g of fresh leaves were immersed in the centrifuge tube with 15 ml of deionized water. The tubes were shaken for 24 h on a shaker table. The conductivity of the solution ( $C_{initial}$ ) was measured using a conductivity meter. Leaves then were boiled at 100 °C for 30 min. The conductivity of boiled tissues ( $C_{max}$ ) was measured. Relative EL was calculated as the percentage of  $C_{initial}$  over  $C_{max}$  (Blum and Ebercon, 1981).



**Fig 7.** The effect of exogenous spermidine on (A) OAT activity, (B) P5CS activity and (B) ProDH activity in wheat clover under water stress. Vertical bars indicate  $\pm$  SE (n=4). The asterisk “\*” indicates LSD values where significant differences were detected ( $P \leq 0.05$ ) for comparison between different treatments at a given day.

#### Antioxidant enzyme activities and malondialdehyde (MDA) content

To analyze the antioxidant enzyme activities, 0.2 g fresh leaves were randomly sampled from each pot at each sampling date, frozen in liquid nitrogen immediately, and stored at  $-80^{\circ}\text{C}$  until use. For extraction, the frozen sample was ground on ice with 4 ml of 50 mM cold phosphate buffer (pH 7.8) containing 1% (w/v) polyvinylpyrrolidone. The homogenate was centrifuged at 12 000 g for 30 min at  $4^{\circ}\text{C}$ . The supernatant was used for assays of antioxidant enzyme activity and content of malondialdehyde (MDA), which was measured as the degree of lipid peroxidation. The SOD activity was measured by recording the rate of p-nitro blue tetrazolium chloride reduction in absorbance at 560 nm (Giannopolities and Rise, 1977). The activity of CAT, POD, APX, MR, DR, and GR was determined by following the changes in absorbance at 240, 470, 290, 340, 265, and 340 nm, respectively (Chance and Maehly, 1955; Nakano and Asada, 1981). Protein content was determined using Bradford's (1976) method. The content of MDA was measured using the method of Dhindsa et al. (1981) with some modification. 0.5 ml enzyme extract and 1.0 ml reaction solution containing 20% w/v trichloroacetic acid and 0.5% w/v thiobarbituric acid were added to the pellet. The mixture was heated in a water bath at  $95^{\circ}\text{C}$  for 15 min, and then cooled quickly in an ice-water bath. The homogenate was centrifuged at 8 000 g for 10 min. The absorbance of the supernatant was measured at 532, 600 and 450 nm. The concentration of MDA was calculated by subtraction of  $\text{OD}_{600}$  from  $\text{OD}_{532}$  and  $\text{OD}_{450}$ .

#### Gene expression analysis

All genes used in this study, except POD gene (GenBank ID: AJ011939) and the reference genes  $\beta$ -actin (GenBank ID: JF 968419), were identified based on sequence similarity after TBLASTX analysis with related genes from white clover expressed sequence tags (ESTs). Primers were manually designed based on the EST sequences. Candidate sequences were further confirmed by sequencing after PCR amplification using the same primers. The white clover homologues of *Cu/ZnSOD*, *CAT* and *APX* genes were identified based on sequence similarity with the red clover *Cu/ZnSOD* gene (GenBank ID: AY434497), broad bean *CAT* gene (GenBank ID: JQ043348) and alfalfa *APX* gene (GenBank ID: XM\_003601995). The putative white clover *Cu/ZnSOD*, *CAT* and *APX* genes displayed 94%, 93% and 95% identity with red clover *Cu/ZnSOD* gene, broad bean *CAT* gene and alfalfa *APX* gene, respectively. Primers used for RT-PCR are presented in Table.1. Gene expression was performed using a reverse transcriptase polymerase chain reaction (RT-PCR). Total RNA was extracted from mature leaves with RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. RNA concentration was calculated from the optical density of the samples at 260 nm. RNA was reverse-transcribed with Revert Aid First Stand Cdna Synthesis Kit (Fermentas). The synthesized cDNA was subjected to PCR using primers of *Cu/ZnSOD*, *CAT*, *POD*, *APX* and  $\beta$ -actin (as internal control). Conditions of the PCR for  $\beta$ -actin, *Cu/ZnSOD*, *CAT* and *POD* genes were as follows: 3 min at  $94^{\circ}\text{C}$  and 30 repeats of



denaturation at 94 °C for 30 s, annealing at 57 °C for 30 s and extension at 72 °C for 1 min. Condition of the PCR for *APX* gene was as follows: 3 min at 94 °C and 30 repeats of denaturation at 94 °C for 30 s, annealing at 62.5 °C for 30 s and extension at 72 °C for 1 min. Aliquots of individual PCR products were resolved through agarose gel electrophoresis and images were captured by Quantity One and the bands were also determined with the Discovery Series Quantity One.

#### **Organic solutes and the key enzyme activities of proline metabolism**

Free proline was quantified spectrophotometrically by the ninhydrin method according to Bates et al. (1973). The activity of OAT, P5CS and ProDH was determined according to the method of Lu and Mazelis (1975), Garcia-Rios et al. (1997) and Sanchez et al. (2001), respectively. Soluble sugars were quantified following the phenolsulfuric acid method described by Robyt and White (1987). The reducing sugar content was determined using the colorimetric assay with anthrone reagent and measuring the green colour intensity at 630nm with respect to the standard curve (Roychoudhury et al., 2008). Betaine was estimated by the colorimetric method according to Grieve and Grattan (1983).

#### **Statistical analysis**

Each treatment had four replicates (four plates) and was arranged in a completely randomized design. The general linear model procedure of SAS 9.1 (SAS Institute, Cary, NC) was used to determine the significance of relationships among the measured variables. Conclusions are based on differences between means significant at  $P \leq 0.05$  by Duncan's multiple range test.

#### **Conclusion**

In summary, the present study confirmed that exogenous Spd effectively alleviated the negative effects in drought-sensitive white clover cv. 'Ladino' under water stress, as demonstrated by lower lipid peroxidation, better cell membrane stability, higher RWC and RGR in Spd-treated plants as compared to untreated plants. Enhanced antioxidant enzyme activities, ASC-GSH cycle and expression of genes encoding antioxidant enzymes induced by exogenous Spd may be one of the critical reasons in acquiring drought tolerance through scavenging ROS. This study also suggests that Spd induced organic solutes accumulation and proline metabolism, which plays an important role in improving drought tolerance associated with osmotic adjustment and osmoprotection. Keeping balance between proline biosynthesis and catabolism is as important as proline accumulation for plants to survive drought.

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