

Improved drought tolerance through drought preconditioning associated with changes in antioxidant enzyme activities, gene expression and osmoregulatory solutes accumulation in White clover (*Trifolium repens* L.)

Zhou Li, Peng Shi and Yan Peng*

College of Animal Science and Technology, Sichuan Agricultural University, Ya'an 625014, China

*Corresponding author: pengyanlee@163.com

Abstract

Improving drought tolerance through drought preconditioning is an important way to understand drought tolerance mechanism in plants. Some limited researches have focused on the gene expression patterns in conjunction with the underlying enzymes promoting drought tolerance and post-drought recovery in white clover. The objective of this study was to identify whether preconditioning-induced drought tolerance is in relation to changes of antioxidant enzyme activities, gene expression and osmoregulatory solutes in white clover. Plants of white clover cultivar, 'Chuanyin Ladino' (drought sensitive), were exposed to two cycles of drought preconditioning (non-preconditioned, well-watered plants, as a control) then exposed to drought stress for 11 days and rewatered for 6 days in a growth chamber with 14 h photoperiod at day/night temperature of 21/16 °C, 70 % relative humidity, and 300 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetic photon flux density. Results showed that drought preconditioning improved drought tolerance and post-drought recovery in white clover, as demonstrated by significantly lower ($p \leq 0.05$) lipid peroxidation, better cell membrane stability and higher relative water content in drought-preconditioned plants as compared to non-preconditioned plants under drought stress and rewatering. Drought-preconditioned plants exhibited higher superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT) and guaiacol peroxidase activities (POD), as well as higher transcript level of *Cu/Zn SOD* gene. Meanwhile, more accumulation of soluble sugars and betain and less accumulated proline were also observed in preconditioned plants. Enhanced antioxidant enzyme activities and significantly higher transcript level of *Cu/Zn SOD* gene may be the critical reasons in acquiring drought tolerance through drought preconditioning. This study also suggests that drought preconditioning improved white clover drought tolerance, which could be related to more accumulation of soluble sugars and betain, while the accumulated proline is associated with the degree of drought stress injury in white clover.

Keywords: Antioxidant enzyme; Drought preconditioning; Gene expression; Osmotic adjustment; Rewatering; White clover (*Trifolium repens* L.).

Abbreviations: APX_Ascorbate peroxidase; CAT_Catalase; D- Drought; EL_Electrolyte leakage; LSD_The least significant difference; MDA_Malondialdehyde; Non_Non-preconditioned; Pre_Drought-preconditioned; R_Rewatered; ROS_Reactive oxygen species; RWC_Relative water content; SOD_Superoxide dismutase.

Introduction

White clover (*Trifolium repens* L.) is an essential cool-season perennial forage legume in temperate pastures of the world, contributing to animal performance, improving the quality of consumed herbage, and improving soil fertility through nitrogen fixation. However, white clover is less drought tolerant than other perennial temperate forage legumes [e.g. alfalfa (*Medicago sativa* L.)], owing to its shallow root system and inability to effectively control transpiration (Annicchiarico and Piano, 2004). Its seedling tap-root does not persist for more than two years under dry conditions (Westbrooks and Tesar, 1955; Charlton, 1984). Therefore, improving drought tolerance and understanding drought tolerance mechanism in white clover are critical for increasing legume content, enhancing pasture quality and animal performance in grazed swards.

Drought tolerance can be acquired through pre-disposition of plants to water stress (drought preconditioning), such as in mediterranean species (*Pistacia lentiscus* L., *Quercus coccifera* L., and *Juniperus oxycedrus* L.) seedlings (Vilagrosa et al., 2003) and *Eucalyptus globulus* subsp. *bicostata* seedlings (Guamaschelli et al., 2006). Various

drought-induced physiological changes may be favorable for subsequent drought tolerance. Renu and Devarshi, (2007) reported that the drought-acclimated wheat cultivar "C306" leaves maintained favorable water relations and lower membrane injury due to low H_2O_2 accumulation than non-acclimated 'C306' plants during severe water stress. The study of Guamaschelli et al. (2003) showed that drought-preconditioned *Eucalyptus globulus* seedlings displayed greater tolerance of water stress than non-conditioned plants and perform better during early establishment (higher survival and early growth) associated with higher values of stomatal conductance, predawn relative water content and water potential and lower mortality under drought stress. However, fewer reports were given about improving drought tolerance through drought preconditioning in white clover; thereby, further studies need to be done.

Drought tolerance in plants involves various physiological responses, including antioxidant defenses and osmolytes (Lu et al., 2009). Drought stress causes imbalance between the generation and quenching of reactive oxygen species (ROS). ROS, such as superoxide radicals (O_2^-), hydrogen peroxide

(H₂O₂) and hydroxyl radicals (·OH), which are highly reactive in the absence of effective protective mechanism. Drought can seriously damage plants by lipid peroxidation, protein degradation, breakage of DNA and cell death (Hendry, 1993; Tambussi et al., 2000). To minimize and eliminate oxidative damage, plants have evolved an antioxidant defense system composed of both non-enzymatic and enzymatic constituents present in plant cells, such as ascorbate peroxidase (APX), catalase (CAT), guaiacol peroxidase (POD), superoxide dismutase (SOD) (Sairam et al., 2011). These antioxidants could remove, neutralize, and scavenge the ROS at different cellular locations, helping to reduce lipid peroxidation and maintaining cell membrane stability (Foyer et al., 1994; Scandalios, 1993). Previous studies generally agreed that maintaining active antioxidant enzymes is important for drought tolerance in various plant species by increasing the protection capacity against oxidative damage (Sharma and Dubey, 2005; Turkan et al., 2005; Xu et al., 2011). Furthermore, general idea is that morphological changes and osmotic adjustments may be a mechanism for long-term acclimation, when plants are under drought stress. The accumulation of these compatible solutes, such as soluble sugar, betaine and free proline, is one of the most common responses of plants to water deficit (Morgan, 1984).

Osmotic adjustment could be involved in plant response to water deficit in two ways. The first, by improving the plant's ability to absorb water when the deficit is moderate; and second, by enhancing osmoprotection and maintaining structural and functional integrity of cell components (Lambers et al., 2006). Osmoprotection is of paramount importance especially when water stress is severe and the plant fights for survival (Hasegawa et al., 2000). Many previous studies also suggested that osmoregulatory solutes in white clover played a cardinal role to survive under drought stress (Turner, 1990; Karsten and Macadam, 2001).

However, limited research has focused on the gene expression patterns in conjunction with the underlying enzymes promoting drought tolerance and post-drought recovery in white clover. Further understanding of association of antioxidant enzyme activity and gene expression under drought stress and rewating condition is important for studying molecular factors controlling antioxidant defense. In addition, effects of drought preconditioning on changes of different osmolytes in white clover have not been well revealed under drought and rewating conditions. The objectives of this study are (i) to determine whether the acquired drought tolerance induced by drought preconditioning is associated with the changes in antioxidant enzymes and osmoregulatory solutes accumulation and (ii) to determine differential SOD gene expression patterns in drought-preconditioned and non-preconditioned plants under drought stress and post-drought recovery. Such information will help further understanding of the interactive effects of plant tolerance to drought stress and gain more insights on the possible mechanisms of the enhanced drought tolerance.

Results

Physiological response to drought stress and post-drought recovery following drought preconditioning

Gradual decrease in relative water content (RWC) for either preconditioned or well-water treated white clovers was observed at various stages of drought stress.

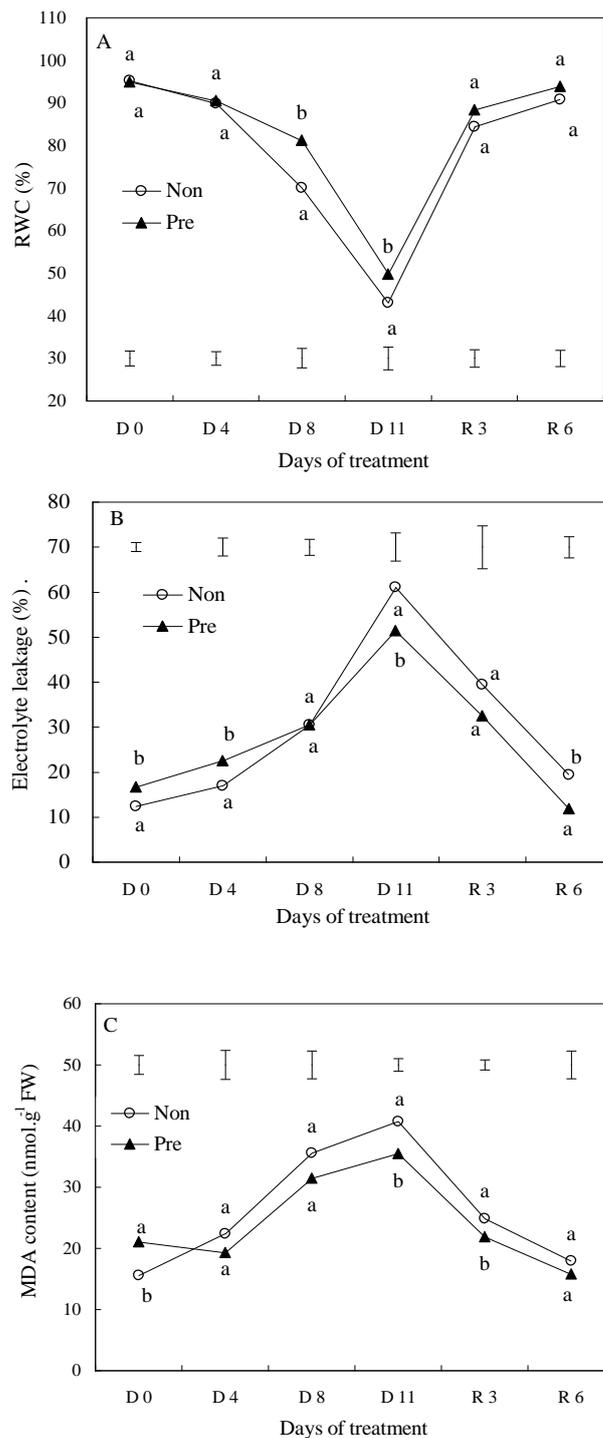


Fig 1. Effects of drought stress and post-drought recovery in white clover: (A) leaf relative water content (RWC), (B) electrolyte leakage (EL), and (C) malondialdehyde (MDA) content. Vertical bars indicate LSD ($P \leq 0.05$) for treatment comparisons and different letters indicate a significant difference at a given day of treatment. Pre, drought-preconditioned; Non, non-preconditioned; D 0-11, drought 0-11 d; R 3-6, rewated 3-6 d.

The drought-preconditioned plants were able to maintain higher RWC level than non-preconditioned plants under the same duration of drought treatment (Fig. 1A). RWC was significantly higher for drought-preconditioned plants after 8 days and 11 days of drought stress as compared to non-preconditioned plants. Meanwhile, RWC of drought-preconditioned plants was recovered to a higher level after 6 days of rewatering, but this difference was not statistically significant (Fig. 1A). Phenotypic effect of drought preconditioning was shown in Fig. 2 and drought-preconditioned plants maintained green and turgid. At the same time it also had less injury in the process of drought stress and recovered better after 6 d of rewatering, as compared to non-preconditioned plants (Fig. 2).

During drought stress, both the EL of drought-preconditioned and non-preconditioned plants increased significantly (Fig. 1B). The EL of drought-preconditioned plants was significantly higher than that non-preconditioned plants at the beginning of drought stress, but the EL of non-preconditioned plants increased steeply and drought-preconditioned plants maintained significantly lower EL level at the end of drought stress. The result indicated that drought-preconditioned plants maintained better membrane stability than non-preconditioned plants. After rewatering, both treatments were rapid decline in EL level and the EL of drought-preconditioned plants was also significantly lower than non-preconditioned plants, which indicated a better ability of membrane repairing in drought-preconditioned plants (Fig. 1B).

Drought preconditioning increased MDA content before exposure of preconditioned plants to drought stress (0 d) (Fig. 1C). However, during drought stress, preconditioned plants had 14%, 12%, and 13% lower MDA content than non-preconditioned plants at 4 d, 8 d and 11 d, respectively. Lipid peroxidation was decreased after rehydration in two treatments. The recovery of both treatments was well, which almost closed to the well-watered level. The differences in MDA content between the two treatments were statistically significant (Fig. 1C).

Effects of drought preconditioning on antioxidant enzyme activity under drought stress and post-drought recovery

At the beginning of drought stress, the activities of SOD were not significantly different between drought-preconditioned and non-preconditioned plants and both were increased from 0 d of drought to 8 d of drought and then started to decrease (Fig. 3A). Drought-preconditioned plants showed significantly higher SOD activity over non-preconditioned plants after 11 d of drought stress and reached to a peak value at 8 d of drought stress. After 3 d of rewatering, the activity of SOD in drought-preconditioned plants maintained significantly higher level than that in non-preconditioned plants (Fig. 3A). In response to drought stress, the activities of CAT showed an increase and reached a peak value at 11 d of drought stress and then started to decrease after 6 d of rewatering in both drought-preconditioned and non-preconditioned plants (Fig. 3B). However, CAT activity of drought-preconditioned plants was significantly higher than that of non-preconditioned plants at 4 d and 11 d of drought. After 6 d of rewatering, the activities of CAT in both treatment plants returned to the well-watered level without statistically significant differences.

During the process of drought stress, APX activities showed similar trend in drought-preconditioned and non-preconditioned plants, where were increased gradually,

with maximum activity at the last day of drought stress and then declined to the lower level in both treatments after 6 d of rewatering (Fig. 3C). However, APX activity of drought-preconditioned was significantly higher than that of non-preconditioned in the whole process of drought stress.

There was a gradual increase in POD activities for both treatments until the end of drought stress and then started to decline after 6 d of rewatering (Fig. 3D). POD activity increased up to about 67% in drought-preconditioned treatment and 41% in non-preconditioned treatment at the last day of drought stress. A significantly higher POD activity was observed in drought-preconditioned plants than that in non-preconditioned plants at 0, 8 and 11 d of drought stress (Fig. 3D).

Differential Cu/Zn superoxide dismutase (SOD) gene expression to drought stress and post-drought recovery between drought-preconditioned and non-preconditioned plants

The expression of *Cu/Zn SOD* was strongly enhanced in both treatments by drought stress and 6 d of rewatering (Fig. 4). The transcript levels of *Cu/Zn SOD* gene were not different between drought-preconditioned and non-preconditioned treatment under well-watered condition (0 d), but there was significantly higher expression of *Cu/Zn SOD* detected in drought-preconditioned plants than in non-preconditioned plants, after 8 d of drought stress. Both treatments still maintained higher transcript level of *Cu/Zn SOD* at 6 d of rewatering. These results were consistent with *SOD* activities in both treatments as discussed previously (Fig. 4).

Effects of drought preconditioning on the accumulation of osmoregulatory solutes under drought stress and post-drought recovery

There are significant higher osmoregulatory solutes (total soluble sugar, free proline and betain) content in drought-preconditioned plants as compared to non-preconditioned plants before drought stress (0 d) (Fig. 5A). Progressive water deficit induced significant increase of soluble sugar content, so that soluble sugar accumulation of drought-preconditioned and non-preconditioned plants in leaves reached their maximum after 11 d and 8 d of drought stress, respectively. More soluble sugar was accumulated in drought-preconditioned plants than in non-preconditioned plants in response to drought stress. As a result, there was a significant difference in soluble sugar content between two treatments after 11 d of drought stress. After 6 d of rewatering, soluble sugar content in both treatments were changed, almost reaching the same final level (Fig. 5A).

Free proline content increased in leaves of both treatments, when RWC decreased and drought stress intensified (Fig. 5B). Non-preconditioned plants, which had a faster increase, maintained higher free proline content than drought-preconditioned plants in response to drought stress. Free proline content became significantly higher in non-preconditioned plants at 8 d and 11 d of drought stress and 3 d and 6 d of rewatering, as compared to pre-condition treated plants. At the last day of drought stress, free proline content increased 2.8 and 4.82 folds in drought-preconditioned and non-preconditioned plants, respectively (Fig. 5B).

Furthermore, changes of betaine content showed similar trends of change in leaves of both treatments under drought stress and rewatering (Fig. 5C). Betaine content increased when drought stress extended. Drought-preconditioned plants

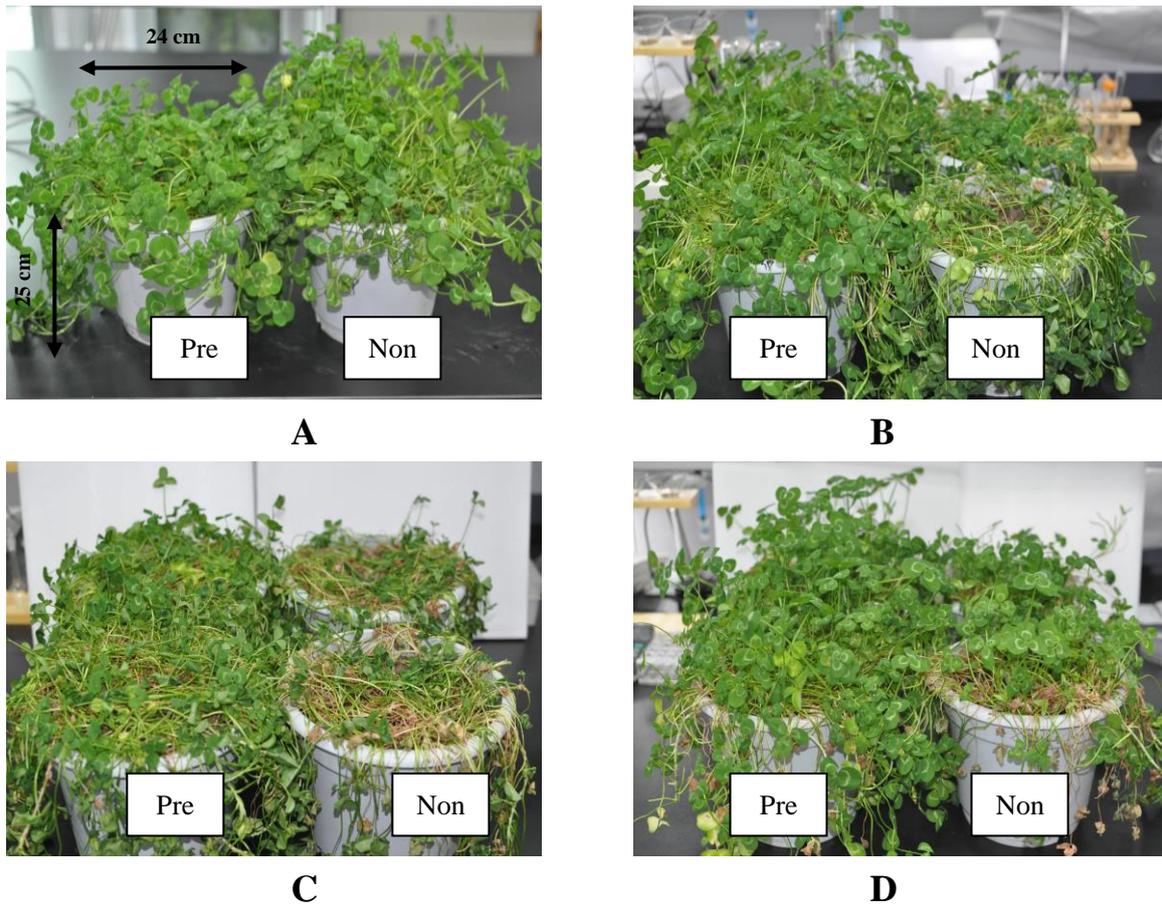


Fig 2. Effects of drought preconditioning on phenotypic responses to drought stress and post-drought recovery in white clover. These Photos were taken at: (A) drought 0 d (after two cycles of drought preconditioning and then rewatering for 3 d), (B) drought 8 d, (C) drought 11 d, and (D) rewatering 6 d. Pre, drought-preconditioned; Non, non-preconditioned.

had obviously higher betaine content than non-preconditioned plants at the start and could also maintain significantly higher betaine content with duration of drought stress and rewatering (Fig. 5C).

Discussion

When plants are subjected to drought stress, they are characterized by an increase in the level of ROS, expression of antioxidant genes and activities of antioxidants meant for ROS scavenging and improved antioxidant defense system results in tolerance against drought (Mano, 2002). In current study, we chose drought-sensitive white clover ‘Chuanyin Ladino’ as material to study the drought tolerance mechanism of drought preconditioning associated with antioxidant defense, according to our early study (Li et al., 2013) that found drought-tolerant white clover cultivar PI 288084 (provided by NPGS) have more active reactive oxygen species (ROS) scavenging system and higher activities of SOD, POD, CAT and APX than drought-sensitive cultivar ‘Chuanyin Ladino’. Those results also indicated that antioxidant defense plays an important role in improvement of drought tolerance in white clover. SOD is regarded as the key enzyme in the active oxygen scavenger system because it catalyzes superoxide free radical dismutation into H_2O_2 and O_2 , which is the first step of scavenging ROS (Bowler et al., 1992). Raul et al. (2006) found that SOD activity significantly increased in resistant white clover plants, but no

significant change was observed in the sensitive biotype under drought stress. Furthermore, higher expressions of antioxidant enzyme genes in drought-tolerant cultivars or transgenic drought-tolerant plants of tobacco, rice and kentucky bluegrass have been reported (Badawi et al., 2004; Prashanth et al., 2008; Xu et al., 2011). The results from this study showed that increased activities of SOD in both treatments during the early period of drought stress could be an adaptation or response to alleviate oxidative stress and improve growth. SOD activity in drought-preconditioned plants showed a higher increase compared to non-preconditioned plants during prolonged period of drought stress, which resulted in a statistically significant difference ($p \leq 0.05$).

The expression of *Cu/Zn SOD* was consistent with SOD activities in both treatments and significantly higher expression of *Cu/Zn SOD* was detected in drought-preconditioned plants than in non-preconditioned plants after 8 d of drought stress. It is suggested that drought-preconditioned plants have higher potential to scavenge superoxide free radical. The better capacity could be attributed to drought preconditioning. CAT and APX are two major enzymes which catalyze the conversion of H_2O_2 to H_2O and O_2 in overcoming drought stress imposed oxidative stress as there is an increase in CAT activities in stressed plants (Asada, 1992; Hrishikesh et al., 2008). Drought-preconditioned plants maintained significantly

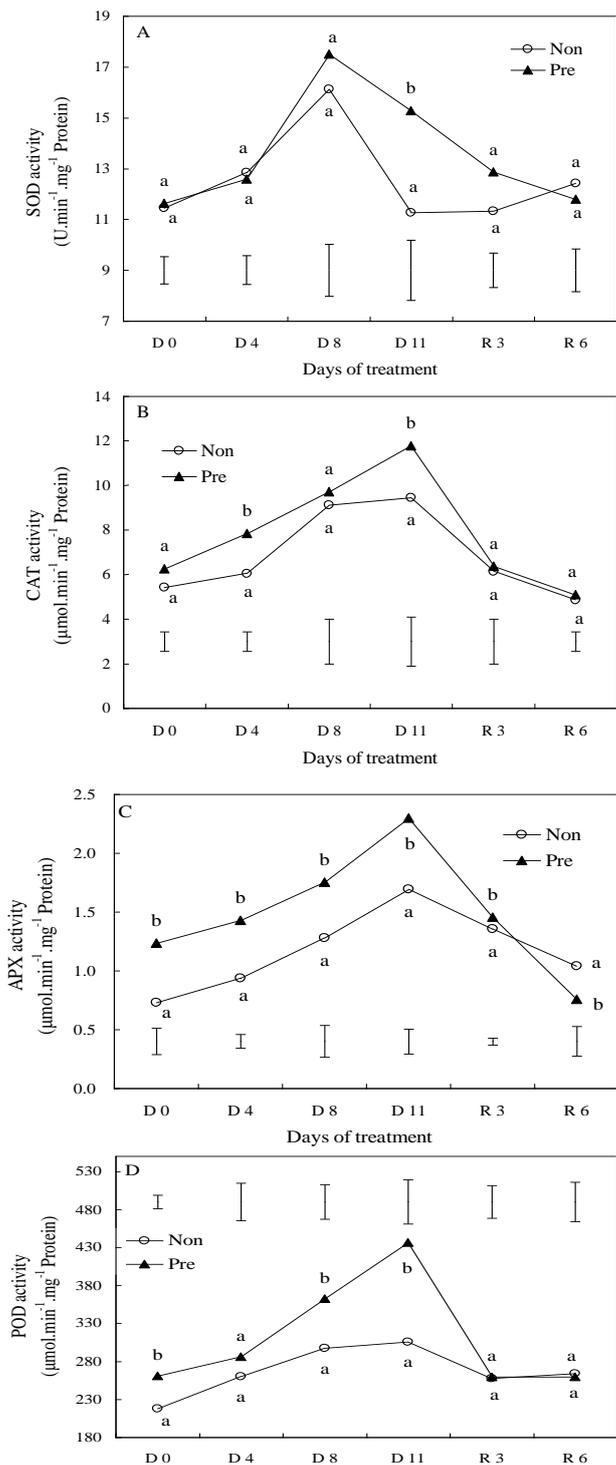


Fig 3. Effects of drought preconditioning on antioxidant enzyme activities under drought stress and post-drought recovery in white clover: (A) Superoxide dismutase (SOD) activity, (B) Catalase (CAT) activity, (C) Ascorbate peroxidase (APX) activity, and (D) Guaiacol peroxidase (POD) activity. Vertical bars indicate LSD ($P \leq 0.05$) for treatment comparisons and different letters indicate a significant difference at a given day of treatment. Pre, drought-preconditioned; Non, non-preconditioned; D 0-11, drought 0-11 d; R 3-6, rewatered 3-6 d.

higher CAT and APX activities as compared to non-preconditioned plants throughout the drought stress process. POD activities also showed similar trend in both treatments of plants. From these results, drought preconditioning improved or enhanced antioxidant defense system in white clover. Conversely, it may suppress drought-induced leaf senescence, as demonstrated by lower lipid peroxidation (lower MDA content) and better cell membrane stability under drought stress. The increase of antioxidant enzyme activities and gene expression may play an important role in protecting grass species from drought stress injury.

Drought-stressed plants have been shown to accumulate organic osmolytes such as sugars and amino acids that are known to contribute to the plant tolerance under water-deficit conditions (Schellenbaum et al., 1998). Betaines, ectoine, and proline are among the compatible solutes that also accumulate in plants as a widespread response against environmental stress (Chen and Murata, 2002). Some investigators observed that more sugars accumulated in drought-tolerant cultivars as compared to that in drought-sensitive cultivars (Turner, 1990; Kerepesi and Galiba, 2000). Early study has reported that under water deficit, soluble carbohydrate accumulation was significantly higher in a drought-tolerance white clover cultivar when compared with a drought-sensitive cultivar (Michael et al., 2000). In our case, drought-preconditioned plants maintained significantly higher soluble sugars, proline and betain content than non-preconditioned plants prior to exposure of plants to drought stress (0 d). The accumulated osmolytes in the process of drought preconditioning may not be used completely in growth or something else after two days of rewatering. Furthermore, both the drought-preconditioned and non-preconditioned plants accumulated soluble sugars, free proline and betain in response to drought stress and these organic osmolytes content changed after 6 d of rewatering, almost reaching the same final level. More soluble sugars and betain were accumulated in drought-preconditioned plants than in non-preconditioned plants. This result suggested soluble sugars and betain in white clover may be involved in osmotic adjustment and osmoprotection, which can explain the higher RWC and better membrane stability in drought-preconditioned plants regarding the response of drought stress. It is worthwhile to further investigate the different function and capacity of osmotic adjustment about soluble sugars and betain induction by drought precondition under drought stress.

Iannucci et al. (2002) suggested that proline levels of white clover played an important role in response to drought stress. But in our results, less accumulated proline was observed in drought-preconditioned treatment. Other similar results also showed that drought-tolerant cultivars accumulated less proline than drought-sensitive cultivars in many crops and grass species under drought stress (Hanson et al., 1979; Premachandra et al., 1995; Sundaresan and Sudhakaran, 1995; Lu et al., 2009). These studies have reported on the relationship between proline accumulation and stress tolerance under drought stress and suggested that proline accumulation caused by drought stress was not an indicator for drought stress resistance, but just a symptom of drought stress injury. Taking into account, the significantly declined RWC and cell membrane stability and increased lipid peroxidation level in non-preconditioned plants compared to drought-preconditioned plants, we highly suggest that non-preconditioned plants with higher free proline under drought condition may reflect a more severe metabolic injury caused by drought stress.

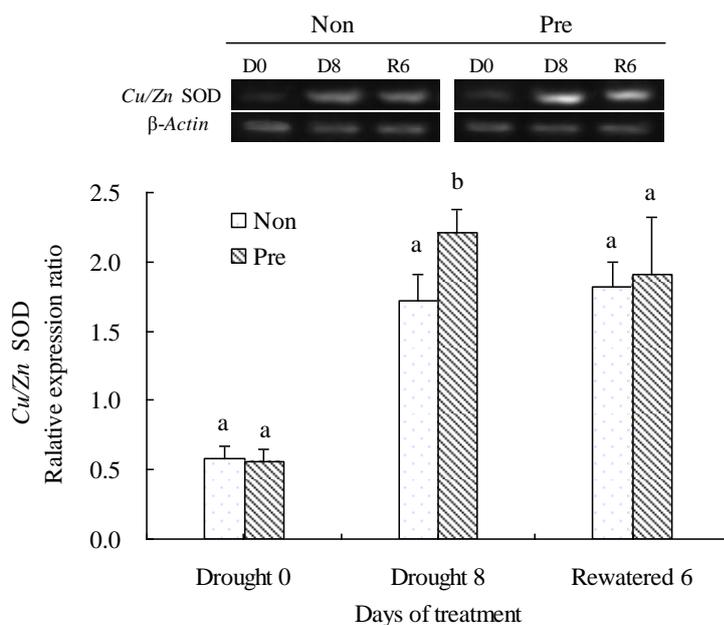


Fig 4. Effects of drought preconditioning on relative expression ratio of Cu/Zn Superoxide dismutase (SOD) gene under 0 d of drought stress (Drought 0), 8 d of drought stress (Drought 8) and 6 d of rewatering (Rewatered 6). Bars indicate \pm SE (n=3). Different letters indicate a significant difference for treatment comparisons at a given day. Pre, drought-preconditioned; Non, non-preconditioned.

Materials and Methods

Plant materials

Our earlier study (Li et al., 2013) showed that white clover cultivar PI 288084 (drought tolerance, provided by NPGS) have more active reactive oxygen species (ROS) scavenging system and higher activities of SOD, POD, CAT and APX than cultivar ‘Chuanyin Ladino’ (drought sensitive). The results suggested that comparatively higher ROS scavenging system could be associated with better drought tolerance and post-drought recovery in white clover. Accordingly, in this study we chose drought-sensitive white clover ‘Chuanyin Ladino’ as material to study the drought tolerance mechanism of drought preconditioning associated with antioxidant defense. Plants of white clover cultivar, ‘Chuanyin Ladino’ (drought sensitive), were collected from the farm of Sichuan Agricultural University, Sichuan, China and were planted in pots (24 cm diameter, 25 cm deep) filled with sterilized mixture of sand and loamy soil (1: 2, v: v). The plants were watered daily and fertilized weekly with Hoagland’s solution (Hoagland and Arnon, 1950) in the greenhouse. After three months of establishment in the greenhouse, plants were transferred to a growth chamber with 14 h photoperiod at day/night temperature of 21/16 °C, 70 % relative humidity, and 300 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetic photon flux density.

Drought preconditioning and stress treatments

One set of plants in four containers (four replicates) were kept well-watered by daily irrigation to serve as a well-watered control or non-preconditioned. Another set of plants in four containers were exposed to two cycles of drought preconditioning. These plants were subjected to drought stress for 7 d until leaf relative water content dropped to around 60 % and then rewatered for 3 d to allow leaf relative water content recovered to the well-watered control

level. The re-watered plants were then subjected to the second cycle of drought stress for 7 d, and then rewatered for 2 d until leaves were fully hydrated or turgid well-watered control. After rewatering, both drought-preconditioned and non-preconditioned plants were exposed to drought stress for 11 d and rewatered for 6 d in growth chambers (21 °C/16 °C, day/night). Drought-preconditioned and non-preconditioned plants were arranged randomly inside chamber. Mature leaves were sampled at 0, 4, 8 and 11 d after the beginning of drought stress and at 3 and 6 d after recovery by optimal watering.

Antioxidant enzymes and lipid peroxidation analysis

0.2 g fresh leaves were randomly sampled from each pot at each sampling date and 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 °C. The supernatant was used for assays of antioxidant enzyme activities and content of malondialdehyde (MDA). 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 and APX was determined by following the changes in absorbance at 240, 470, 290 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).

Cu/Zn superoxide dismutase (SOD) gene expression analysis

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

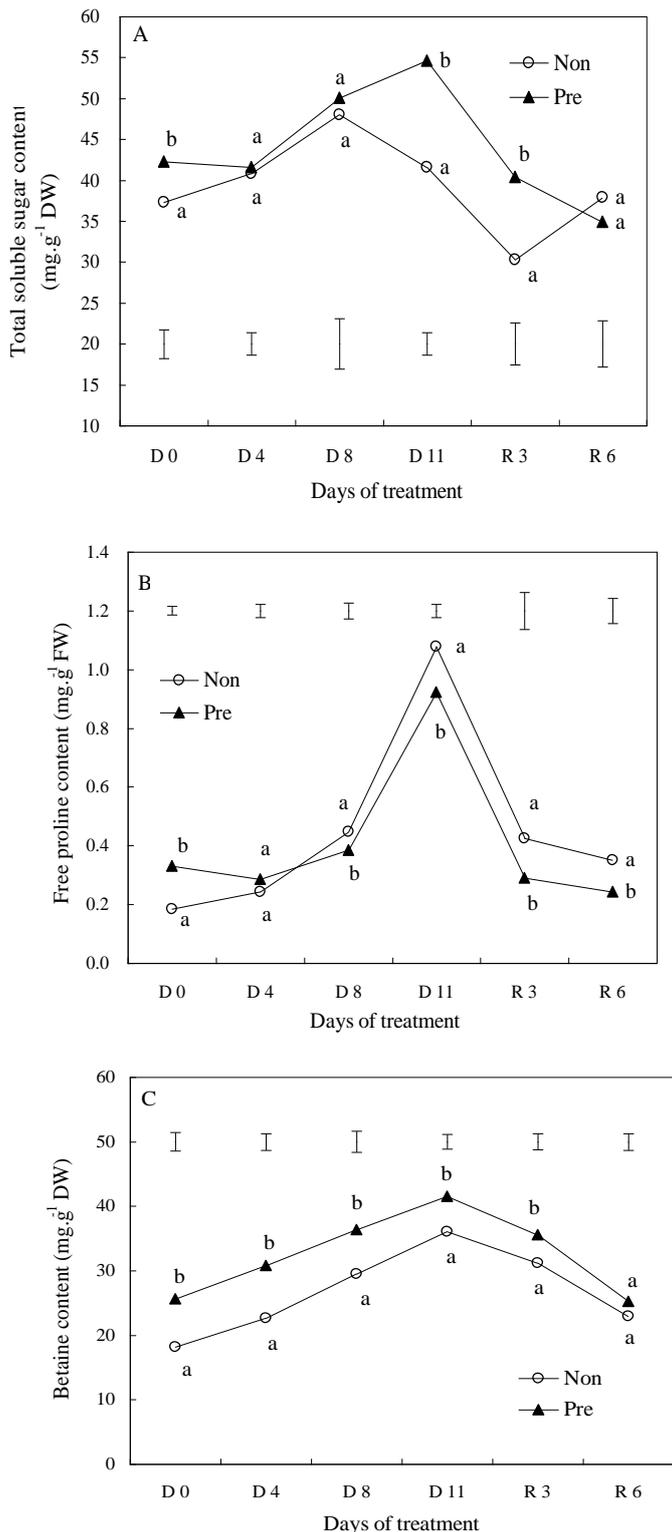


Fig 5. Effects of drought preconditioning on the accumulation of osmoregulatory solutes under drought stress and post-drought recovery in white clover: (A) total soluble sugar content, (B) free proline content, and (C) betaine content. Vertical bars indicate LSD ($P \leq 0.05$) for treatment comparisons and different letters indicate a significant difference at a given day of treatment. Pre, drought-preconditioned; Non, non-preconditioned; D 0-11, drought 0-11 d; R 3-6, rewatered 3-6 d.

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/Zn SOD and β -Actin (as internal control).

The β -Actin gene (GenBank ID: JF 968419) and Cu/Zn SOD gene (AY434497.1) were amplified with specific primers, respectively. The specific primers of β -Actin gene were: forward: 5'-TTACAATGAATTGCGTGTG-3'; reverse: 5'-AGAGGACAGCCTGAATGG-3'. The specific primers of Cu/Zn SOD genes were: forward: 5'-GCCTCTTACTGTCTGTTGC-3'; reverse: 5'-CCTGTTG-AGATACACCCATT-3'. The ORF region is chosen for primer pairs designed. The conditions of the PCR protocol for β -Actin gene and Cu/Zn SOD genes were both as follows: 3 min at 94 °C and 27 repeats of denaturation at 94 °C for 30 s, annealing at 57 °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.

Measurement of osmoregulatory solutes

Free proline was quantified spectrophotometrically by the ninhydrin method according to Bates et al. (1973). Soluble sugars were quantified following the phenolsulfuric acid method described by Robyt and White (1987). Betaine was estimated by the colorimetric method according to Grieve and Grattan (1983).

Statistical analysis

A completely randomized design with four replicates was used for two treatments and six sampling dates for leaves. 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 $P \leq 0.05$.

Conclusion

In summary, these results imply that drought preconditioning improved drought tolerance in drought-sensitive white clover cultivar of 'Chuanhui', as demonstrated by lower lipid peroxidation, better cell membrane stability and higher RWC in drought-preconditioned plants as compared to non-preconditioned plants. Enhanced antioxidant enzyme (SOD, CAT, APX and POD) activities and higher transcript level of Cu/Zn SOD gene may be one of the critical reasons in acquiring drought tolerance through drought preconditioning. This study also suggests that drought preconditioning enhanced white clover drought tolerance could be related to higher accumulation of soluble sugars and betaine, while the accumulated proline is associated with the degree of drought stress injury in white clover. The obtained results will help better understand physiological mechanism of plants under drought stress and post-drought recovery.

Acknowledgements

This study was supported by grants from Ministry of Education 'Chunhui' Program (Grant No. Z2010090), National Support Program (2011BAD17B03) and Sichuan Province Breeding Program (Grant No. 2011 NZ0098-11).

References

- Asada K (1992) Ascorbate peroxidase-A hydrogen peroxide-scavenging enzyme in plants. *Physiol Plantarum*. 85:235–241.
- Annicchiarico P, Piano E (2004) Indirect selection for root development of white clover and implications for drought tolerance. *J Agron Crop Sci*. 190:28–34.
- Barrs HD, Weatherley PE (1962) A re-examination of the relative turgidity technique for estimating water deficits in leaves. *Aust J Biol Sci*. 15 (3):413–428.
- Bates S, Waldren RP, Teare ID (1973) Rapid determination of the free proline in water stress studies. *Plant Soil*. 39:205–208.
- Blum A, Ebercon A (1981) Cell membrane stability as a measure of drought and heat tolerance in wheat. *Crop Sci*. 21:43–47.
- Bowler C, Montagu MV, Inze D (1992) Superoxide dismutase and stress tolerance. *Annu Rev Plant Physiol Plant Mol Biol*. 43:83–116.
- Badawi GH, Yasuo Y, Emi S, Ryozyo S, Naoyoshi K, Kunisuke T, Kiyoshi T (2004) Enhanced tolerance to salt stress and water deficit by overexpressing superoxide dismutase in tobacco (*Nicotiana tabacum*) chloroplasts. *Plant Sci*. 166:919–928.
- Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*. 72:248–254.
- Chance B, Maehly AC (1955) Assay of catalase and peroxidase. *Method Enzymol*. 2:764–775.
- Charlton JFL (1984) Persistence of Grasslands Huia white clover (*Trifolium repens* L.) in hill country pastures. *Proc N Z Grassland Assoc*. 45:131–139.
- Chen THH, Murata N (2002) Enhancement of tolerance of abiotic stress by metabolic engineering of betaines and other compatible solutes. *Curr Opin Plant Biol*. 5:250–257.
- Dhindsa RS, Dhindsa PP, Thorpe TA (1981) Leaf senescence: correlated with increased leaves of membrane permeability and lipid peroxidation, and decreased levels of superoxide dismutase and catalase. *J Exp Bot*. 32:93–101.
- Foyer CH, Descourvieres P, Kunert KJ (1994) Photooxidative stress in plants. *Physiol Plantarum*. 92:696–717.
- Giannopolitis CN, Ries SK (1977) Superoxide dismutase. I. Occurrence in higher plants. *Plant Physiol*. 59:309–314.
- Grieve CM, Grattan SR (1983) Rapid assay for determination of water soluble quaternary ammonium compounds. *Plant Soil*. 70:303–307.
- Guamaschelli AB, Lemcoff JH, Prystupa P, Basci SO (2003) Responses to drought preconditioning in *Eucalyptus globulus* Labill procenances. *Trees Struct Funct*. 17:501–509.
- Guamaschelli AB, Prystupa P, Lemcoff JH (2006) Drought conditioning improves water status, stomatal conductance and survival of *Eucalyptus globulus* subsp. *bicostata* seedlings. *Ann For Sci*. 63:941–950.
- Hoagland CR, Arnon DI (1950) The solution culture method for growing plants without soil. *California Agric Exp Circ*. 347:1–32.
- Hanson AD, Nelsen CE, Pedersen AR, Everson EH (1979) Capacity for proline accumulation during water stress in barley and its implications for breeding for drought resistance. *Crop Sci*. 19:489–493.
- Hendry GAF (1993) Oxygen free radical process and seed longevity. *Seed Sci Res*. 3:141–153.
- Hasegawa PM, Bressan RA, Zhu JK, Bohnert HJ (2000) Plant cellular and molecular responses to high salinity. *Annu Rev Plant Physiol Mol Biol*. 51:463–499.
- Hrishikesh U, Sanjib KP, Biman KD (2008) Variation of physiological and antioxidative responses in tea cultivars subjected to elevated water stress followed by rehydration recovery. *Acta Physiol Plant*. 30:457–468.
- Iannucci A, Russo M, Arena L (2002) Water deficit effects on osmotic adjustment and solute accumulation in leaves of annual clovers. *Eur J Agron*. 16(2):111–122.
- Kerepesi I, Galiba G (2000) Osmotic and salt stress-induced alteration in soluble carbohydrate content in wheat seedlings. *Crop Sci*. 40:482–487.
- Karsten HD, Macadam JW (2001) Effect of drought on growth, carbohydrates, and soil water use by perennial ryegrass, tall fescue, and white clover. *Crop Sci*. 41:156–166.
- Lambers H, Shane MW, Cramer MD, Pearse SJ, Veneklaas EJ (2006) Root structure and functioning for efficient acquisition of phosphorus: matching morphological and physiological traits. *Ann Bot (Lond)*. 98:693–713.
- Li Z, Peng Y, Ma X (2013) Different response on drought tolerance and post-drought recovery between the small-leafed and the large-leafed white clover (*Trifolium repens* L.) associated with antioxidative enzyme protection and lignin metabolism. *Acta Physiol Plant*. 35:214–222.
- Lu, SY, Chen CH, Wang ZC, Guo ZF, Li HH (2009) Physiological responses of somaclonal variants of triploid bermudagrass (*Cynodon transvaalensis* × *Cynodon dactylon*) to drought stress. *Plant Cell Rep*. 28:517–526.
- Morgan JM (1984) Osmoregulation and water in higher plants. *Annu Rev Plant Physiol* 35:299–319.
- Michael TM, Rod LB, John RC, Barker D (2000) Pinitol accumulation in mature leaves of white clover in response to a water deficit. *Environ Exp Bot*. 43:11–18.
- Mano J (2002) Early events in environmental stresses in plants—induction mechanisms of oxidative stress. In: Inze D, Montago MV, eds, *Oxidative stress in plants*. New York, USA, pp 217–245.
- Nakano Y, Asada K (1981) Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. *Plant Cell Physiol*. 22(5):867–880.
- Premachandra GS, Hahn DT, Rhodes D, Joly RT (1995) Leaf water relations and solute accumulation in two grain sorghum lines exhibiting contrasting drought tolerance. *J Exp Bot*. 46:1833–1841.
- Prashanth SR, Sadhasivam V, Parida A (2008) Over expression of cytosolic copper/zinc superoxide dismutase from a mangrove plant *Avicennia marina* in indica rice var Pusa Basmati-1 confers abiotic stress tolerance. *Transgenic Res*. 17: 281–291.
- Robynt JF, White BJ (1987) *Biochemical techniques—theory and practice*. Books/Cole Publishing Company, Monterey, pp 267–275.
- Raul B, Juan JI, Jesus MS (2006) Short-term drought response of two white clover clones, sensitive and tolerant to O₃. *Physiol Plantarum*. 127:658–669.
- Renu KC, Devarshi SS (2007) Acclimation to drought stress generates oxidative stress tolerance in drought-resistant than -susceptible wheat cultivar under field conditions. *Environ Exp Bot*. 60:276–283.
- Sairam RK, Vasanthan B, Arora A (2011) Calcium regulates *Gladiolus* flower senescence by influencing antioxidative enzymes activity. *Acta Physiol Plant*. 33:1897–1904.
- Scandalios JG (1993) Oxygen stress and superoxide dismutases. *Plant Physiol*. 101:7–12.

- Sundaresan S, Sudhakaran PR (1995) Water stress induced alterations in the proline metabolism of drought-susceptible and -tolerant cassava (*Manihot esculenta*) cultivars. *Physiol Plantarum*. 94:635–642.
- Schellenbaum L, Muller J, Boller T, Wiemken A, Schuepp H (1998) Effects of drought on non-mycorrhizal and mycorrhizal maize: changes in the pools of non-structural carbohydrates, in the activities of invertase and trehalase, and in the pools of amino acids and imino acids. *New Phytologist*. 138:59–66.
- Sharma P, Dubey RS (2005) Modulation of nitrate reductase activity in rice seedlings under aluminium toxicity and water stress: Role of osmolytes as enzyme protectant. *J Plant Physiol*. 162:854–864.
- Turner LB (1990) The extent and pattern of osmotic adjustment in white clover (*Trifolium repens* L.) during the development of water stress. *Ann Bot*. 66(6):721–727.
- Tambussi EA, Bartoli CG, Beltrano J, Guamet JJ, Arans JL (2000) Oxidative damage to thylakoid proteins in water stressed leaves of wheat (*Triticum aestivum*). *Physiol Plantarum*. 108:398–404.
- Turkan I, Bor M, Ozdemir F, Koca H (2005) 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*. 168:223–231.
- Vilagrosa A, Cortina J, Pelegrin EG (2003) Suitability of drought-preconditioning techniques in mediterranean climate. *Restor Ecol*. 11:208–216.
- Westbrooks FE, Tesar MB (1955) Tap-root survival of ladino clover. *Agron J*. 47:403–410.
- Xu LX, Han LB, Huang BR (2011). Antioxidant enzyme activities and gene expression patterns in leaves of Kentucky bluegrass in response to drought and post-drought recovery. *J Amer Soc Hort Sci*. 136:247–255.