

Photosynthetic characteristics and variation of osmoregulatory solutes in two white clover (*Trifolium repens* L.) genotypes in response to drought and post-drought recovery

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Abstract

This study was conducted to analyze responses of the small-leafed white clover (SL) and the large-leafed white clover (LL) associated with photosynthetic characteristics and osmoregulatory solutes accumulation under drought stress and rewatering. The plants of two genotypes were subjected to drought stress for 12 days by withholding irrigation and then rewatering for 6 days in a greenhouse. Their relative water content (RWC), electrolyte leakage (EL), malondialdehyde content (MDA), water use efficiency (WUE) in photosynthetic process and osmoregulatory capacity in response to water stress and rewatering were comparatively measured. Results showed that SL plants maintained significantly higher RWC and membrane stability ($P \leq 0.05$) expressed as EL level and content of MDA with the increased drought. Following rewatering, SL recovered more rapidly in all parameters than LL. Progressive water deficit induced the decline of net photosynthetic rate (Pn) and stomatal conductance (g_s) in both genotypes. However, intercellular CO₂ concentration (Ci) of both clovers gradually increased with the decline of RWC and was not affected by a decline of g_s . It could be referred that limit photosynthesis of the two white clovers was caused by non-stomatal factors during drought stress. Meanwhile, WUE in the SL was significantly higher ($P \leq 0.05$) than that in the LL under drought stress although the WUE trend was totally opposite while being well watered and rewatered. Furthermore, significantly higher ($P \leq 0.05$) osmoregulatory solutes, such as free proline, total soluble sugar and betaine, were observed in the SL leaves as compared with that in the LL under treatment conditions. The results suggested that drought tolerance and post-drought recovery in different white clover genotypes were associated with their ability to accumulate the osmoregulatory solutes, which might play an important role in protecting cellular membrane stabilization from water loss and improving water use efficiency in photosynthetic process during drought stress.

Keywords: drought stress; leaf size; osmotic adjustment; photosynthetic; rewatering; white clover (*Trifolium repens* L.).

Abbreviations: Ci - intercellular CO₂ concentration; EL - electrolyte leakage; g_s - stomatal conductance; LL - the large-leafed white clover; MDA - malondialdehyde; OAT - ornithine aminotransferase; Pn - net photosynthetic rate; RWC - relative water content; SL - the small-leafed white clover; WUE - water use efficiency.

Introduction

White clover (*Trifolium repens* L.) is a crucial legume herbage for temperate pastures because of its contributions to feed quality, Nitrogen fixation and complementary growth patterns. It is also widely used for turfs and urban parks due to its ornamental value. In practice, white clovers rarely reach their potential yields because of various abiotic and biotic restraints (Mercer and Watson, 2007). Drought stress has become one of the most detrimental abiotic stresses for their growth and development. Identification of white clovers with enhanced drought tolerance could increase legume content in grazed swards, enhance pasture quality and animal performance (Van et al., 1993). Acclimation to water stress may comprise responses involving gene expression and modification of plant physiology and morphology, taking place in days to weeks, which lead to a homeostatic compensation for the initial negative effects of water stress on photosynthesis (Jaume et al., 2009). In generally, photosynthesis limitation is firstly and predominantly driven by a decline in stomatal conductance. When stomatal conductance drops below a certain threshold ($<50 \text{ mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$), limitations of non-stomatal processes become more important, in particular, decreased mesophyll conductances (g_m) and impaired photobiochemistry (Zhou et al., 2007; Flexas and Medrano, 2002). Furthermore, as drought stress mainly affects the plant carbon balance, in particular,

photosynthesis and respiration adjustments at the leaf level are of primary importance (Chaves et al., 2003). Adjustment of leaf diffusion components for CO₂ is one way for plants to cope with situation of limited water supply and concurrently to improve their water use efficiency (Alexander et al., 2010). It is known that during drought stress, plants with C₄ photosynthesis increase water use efficiency and suppress photorespiration, thus, C₄ plants are often more competitive than C₃ plants in drought-prone areas (Edwards and Ku, 1987). According to the study of Carolina et al. (2006), drought caused a decrease in stomatal conductance in two varieties of common bean, but drought-tolerant 'Orfeo' maintained lower conductances than drought-sensitive 'Arroz' for a given P_{\max} value, indicating higher water use efficiency in Orfeo. Beke and Wu (2012) showed a similar conclusion studying on photosynthetic response of three climber plant species to osmotic stress induced by polyethylene glycol 6000. Moreover, Eleni et al. (2008) found that the maintenance of higher photosynthetic and WUE may have contributed to the improved drought tolerance in hybrid bluegrass. A general idea is that morphological changes and osmotic adjustments may be a long-term acclimation when plants are under drought stress. 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; 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). Proline is thought to play a cardinal role as an osmoregulatory solute in plants subjected to hyperosmotic stresses, primarily drought and soil salinity (Ashton and Desh, 1993). A variety of other 'compatible' organic solutes including glycine betaine, soluble sugar and glycerol have been shown to accumulate in osmotically stressed plants cells. They are to help maintain plant water status, particularly turgor (Cassandra and Derrick, 2000). Iannucci et al. (2002) who used four clovers in Italy to evaluate the osmotic adjustment capability and the relevant contribution of inorganic and organic solutes in response to water deficits found that water-stressed plants showed an increase in potassium, reducing sugars and proline concentrations and a decrease in non-reducing sugar contents. Generally, about 80% of the measured cellular osmotic potential was attributable to assayed osmotically active solutes. Other studies also suggested that osmotic adjustment in white clover stolons conserves the stolons until water is available, and the plant can regrow from the stolon (Turner, 1990; Karsten and Macadam, 2001). Such a strategy (osmoregulatory solutes accumulation) could explain how white clover plants survive a drought better than perennial ryegrass and dominate a pasture over time. However, current knowledge is scarce on physiological limitations to photosynthesis during short-term acclimation to different water-stress intensities and recovery after rewatering, but crucial to improve the understanding of plant responses to drought and for the development of water-saving irrigation schedules in agriculture (Flexas et al., 2006). Moreover, it is worth discussing whether active osmotic adjustment is associated with better photosynthetic ability and whether different drought tolerance has a correlation with water use efficiency and osmotic adjustment between the large-leafed and the small-leafed white clover. Better understanding of physiological mechanisms of stress tolerance and physiological effects of post-drought recovery in white clovers would benefit clover breeding and management programs. The objectives of the current study were (i) to evaluate the drought tolerance and post-drought recovery in two white clover genotypes associated with photosynthetic ability and osmotic adjustment and (ii) to compare difference of water use efficiency when white clovers with different leaf sizes are exposed to drought stress and rewatering. Such information will help further understand the interactive mechanisms of plant tolerance to drought stress and prepare for selecting better white clover cultivars.

Results

Physiological analysis of drought tolerance and post-drought recovery in leaves

A gradual decrease in RWC for both white clover genotypes was observed at various stages of drought stress. However, the SL was able to maintain higher RWC level than the LL under the same days of drought treatment (Fig 1A). RWC was significantly higher ($p \leq 0.05$) for the SL at 12 d of drought stress as compared to the LL. Meanwhile, RWC of the SL recovered to a higher level after rewatering for 6 d (Fig 1A). Cellular membrane stability was estimated by the EL level and MDA content. As is shown in Fig 1B and 1C, the similar variation of the EL level and MDA content were observed in two clovers during the whole period of treatment. Their EL and MDA almost maintained at the same initial level under normal watering condition (0 d) and increased continuously with the

duration of drought stress, respectively. However, the EL and MDA of the SL were significantly lower ($p \leq 0.05$) than those of the LL after 12 d of drought stress. Correspondingly, the EL and MDA for both genotypes declined fast after rewatering, but the EL and MDA in the SL were also significantly lower ($p \leq 0.05$) than those in the LL (Fig 1B and 1C). The results implied that the SL has a better ability of refraining from drought damage or repairing the integrality of cell membrane system.

Photosynthetic characteristics in response to drought and rewatering in leaves

Two white clover genotypes, which showed a different net photosynthetic rate (P_n) in fully watering (0 d), experienced reduction in P_n in response to drought stress. This reduction was more quickly for the LL (77%) than that for the SL (56%) after 5 d of drought stress, which resulted in a significantly higher ($p \leq 0.05$) P_n in SL than that in LL at the same time. The P_n of two clovers decreased almost to the same level after 12 d drought stress, but the SL exhibited a significantly higher ($p \leq 0.05$) P_n than the LL after 6 d of rewatering (Fig 2A). Water use efficiency (WUE) in photosynthesis declined in both genotypes in response to drought stress and then increased after 6 d of rewatering (Fig 2B). WUE declined by about 70% in the SL and 86% in the LL by the end of drought stress, respectively. The LL genotypes showed a significantly higher ($p \leq 0.05$) WUE than the SL under well-watered condition and after 6 d of rewatering, while this situation was the opposite at 5 and 12 d of drought stress. Accordingly, the SL maintained a significantly higher ($p \leq 0.05$) WUE in response to drought stress (Fig 2B). Stomatal conductance (g_s) of two genotypes progressively declined during the experiment, from about $0.615 \text{ mol H}_2\text{O m}^{-2} \text{ s}^{-1}$ (0 d) to $0.152 \text{ mol H}_2\text{O m}^{-2} \text{ s}^{-1}$ (12 d) in the SL, and about $0.567 \text{ mol H}_2\text{O m}^{-2} \text{ s}^{-1}$ (0 d) to $0.130 \text{ mol H}_2\text{O m}^{-2} \text{ s}^{-1}$ (12 d) in the LL (Fig 2C). The differences in g_s between the SL and the LL were not statistically significant ($p \leq 0.05$). Upon rewatering, the g_s in both clovers began to increase again (Fig 2C). With the development of drought stress, intercellular CO_2 concentration (C_i) in both genotypes increased gradually and dropped after 6 days of rewatering (Fig 2D). The LL maintained significantly higher ($p \leq 0.05$) C_i than the SL during the whole treatments (Fig 2D).

Osmoregulatory solutes and OAT activity in response to drought and rewatering in leaves

Progressive water deficit induced significant increase of proline content, so that proline accumulation of both genotypes in leaves reached their maximum after 12 d of drought stress. Proline content increased by about 100% in the SL and by about 44% in the LL respectively after 12 d of drought stress as compared with well-watered level (0 d), which resulted in a significant difference ($p \leq 0.05$) of proline content between two genotypes. After 6 d of rewatering, proline content in both genotypes almost declined to the same level (Fig 3A). Ornithine aminotransferase (OAT) is a key enzyme in the synthesis of proline metabolism process. OAT activity in the LL was significantly higher ($p \leq 0.05$) compared to that in the SL in the beginning of drought stress, but both maintained at a lower level (Fig 3B). OAT activities in both genotypes reached to their peak value after 5 d of drought stress and at this time, more than 3.8 times of increased OAT activity was in the SL and 3.1 times was in the LL as compared with the initiation of stress (0 d), respectively. With the continuation of drought,

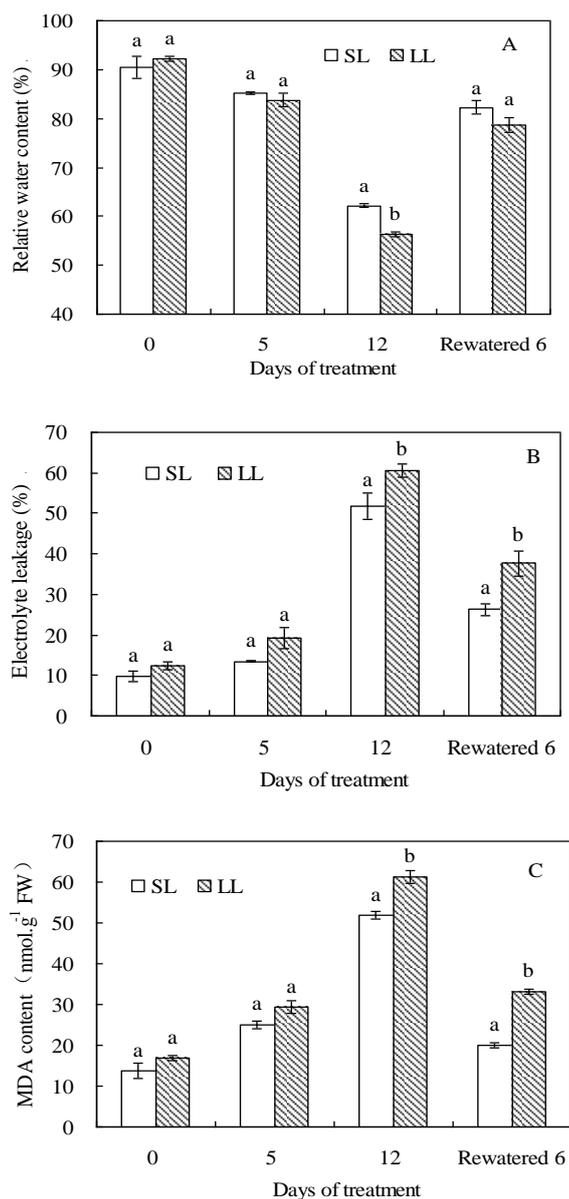


Fig 1. Changes in relative water content (RWC), electrolyte leakage (EL) and MDA content of the large-leaved (LL) and the small-leaved (SL) white clover at 0, 5 and 12 days of drought stress and 6 days of rewatering after treatment. (A) RWC, (B) EL and (C) MDA content. Vertical bars indicate \pm SE of mean ($n=4$). Different letters above columns indicate a significant cultivar differences. LSD ($P \leq 0.05$)

OAT activity began to decline quickly in both materials, nevertheless, the SL had a significantly higher ($p \leq 0.05$) OAT activity than the LL at 12 d of drought stress. After rewatering, OAT activities of both treatments dropped back to the similar level (Fig 3B). Total soluble sugar content in leaves of both clovers accumulated or decreased with the declining or rising of RWC during the different period of treatments. From 5 d to 12 d of drought stress, the SL and the LL displayed 60% and 50% growth in total soluble sugar, respectively. Meanwhile, total soluble sugar content between two treatments was evidently different ($p \leq 0.05$) after 12 d of drought stress and 6 d of rewatering (Fig 3C). Furthermore, betaine content in leaves of both genotypes tended to the similar changes under drought stress and rewatering expressed by accumulatively

increasing when drought stress extended and then slightly reducing after rewatering. The SL not only had obviously higher betaine content than the LL before drought stress, but also could still maintain significantly higher ($p \leq 0.05$) level with the duration of drought stress and after rewatering (Fig 3D).

Discussion

Photosynthetic inhibition is one of the primary harmful effects of drought stress. Drought could decrease CO_2 supply caused by diffusion limitations through stomata factors or reduce net photosynthesis through non-stomatal factors such as decrease carboxylation efficiency (Chaves et al., 2009; Rouhi et al., 2007). Blaikie et al. (1988) reported that white clover was more sensitive to soil water deficit than paspalum. When water deficit stress reduced the maximum photosynthetic rate of white clover by 50%, the photosynthetic rate of paspalum did not decline. The research of Grieu et al. (1993) showed a similar result that white clover, which stomata closed dramatically and net CO_2 assimilation rate decreased steeply in response to water supply being withheld, was rather resistant to drought. In this study, both genotypes experienced reduction in net photosynthetic rate (P_n) under drought stress. But, P_n was significantly higher ($P \leq 0.05$) in the SL than that in the LL in the beginning of drought stress and after 6 days of rewatering, which indicated that the SL could have a better photosynthetic adaptation and recovery mechanisms under drought stress and rewatering. On the other hand, progressive water deficit induced decline of stomatal conductance (g_s) in both genotypes. However, intercellular CO_2 concentration (C_i) of both genotypes increased with the decline of RWC and was not affected by a decline of g_s . Considering the reduction in cell membrane stability and the rise in lipid peroxidation, we may therefore suggested that limit photosynthesis in this two white clovers could be caused by non-stomatal factors such as metabolic impairment associated with inhibition of photochemical reactions and carbon fixation, which decreased net CO_2 assimilation rate and led to CO_2 accumulation in plant cells as well. Water use efficiency (WUE) is an important index of a plant to adapt to an arid environment (Beke and Wu, 2012). During water stress plants generally close their stomata with resultant reduced water loss, decreased photosynthesis and an overall apparent increase in water use efficiency (Barbour et al., 1996). Hafid et al. (1997) stated that WUE was strongly positively associated with net CO_2 uptake, transpiration ratio and osmoregulation capacity by studying drought resistance in spring durum wheat (*Triticum L.*) cultivars. Awada et al (2003) suggested that higher WUE in big bluestem may become advantageous where water is a limiting factor. The results in our work demonstrated that although WUE of the LL was significantly higher ($P \leq 0.05$) than WUE of the SL in well-watered condition, WUE decreased more steeply in the LL (86%) than that in the SL (70%) after 12 d of drought stress. The SL maintained significantly higher WUE ($P \leq 0.05$) than the LL under drought stress suggested that the SL was able to keep balance between photosynthesis and transpiration, which could help it to survive drought stress through efficient use of water without loss of the photosynthetic capacity. Accordingly, the SL has a long-term adaptability to drought stress. 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). Turner (1990) found that water-

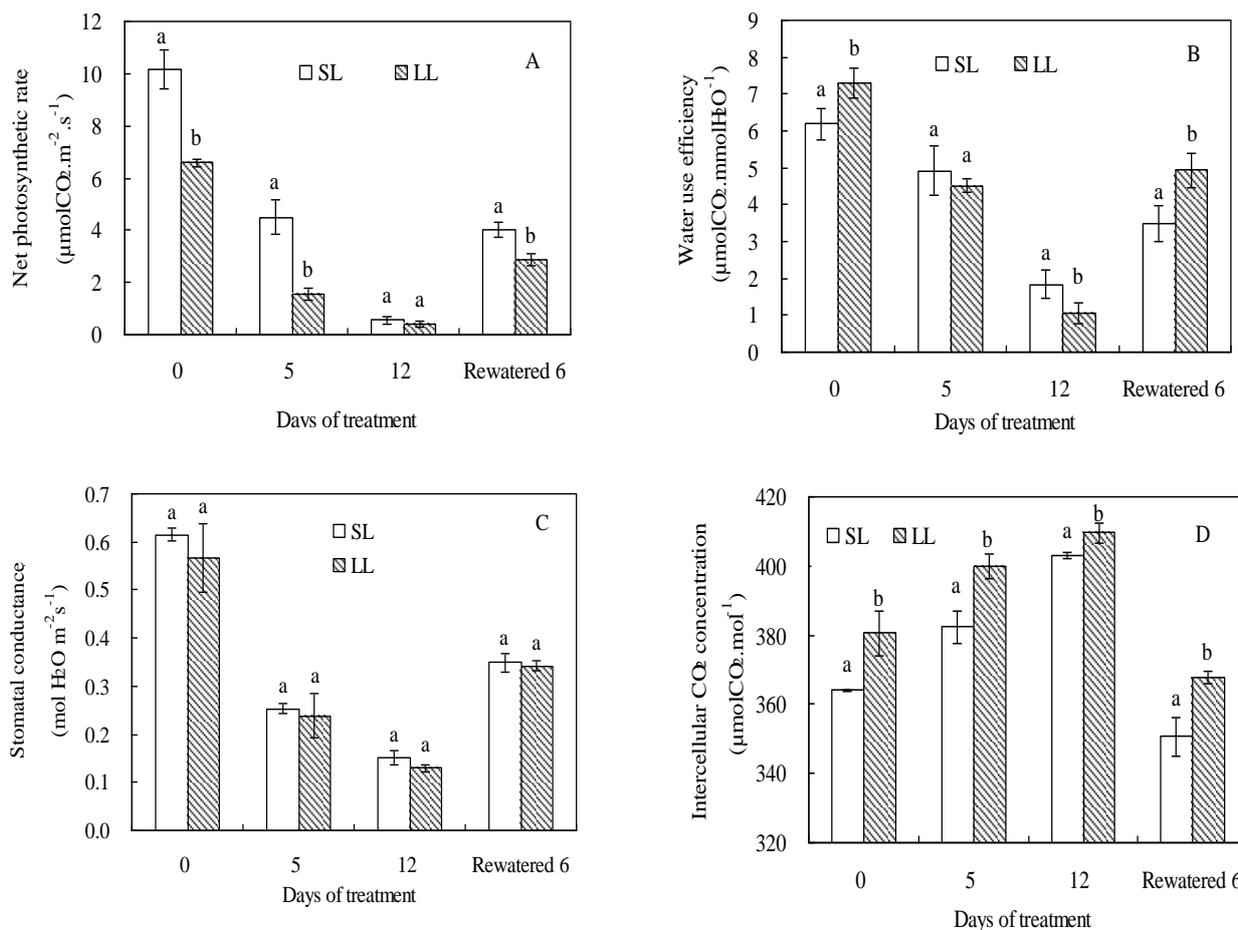


Fig 2. Changes in net photosynthetic rate (Pn), water use efficiency (WUE), stomatal conductance (g_s) and intercellular CO_2 concentration (C_i) of the large-leaved (LL) and the small-leaved (SL) white clover at 0, 5 and 12 days of drought stress and 6 days of rewatering after treatment. (A) Pn, (B) WUE, (C) g_s and (D) C_i . Vertical bars indicate \pm SE of mean ($n=4$). Different letters above columns indicate a significant cultivar differences. LSD ($P \leq 0.05$).

soluble carbohydrates and proline accumulated in white clover during water stress and the increase in proline level in leaves did not follow the same pattern as that in stolon tips, although toward the end of the water stress period the level had increased by a similar extent in both parts of the plant. Iannucci et al. (2002) also suggested that proline levels played an important role in white clover in response to drought stress. In this case, the SL showed a more efficient and resilient osmotic adjustment than the LL under drought stress and rewatering conditions. Free proline, soluble sugar and betain content began to accumulate in both genotypes at the initial stage of drought treatment and then reached their maxima at the last day of drought stress. The SL showed a higher variety of free proline content and OAT activity in response to drought stress as compared to the LL that suggested a more sensitive and rapid proline metabolic system consisted in the SL. During the periods of drought and rewatering treatment, the SL also had the advantage of accumulating more total soluble sugar and betaine solutes. This result agreed with earlier study of Michael et al. (2000) that proline and soluble carbohydrate accumulation were significantly higher in a drought-tolerance white clover cultivar when compared with a drought-sensitive cultivar under water deficit. Therefore, a more sensitive osmotic adjustment system could explain the higher RWC and better membrane stability in the SL regarding the response of drought stress. Furthermore, the higher osmoregulatory solutes

accumulation in the SL was also able to adjust osmotically to maintain photosynthesis and other metabolic functions. These differences could offer the SL better protection to minimize and eliminate drought effect.

Materials and methods

Plant material and treatments

Two genotypes of white clover The large-leaved drought-sensitive “Chuanyin Ladino” genotype was collected from the farm of Sichuan Agricultural University, Sichuan, China and the small-leaved drought-tolerant PI 288084 genotype was provided by NPGS were planted in pots (24 cm diameter, 30 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 with 14 h photoperiod at day/night temperatures of 24/ 20 °C for six months. The pot experiment was arranged in a completely randomized block design with two treatments (the SL genotype and the LL genotype) and four replications each treatment (4 pots). The plants of two genotypes were exposed to drought stress for 12 d and then rewatering for 6 d. And the leaves were sampled at 0, 5 and 12 d of drought stress and at 6 d of rewatering. Tissue samples were immediately frozen in liquid nitrogen until analyses.

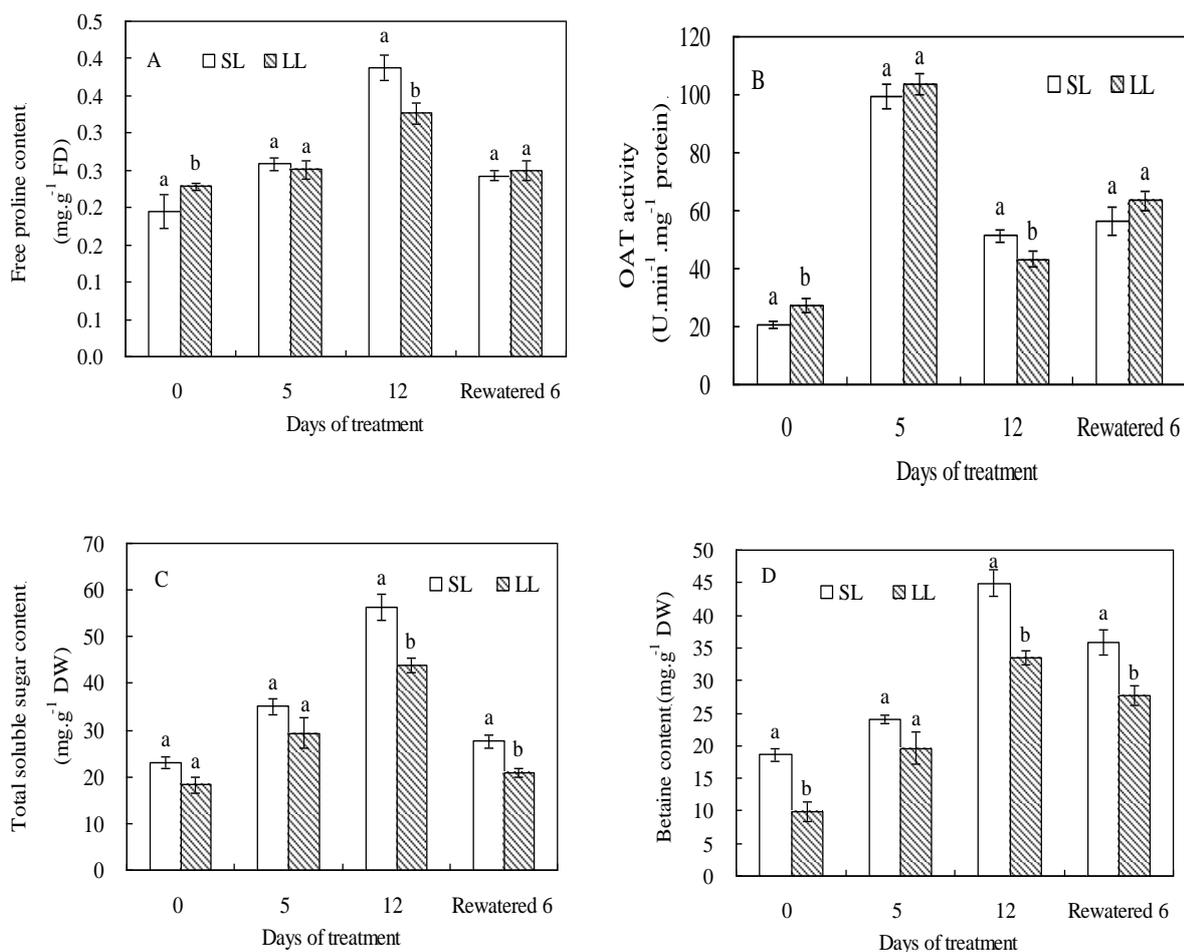


Fig 3. Changes in free proline content, OAT activity, total soluble sugar content and betaine content of the large-leaved (LL) and the small-leaved (SL) white clover at 0, 5 and 12 days of drought stress and 6 days of rewatering after treatment. (A) free proline content, (B) OAT activity, (C) total soluble sugar content and (D) betaine content. Vertical bars indicate \pm SE of mean (n=4). Different letters above columns indicate a significant cultivar differences. LSD ($P \leq 0.05$).

Physiological analysis

Physiological changes of the two genotypes in response to drought stress and rewatering were evaluated by leaf relative water content (RWC), cellular membrane stability expressed as electrolyte leakage (EL) and the degree of lipid peroxidation was estimated by the content of malondialdehyde (MDA). RWC, EL and MDA were determined according to methods of Barrs and Weatherley (1962), Blum and Ebercon (1981) and Dhindsa et al. (1981), respectively.

Determination of photosynthetic characteristics

Maximum net photosynthetic rates (P_n), transpiration rates (T_r), stomatal conductance (g_s) and intercellular CO_2 concentration (C_i) were measured using a portable photosynthetic system (LI-6400, LICOR Inc., Lincoln, NE, USA). The photosynthetic active radiation (PAR), temperature and CO_2 concentration during the measurements were $1200 \mu mol m^{-2} s^{-1}$, $23^\circ C$ and $390 \mu mol mol^{-1}$, respectively. Water use efficiency (WUE) was calculated according to the following equation: $WUE = P_n / T_r$. Measurements were made at 10:30 in the morning at 0, 5 and

12 d of drought stress and at 6 d of rewatering, respectively. 20 leaves were selected randomly in each plot for measurement each time.

Determination of osmoregulatory solutes and OAT activity

Free proline was quantified spectrophotometrically by the ninhydrin method according to Bates et al. (1973). The activity of OAT was determined according to the method of Lu and Mazelis (1975). 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 was used with four replicates for two water treatments and four 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 $P \leq 0.05$ by Duncan's multiple range test.

Conclusion

In conclusion, white clovers have developed various strategies to survive water-deficit and to improve their drought tolerance. However, drought resistance in white clover is different among different leaf size genotypes. The results imply that the small-leaved white clover (SL) could have more active and resilient osmotic adjustment mechanism, which might play an important role in protecting membrane system and improving RWC of leaves and WUE in photosynthesis under drought stress. Such results will help to better understand osmoprotection and physiological mechanism of white clovers under drought stress and post-drought recovery. Further studies are needed to explore the interactions of WUE and osmotic adjustment under limited water supply, as well as under other different kinds of stress.

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