Full Length Research Paper

Metabolic responses of alfalfa (*Medicago Sativa* L.) leaves to low and high temperature induced stresses

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To explore adaptation mechanism of alfalfa to cold and heat stresses, status of sucrose metabolism and relative water content (RWC) in leaves under low and high temperature treatments were studied. Seedlings (35 day old) were transferred to chambers for treatments. First group was subjected to 5°C as low temperature (LT) stress, second group at 33°C as high temperature (HT) stress and third group at 25°C as the control (CK). Results indicated that, both stresses led to an increase in degree and duration of genes expression and corresponding enzymes activities of sucrose phosphate synthase (SPS) and sucrose synthase (SS), but LT showed a more significant effect. As a result, lower starch content and higher contents of soluble reducing sugar, fructose and sucrose were observed under LT stress. Moreover, LT stress lowered malondialdehyde (MDA) content and electrolyte leakage in leaves, thus achieving a higher RWC. It was suggested that, relatively higher RWC in leaves by LT stress resulted from lowered water potential, and transpiration can explain the reason that alfalfa plants are cold-tolerant but heat-sensitive.

Key words: Alfalfa, temperature stress, sucrose, sucrose phosphate synthase (SPS), relative water content.

INTRODUCTION

Legume plants belong to the main single source of vegetable protein in human diets and livestock feed, having major impacts on agriculture, environment and even health (Graham and Vance, 2003; Dita et al., 2006). Due to the remarkable capacity of symbiotic nitrogen fixation, these legume plants were often used to improve soil organic fertility and nitrogen economy (Somers et al., 2003; Howieson and Ballard, 2004). Alfalfa (*Medicago sativa* L.), as an important forage legume with high feed-

ing quality, is well adapted to semi-arid condition. However, owing to its cold tolerant but heat sensitive characteristics, alfalfa can only be grown in north China rather than in tropical south area. As a result, alfalfa plantation scope was heavily limited and led to the shortage of its supply as pasture, thus affecting development of animal husbandry.

Temperature as a notable factor, can largely affect plants growth, productivity and even distribution scope. As it is known, the main reason that limits alfalfa plantation in south China can be attributed to the relatively high temperature. Responses of alfalfa to temperature stress have drawn wide attention in recent years (Schubert et al., 1995; Erice et al., 2007). For example, it was reported that the optimal temperature for alfalfa growth was about 20°C (Craufurd et al., 2003). In addition, it was demonstrated that, elevating temperature by 4°C can promote photosynthetic rate and thus dry matter accumulation, but further increased temperature exceeding 20°C will do harm to alfalfa growth (Aranjuelo et al., 2006). During the past decades, there have been some

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publications on elucidating the reasons that alfalfa can not be grown under high temperature condition. For instance, it was believed that, high temperature lowered photosynthetic rate, destroyed plasma membrane structure and thus accelerated the process of alfalfa aging (Marcum, 1998; Karim et al., 1999; Aranjuelo et al., 2007). Meanwhile, it was demonstrated that, cold or chilling stress can cause several dysfunctions to some plants species, including damage to cell membranes, generation of reactive oxygen species (ROS), protein denaturation and even accumulation of toxic products (Bowers, 1994; Shinozaki and Yamaguchi-Shinozaki, 1996; Zhu, 2001; Shao et al., 2008). Unfortunately, as far as alfalfa is concerned, its metabolic response to cold or chilling stress was scarcely known (Ivashuta et al., 2002). Moreover, till date, there was no publication on exploring the reason why alfalfa belongs to cold but not heat tolerant plant at enzymatic or molecular level. As it is known, it is vital to understand alfalfa characteristics of cold tolerance and heat sensitivity for its stable production and quality improvement as well as provision of theoretical guidelines for breeding in tropical area.

The main objective of this study is to investigate and compare the effects of predicted low and high temperature stresses on alfalfa growth. In a concrete way, status of sucrose metabolism and relative water content (RWC) in alfalfa leaves under temperature induced stress were investigated. Moreover, the relationship of sucrose metabolism and RWC in leaves was fully explored, aimed at explaining the reason why alfalfa can endure cold but not heat stress, thus providing theoretical guidelines for breeding in high temperature areas of south China.

MATERIALS AND METHODS

Plant materials

Seeds of alfalfa (*M. sativa* L., LongDong MuXu) were sown in November, in a mixture of peaty soil and perlite (with a ratio of 4:1) in pots. The seedlings were cultivated in a greenhouse under natural light and at an ambient temperature. During the process of cultivation, seedlings were fertilized once a week with 1/2 strength Hoagland's nutrient solution and watered once a day. After 35 days cultivation, the seedlings were transferred into different controlled environment chambers and randomly assigned to three treatments (ten pots for each treatment). The control, high temperature (HT) and low temperature (LT) were treated at a temperature of 25, 33 and 5°C for 6 days, respectively. A cycle of 14 / 10 h light/dark was applied for these treatments. Relative humidity (RH) for three treatments was kept at 70% and light intensity was 300 mmol m^{-2} s^{-1}. Fully expanded leaves at the same developmental stage were selected for experimental analysis.

RNA isolation and real time polymerase chain reaction (RT-PCR) analysis of sucrose phosphate synthase (SPS) and sucrose synthase (SS) gene expression

Total RNA isolation from alfalfa leaves was based on the method described by Chomczynski and Sacchi (1987). RNA pellet was suspended in 1% diethylpyrocarbonate and its concentration was estimated spectrophotometrically at the wavelength of 260 nm. A two step semi-quantitative real time polymerase chain reaction (RT-PCR) method was used for assaying genes expression of sucrose phosphate synthase (SPS) and sucrose synthase (SS). The first cDNA strand was synthesized using 2.5 μg of total RNA as template in 25 μl reaction mixture which consists of 0.5 μg of oligo-(dT)18 n primer and 200 units of Moloney murine leukemia virus RT (Promega). And then 3 μl samples from first strand cDNA were used to amplify fragments specific for SPS or SS. The primers (0.5 μM each) were designed according to AF049487, AY851389 and EU664318 on the National Center for Biotechnology Information (NCBI) database, including 5'-ATAAGAATACCCTTCGGTCCACG-3' (SPS 5'), 5'-CACTGTCACCAGCATCAGCATAG-3' (SPS 3'), 5'-TTCCCTTACACCGAAAC TAGCCG-3' (SS 5'), 5'-GCATTCTTGC CGTACCACTCCAC- 3' (SS 3'), 5'-CGTGGATAC TCTTTCAC CACAAC-3' (Act 5') and 5'-ATACCAGGAGCTTCCATACCAAT-3' (Act 3'). The cDNA yield was measured according to PCR signal generated from internal standard ranging from 24 to 30 cycles and starting with 2 μl cDNA solution. Volume of each cDNA pool was adjusted to give same exponential phase polymerase chain reaction (PCR) signal for SPS and SS after 24 cycles. An aliquot (10 μl) of each PCR reaction mixture was used for electrophoresis in agarose gel and ethidium bromide stained band was quantified using a Molecular Imager system (Bio-Rad Laboratories).

Extraction and assay of SPS and SS

Leaf tissue (0.5 g) was ground with a pestle in a chilled mortar and enzymes were extracted with 5 ml of 100 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH 8.2). The buffer contained ethylenediaminetetraacetic acid (EDTA) (10 mM), KCl (15 mM), $MgCl_2$ (5 mM), sodium diethyl dithiocarbamate (2 mM), ß-mercaptoethanol (5 mM) and 1% polyvinyl polypyrrolidone (Kerr et al., 1987). After centrifugation at 10 000 g (4°C) for 15 min, the supernatants were eluted through a Sephadex G-25 column using 10 mM HEPES buffer (pH 7.0, containing 5 mM $MgCl_2$) to remove low molecular weight components (Sharma et al., 1998).

The solution for SS assay consisted of 100 μmoles HEPES buffer (pH 6.5) containing 2 μ moles of UDP and 50 μ moles of sucrose in a total volume of 0.4 ml. Reaction was initiated by adding 0.1 ml of enzyme. In the control assay, UDP was absent. Assay mixture was incubated for 15 min at 37°C and fructose release was estimated (Sharma et al., 1998).

The solution for SPS assay consisted of 2.2 μ moles UDP-glucose, 4.4 μ moles fructose-6-phosphate, 20 μ moles HEPES buffer (pH 8.2) containing 1 μ mole $MgCl_2$ and 2 μ moles NaF in a total volume of 40 μl. To initiate the reaction, 100 μl of enzyme preparation was added and reaction was run at 37°C for 15 min. After stopping reaction by adding 20 μl of 30% NaOH and boiling for 10 min, sucrose formed was determined by anthrone reagent (Van Handel, 1968). The rate of product formation in SS was linear for 40 min and that of SPS was for 25 min (Sharma et al., 1998).

Extraction and assay of soluble reducing sugars and sucrose content in leaves

Extraction and assay of soluble reducing sugar and sucrose were based on the methods (Sharma et al., 1998) with minor modification. Fresh leaves was weighed and dipped in 80% ethanol solution to stop all enzymatic activities and then ground in mortar. The soluble sugars were extracted twice with 20 ml of 80% ethanol and then twice with 70% ethanol. After centrifugation at 5000 g for 10 min, the liquid was decolorized with activated carbon and filtered, and then the supernatants were pooled and concentrated by evaporating ethanol at 50°C under vacuum. The soluble reducing sugars were determined according to the method (Nelson,

1944). Sucrose content was determined by the method (McRary and Slattery, 1945).

Extraction and assay of starch content in leaves

Starch was extracted and assayed according to the method of Djilianov et al. (2003). Insoluble pellets remained from carbohydrate extraction were resuspended in 0.2 ml NaOH (0.5 M) and incubated at 60°C for 1 h. Subsequently, 0.2 ml HCl (0.5 M) was added. After cooling down to room temperature, 0.6 ml sodium acetate buffer (pH 4.5, 0.2 M) containing 1 U amyloglucosidase (special quality for starch determination, Roche Diagnostics, Basel, Switzerland) was added and samples were incubated overnight at 37°C. Reaction was stopped with 2 min boiling. Samples were centrifuged at 10000 g for 10 min and the supernatants were analyzed for glucose formation using a commercial assay (Roche Diagnostics).

Assay of relative water content (RWC) in leaves

Relative water content (RWC) was calculated as follows:

$$RWC = \frac{Wi - Wd}{Wf - Wd} \times 100$$

Where, Wi, was fresh weight of harvested leaves; Wf, weight of leaf segments soaked in water at 25°C in darkness for 24 h; Wd, dry weight of the segments dried at 80°C for 24 h.

Assay of cells lipid peroxidation in leaves

A modified thiobarbituric acid reactive substance assay was used to assess degree of lipid oxidation based on the method of Hodges et al. (1999). 1.0 g fresh leaves tissue was ground in 80:20 ethanol and methanol (v/v) containing 0.01% (w/v) butylated hydroxytoluene (BHT). After centrifugation at 13 000 g for 15 min at 4°C, the supernatants were recovered (200 ml) and added to 1 ml of 20% (w/v) trichloroacetic acid (TCA) and 0.01% (w/v) BHT containing 0.65% (w/v) thiobarbituric acid for 25 min at 95°C. After centrifugation, sample absorbance was measured at 532 nm. Blank measurements were performed using reagent solution without thiobarbituric acid. Nonspecific turbidity was subtracted from 532 nm signals by using the measurements at 600 and 440 nm. The results were expressed as malondialdehyde (MDA) equivalent.

Assay of electrolyte leakage in leaves

Electrolyte leakage was determined according to the method of Lu et al. (2003) with minor modification. 0.3 g fresh leaves tissue were rinsed with distilled water and immersed in 20 ml distilled water overnight. Conductivity of solution (R1) was measured using a conductivity meter. Samples were then heated in boiling water for 15 min and cooled to room temperature. Conductivity of killed tissues (R2) was measured again. Electrolyte leakage was calculated as the ratio of R1 to R2.

Statistical analysis

Data from three replications of all treatments were subjected to analysis of variance using the Statistical Package for the Social Sciences (SPSS) 13.0 Windows for all statistical analysis. Differences between means at 5% ($P < 0.05$) level were considered as significant.

RESULTS

Effects of LT and HT stresses on genes expression of SPS and SS and their enzymes activities in leaves

The effects of LT and HT stresses on genes expression of SPS and SS and their corresponding enzymes activities in alfalfa leaves were investigated and main results were indicated in Figure 1. Evidently, although both stresses led to an increase in SS expressions, they had different effects (Figure 1a). For example, LT stress induced SS expression increasing through the whole period of treatment and exhibiting a peak on the fourth day (climacteric peak). In contrast, SS expression also began to increase after HT treatment and reached a maximum on the second day and then decreased markedly afterwards. In addition, as shown in Figure 1b, a similar phenomenon was also observed in SPS gene expression in contrast to SS. Although both stresses induced an increase in genes expression, LT showed a more significant effect on their expression degree and duration. Meanwhile, corresponding enzyme activities of SS and SPS shown in Figures 1c and d, further documented the statuses of above genes expressions. For instance, SPS activity reached a maximum on the 4th day after LT treatment, increasing by 58.1% when compared with the control. In comparison, SPS activity reached the highest value on the 2nd day of HT treatment, which was 15.4% higher than the control. In all, on the 4th day, SPS and SS activities under LT treatment were 42.1 and 35.4% higher than those of HT treatments, which indicate that, SPS and SS activities in alfalfa leaves increased accordingly in response to both stresses, but LT showed a longer duration and higher degree.

Effects of LT and HT stresses on contents of sucrose, starch, fructose and soluble reducing sugar in leaves

Parameters of sucrose, starch, fructose and soluble reducing sugar in alfalfa leaves under LT and HT stresses were determined and shown in Figure 2. It was evident that, sucrose content in leaves increased under both LT and HT treatments, but LT stress showed a more remarkable effect. For instance, when compared with the control, sucrose content on the 4th day of LT treatment increased by 167.1%, while corresponding value was 72.5% by HT stress (Figure 2a). Also, as indicated in Figure 2 b both LT and HT treatments decreased starch content in alfalfa leaves. For example, in comparison to the control, starch content on the 2nd, 4th and 6th day of LT treatment were decreased by 39.0, 65.8 and 75.5%; and corresponding values were 23.0, 33.9 and 38.8% for HT treatment. In addition, compared to the control, relatively higher and lower fructose contents were obtained by LT and HT treatments, respectively (Figure 2c). Moreover, an increase in soluble reducing sugar was observed
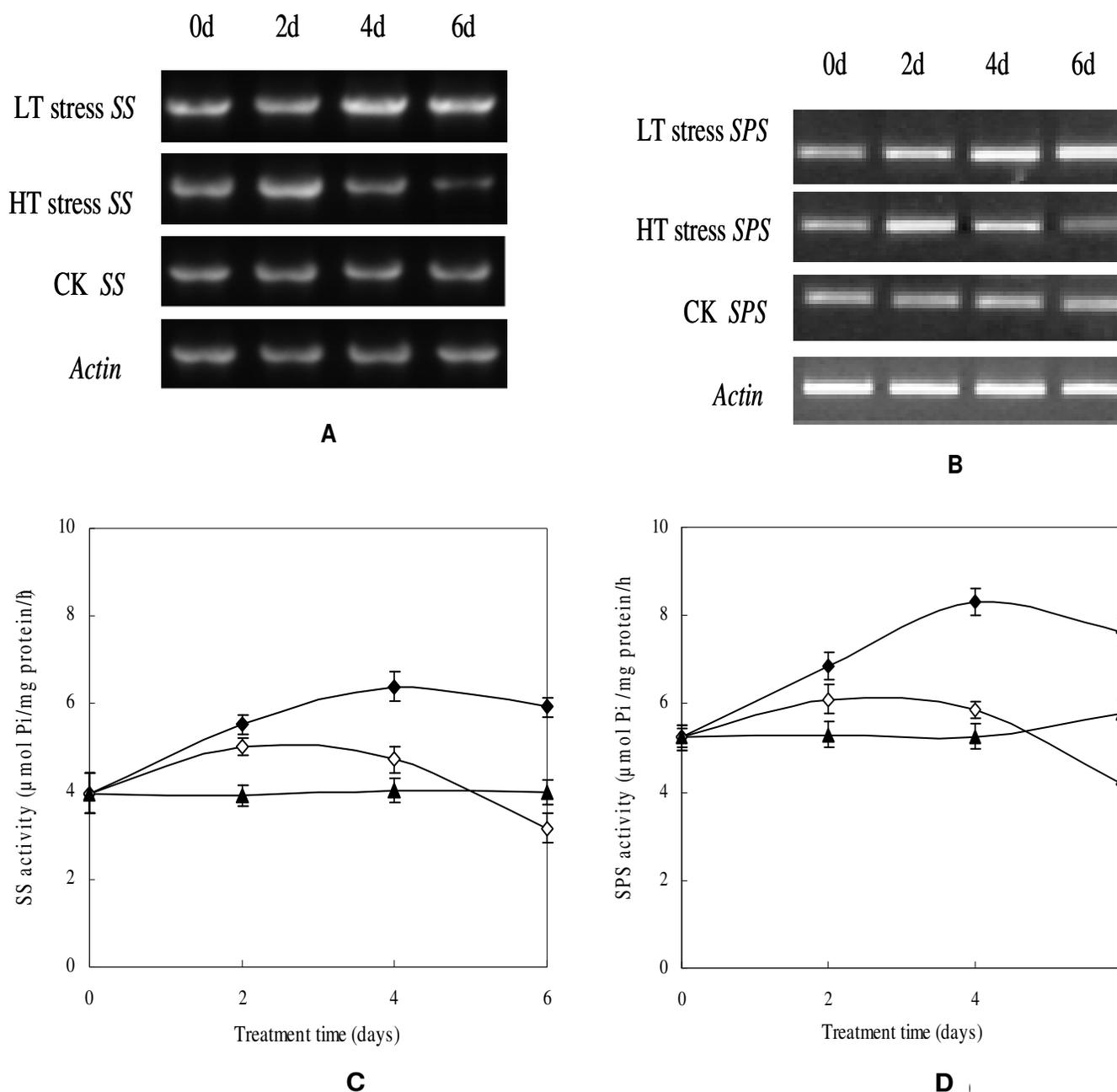
Figure 1. Effects of LT and HT stresses on gene expressions of SPS and SS and their enzymes activities in leaves. ▲, Control; □, LT stress; ◇, HT stress.

under both stresses, but increment degree under LT was higher than HT (Figure 2d). For instance, the content of soluble reducing sugar on the 2nd, 4th and 6th day of LT treatment was 55.8, 75.7 and 109.5% higher than the control. In contrast, the corresponding value was increased by 27.6, 34.4 and 39.3% for HT. In short, it can be concluded that, both stresses resulted to a decrease in starch content and an increase in the contents of sucrose and soluble reducing sugar in alfalfa leaves, but LT had a more significant impact.

Effects of LT and HT stresses on MDA content and electrolyte leakage in leaves

To investigate injuries degree of LT and HT stresses to alfalfa leaves, MDA content and electrolyte leakage were determined. As shown in Figure 3a when compared with the control, MDA content increased continuously in treated leaves during whole treatment period. However, HT had a more significant effect than LT. For example in comparison to the control, on the 2nd, 4th and 6th days
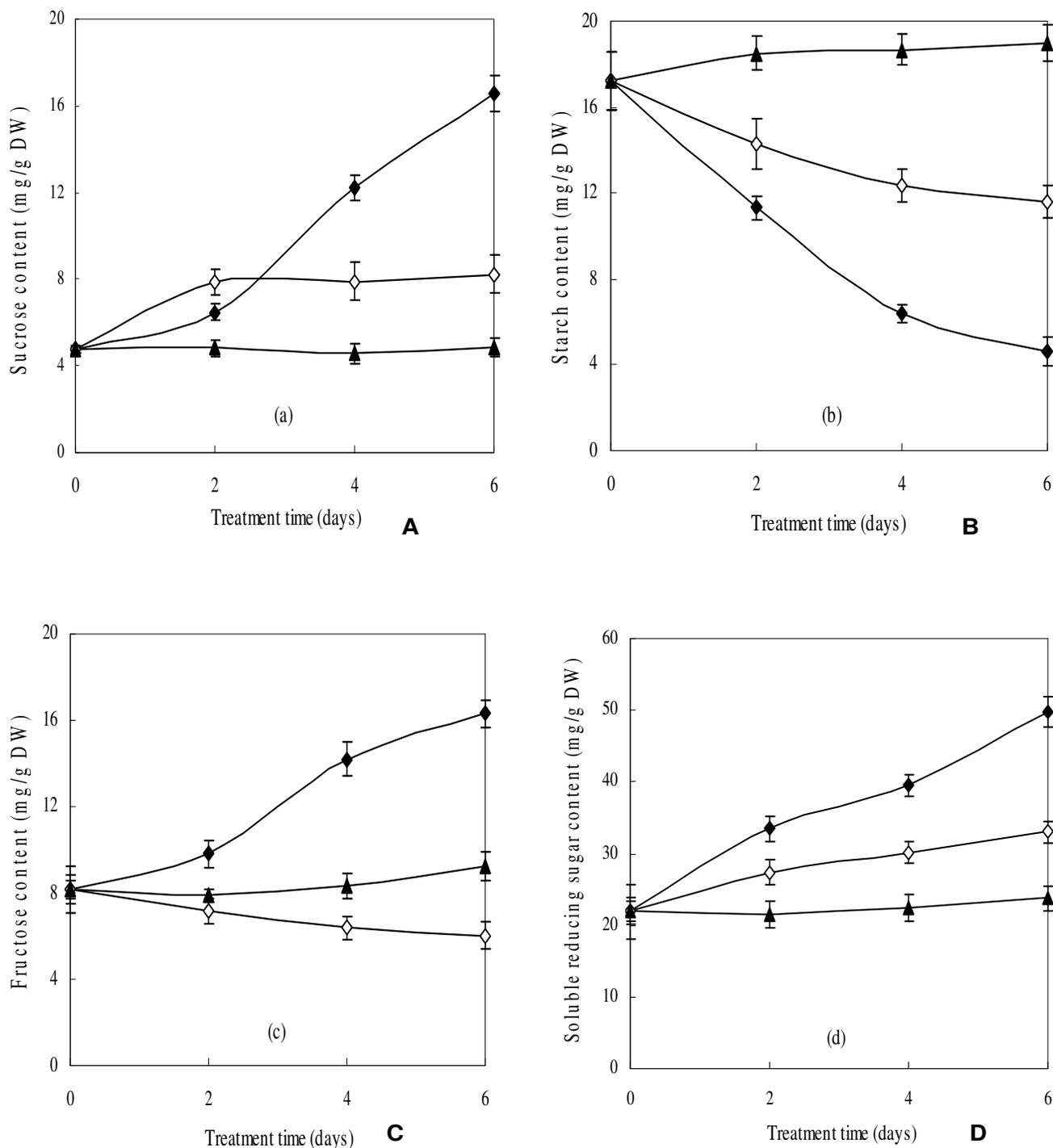
Figure 2. Effects of LT and HT stresses on contents of sucrose, starch, fructose and soluble reducing sugar in leaves. ▲, Control; ◇, LT stress; ◆, HT stress.

after HT treatment, MDA contents were increased by 94.9, 143.8 and 177.9%, respectively. In contrast, corresponding values were 16.3, 45.8 and 61.2% for LT treatment. In addition, it was observed that, HT and LT induced an increase in electrolyte leakage, but HT had a more remarkable impact (Figure 3b). For instance, when compared with the control, electrolyte leakage in leaves on the sixth day of LT treatment was increased by 44.2%. In contrast, it was increased by 91.0, 120.9 and 143.3% on the 2nd, 4th and 6th day of HT treatment. As it is known,
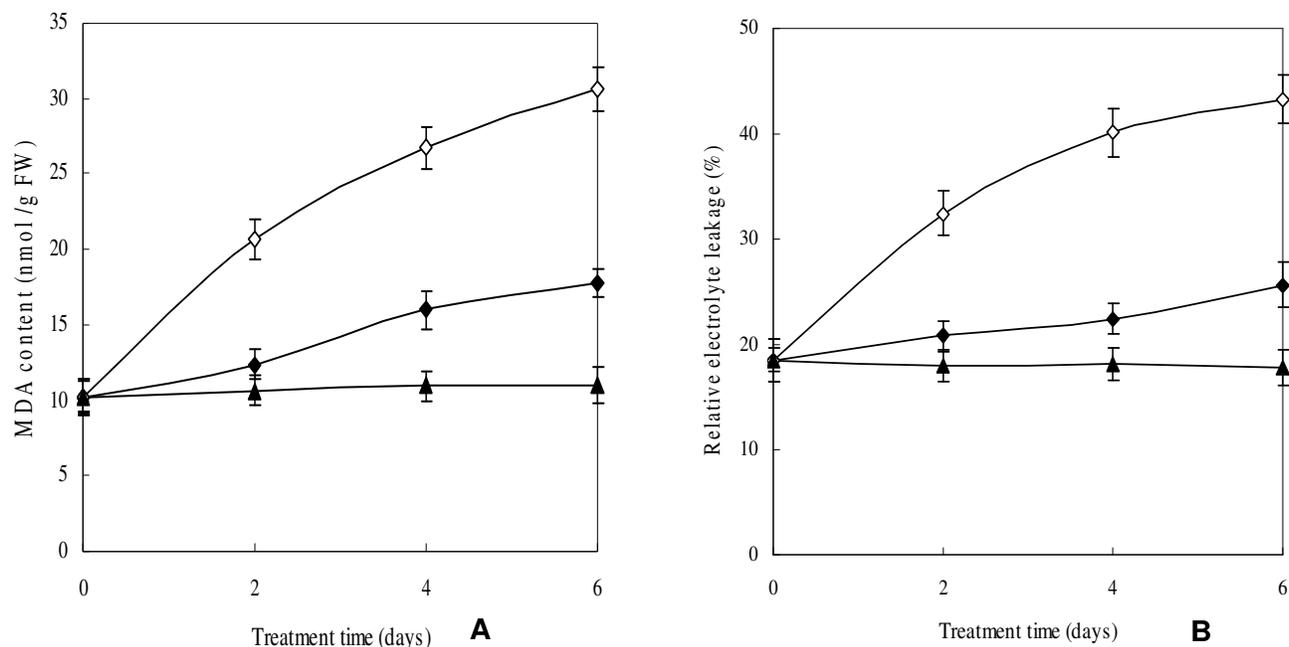
Figure 3. Effects of LT and HT stresses on MDA content and electrolyte leakage in leaves. ▲, Control; □, LT stress; ◇, HT stress.

MDA and electrolyte leakage can indirectly reflect the degree of cell membrane integrity in leaves. Our results indicated that, relatively lower MDA and electrolyte leakage were observed in LT treated leaves, suggesting higher degree of membrane integrity still maintained when compared with HT.

Effects of LT and HT stresses on relative water content (RWC) in leaves

As it is known that under temperature induced stress, RWC will be inevitably lowered in plant leaves, thus leading to injuries in leaves and even stems or roots system. As shown in Figure 4 in the control leaves, RWC was almost kept at a constant value during whole treatment period. In comparison, in the treated leaves\ RWC was decreased continually with extension of treatment times, but HT had a more remarkable influence. For example, on the 2nd, 4th and 6th days after HT treatments, RWC was decreased by 11.7, 17.4 and 18.9%, respectively and corresponding values were 1.3, 3.8 and 4.2% for LT treatment when compared with the control.

DISCUSSION

It is well known that, drought stress that originated from high temperature or water deficit can heavily restrict plants growth and thus limit their plantation scope (Flexas and Medrano, 2002; Lawlor, 2002; Lawlor and Cornic,

2002). Alfalfa, as an important legume is only grown in north China because of its cold tolerant but heat sensitive characteristics. Although the impact of high temperature stress on alfalfa growth was investigated, the reasons leading to its limited plantation area resulted from heat sensitivity which was still little known. It has been documented that, sucrose plays a positive role in protecting cells from injuries under cold or high temperature in some plants (Xu and Zhou, 2006; Firon et al., 2006; Junker et al., 2006). Unfortunately, till date there is no publication on response of sucrose to cold or heat stresses in alfalfa leaves.

In accordance with this study, effects of LT and HT treatments on sucrose metabolism and status of RWC in alfalfa leaves were fully explored. Our resulted indicated that, both LT and HT stresses induced an increase in the degree and duration of genes expression and thus, corresponding enzymes activities of SPS and SS, which were consisted with the previous reports in other plants (Calderón and Pontis, 1985; Sasaki et al., 2001; Shin et al., 2002). As activities of SPS and SS in treated alfalfa leaves were improved, higher sucrose content was observed correspondingly. Moreover, it was fascinating to observe that LT had a more significant effect in comparison to HT, which was in agreement with the previous studies in spinach, chickpea and raspberry (Guy et al., 1992; Palonen et al., 2000; Nayyar et al., 2005). In addition, our results documented that, LT and HT lowered starch content and enhanced soluble reducing sugar in alfalfa leaves and LT showed a remarkable impact. It was believed that, LT and HT can increase β-amylase activity,
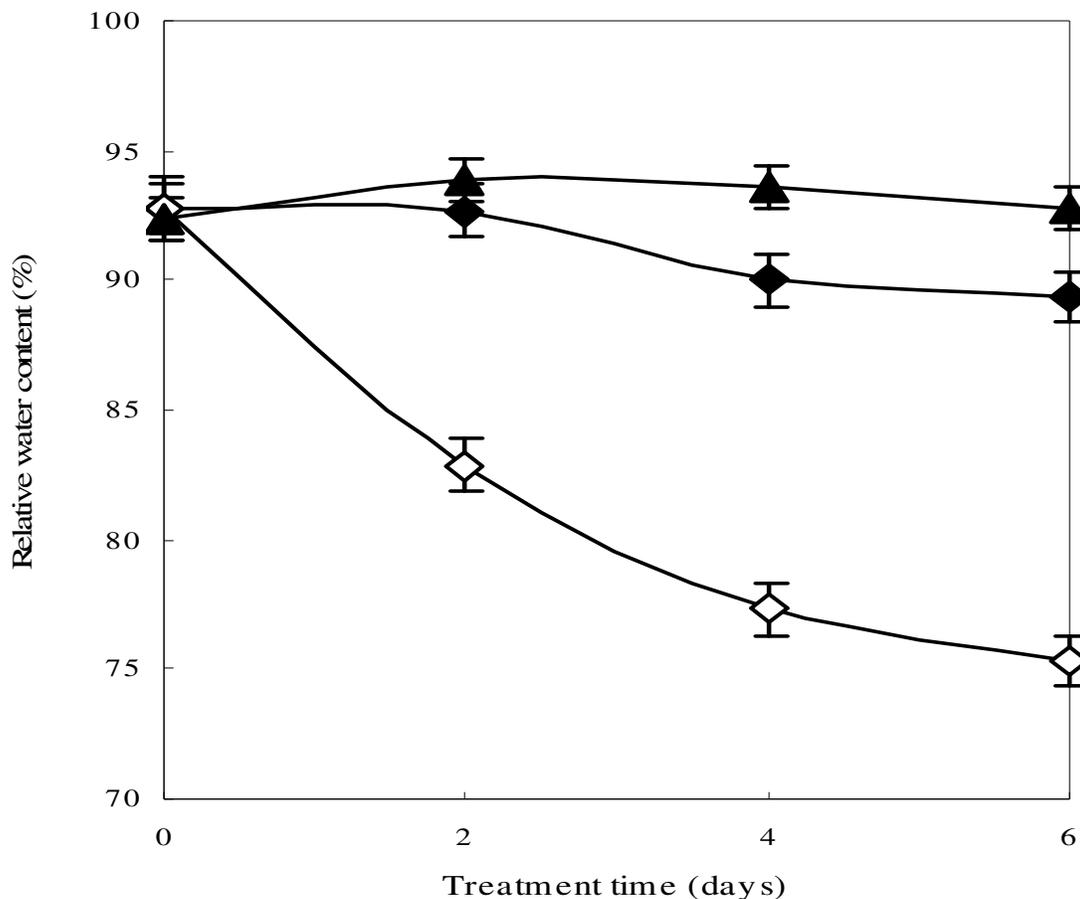
Figure 4. Effects of LT and HT stresses on relative water content in leaves. ▲, Control; □, LT stress; ◇, HT stress.

thus leading to an increase in soluble reducing sugar level in *Arabidopsis thaliana* (Kaplan and Guy, 2004). As it is known, starch degradation will inevitably lead to increased glucose level in alfalfa leaves, which is then used as the precursor for sucrose and other soluble sugars synthesis. In short, increased soluble reducing sugars and sucrose in treated leaves can be attributed to lowered starch content and improved SPS and SS activities.

Previous publications demonstrated that, when compared with other soluble sugars, sucrose had a more significant effect on increasing osmotic potential, thus causing more water being transported from other parts, such as stems or roots system to the leaves, which result in a relatively higher water potential (Wolkers et al, 1998; Norwood et al., 2000; Bray, 2002; Desplats et al., 2005). As mentioned above, LT induced an increase in sucrose level in alfalfa leaves, indicating a lowered water potential being achieved. Therefore, water especially from alfalfa roots will be continually transported to the leaves. As a result, a higher water potential was achieved at the end. Higher water potential in the leaves will inevitably benefit integrity of cell membrane. Although an increase in

sucrose content was also observed by HT stress, much water will be inevitably lost because of relatively higher transpiration rate. Our results indicated that, when compared with HT treatment, a higher RWC maintained in LH treated leaves perhaps resulted from higher sucrose level and lower transpiration rate, which can explain why alfalfa is cold tolerant but sensitive to heat stress.

In all, compared to HT stress, LT was more favorable for conversion of starch into soluble sugars. Moreover, higher activities of SPS and SS further contributed to too much sucrose being accumulated in the alfalfa leaves. As a result, higher sucrose content achieved relatively lower water potential in the leaves, resulting to much water transportation to the leaves. Meanwhile, decreased transpiration rate under LT save much water being lost than HT, thus preventing cell from injuries under LT stress. This might be one of the reasons why alfalfa can endure LT but not HT stress. Due to this, it was suggested that alfalfa can be extended to grow in the tropical area of south China, provided that a gene engineered alfalfa with over- expression of SPS and SS is being constructed.

ACKNOWLEDGEMENTS

REFERENCES

Aranjuelo I, Irigoyen JJ, Perez P, Martinez-Carrasco R, Sanchez-Diaz M (2006). Response of nodulated alfalfa to water supply, temperature and elevated CO2: productivity and water relations. Environ. Exp. Bot. 55: 130-141.

Aranjuelo I, Irigoyen JJ, Sanchez-Diaz M (2007). Effect of elevated temperature and water availability on CO$_2$ exchange and nitrogen fixation of nodulated alfalfa plants. Environ. Exp. Bot. 59: 99-108.

Bowers MC (1994). Environmental effects of cold on plants. In:Wilkinson, R.E. (Ed.), Plant-Environment Interactions. Marcel Dekker, New York, pp. 391-411.

Bray EA (2002). Classification of genes differentially expressed during water-deficit stress in *Arabidopsis thaliana*: an analysis using microarray and differential expression data. Ann. Bot. 89: 803-811.

Calderón P, Pontis HG (1985). Increase of sucrose synthase activity in wheat plants after a chilling shock. Plant Sci. 42: 173-176.

Chomczynski P, Sacchi N (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162: 156-159.

Craufurd PQ, Prasad PVV, Kakani VG, Wheeler TR, Nigam SN (2003). Heat tolerance in ground nut. Field Crop Res. 1: 63-771.

Desplats P, Folco E, Salerno GL (2005). Sucrose may play an additional role to that of an osmolyte in Synechocystis sp. PCC 6803 salt-shocked cells. Plant Physiol. Biochem. 43: 133-138.

Dita MA, Rispail N, Prats E, Rubiales D, Singh KB (2006). Biotechnology approaches to overcome biotic and abiotic stress constrains in legumes.Euphytica. 147:1-24.

Djilianov D, Prinsen E, Oden S, Onckelen H, Müller J (2003). Nodulation under salt stress of alfalfa lines obtained after in vitro selection for osmotic tolerance. Plant Sci. 165: 887-894.

Erice G, Irigoyen JJ, Sánchez-Díaz M, Avice JC, Ourry A (2007). Effect of drought, elevated CO2 and temperature on accumulation of N and vegetative storage proteins (VSP) in taproot of nodulated alfalfa before and after cutting. Plant Sci. 172: 903-912.

Firon N, Shaked R, Peet MM, Pharr DM, Zamski E, Rosenfeld K, Althan L, Pressman E(2006). Pollen grains of heat tolerant tomato cultivars retain higher carbohydrate concentration under heat stress conditions. Scientia Horticulturae, 109: 212-217.

Flexas J, Medrano H (2002). Drought-inhibition of photosynthesis in C3 plants: stomatal and non-stomatal limitations revisited. Ann. Bot. 89: 183-189.

Graham PH, Vance CP (2003). Legumes: importance and constraints to greater use. Plant Physiol. 131: 872-877.

Hodges DM, DeLong JM, Forney CF, Prange RK (1999). Improving the thiobarbituric acid-reactive-substances assay for estimating lipid peroxidation in plant tissues containing anthocyanin and other interfering compounds. Planta, 207: 604-611.

Howieson J, Ballard R, (2004). Optimizing the legume symbiosis in stressful and competitive environments within southern Australia: some contemporary thoughts. Soil Biol. Biochem. 36: 1261-1273.

Ivashuta S, Imai R, Uchiyama K, Gau M, Shimamoto Y (2002). Changes in chloroplast FtsH-like gene during cold acclimation in alfalfa (*Medicago sativa*). J. Plant Physiol. 159: 85-90.

Junker BH , Wuttke R, Nesi AN, Steinhauser D, Schauer N, Büssis D, Willmitzer L, Fernie AR (2006). Enhancing Vacuolar Sucrose Cleavage Within the Developing Potato Tuber has only Minor Effects on Metabolism. Plant Cell Physiol. 47: 277-289.

Kaplan F, Guy CL (2004). β-Amylase Induction and the Protective Role of Maltose during Temperature Shock. Plant Physiol. 135: 1674-1684.

Karim MA, Fracheboud Y, Stamp P (1999). Photosynthetic activity of developing leaves of Zea mays is less affected by heat stress than that of developed leaves. Physiol. Plant, 105: 685-693.

Kerr PS, Torres WK, Huber SC (1987). Resolution of two molecular forms of sucrose phosphate synthase from maize soybean and spinach leaves. Planta, 170: 515-519.

Lawlor DW (2002). Limitation to photosynthesis in water-stressed leaves: stomata vs. metabolism and the role of ATP. Ann. Bot. 89: 871-885.

Lawlor DW, Cornic G (2002). Photosynthetic carbon assimilation and associated metabolism in relation to water deficits in higher plants. Plant Cell Environ. 25: 275-294.

Lu S, Guo Z, Peng X (2003). Effects of ABA and S-3307 on drought resistance and antioxidative enzyme activity of turfgrass. J. Hortic. Sci. Biotechnol. 78: 663- 666.

Marcum KB (1998). Cell membrane thermostability and whole-plant heat tolerance of Kentucky bluegrass. Crop Sci. 38: 1214-1218.

McRary WL, Slattery M (1945). The colorimetric determination of fructosan in plant material. J. Biol. Chem. 157: 161-167.

Nayyar H, Bains TS, Kumar S (2005). Chilling stressed chickpea seedlings: effect of cold acclimation, calcium and abscisic acid on cryoprotective solutes and oxidative damage. Environ. Exp. Bot. 54: 275-285.

Nelson N (1944). A photometric adaptation of Somogyi method for the determination of glucose. J. Biol. Chem. 153: 375-380.

Norwood M, Truesdale MR, Richter A, Scott P (2000). Photosynthetic carbohydrate metabolism in the resurrection plant *Craterostigma platagenium*. J. Exp. Bot. 51: 159-165.

Palonen P, Buszard D, Donnelly D (2000). Changes in carbohydrates and freezing tolerance during cold acclimation of raspberry cultivars grown *in vitro* and *in vivo*. Physiol Plant. 110: 393-401.

Sasaki H, Ichimura K, Imada S, Yamki S (2001). Sucrose synthase and sucrose phosphate synthase, but not acid invertase, are regulated by cold acclimation and deacclimation in cabbage seedlings. J. Plant Physiol. 158: 847-852.

Schubert S, Serraj R, Plies-Balzer E, Mengel K(1995). Effect of drought stress on growth, sugar concentrations and amino acid accumulation in N2-fixing alfalfa. Plant Physiol. 146: 541-546.

Shao HB, Chu LY, Shao MA (2008). Calcium as a versatile Plant Signal Transducer under Soil Water Stress. BioEssays, 30: 634-641.

Sharma N, Kaur N, Gupta AK (1998). Effect of chlorocholine chloride sprays on the carbohydrate composition and activities of sucrose metabolizing enzymes in potato (*Solanum tuberosum* L.). Plant Growth Regul. 26: 97-103.

Shin KS, Chakrabarty D, Paek KY (2002). Sprouting rate, change of carbohydrate contents andrelated enzymes during cold treatment of lilybulblets regenerated *in vitro*. Sci. Hortic. 96: 195-204.

Shinozaki K, Yamaguchi-Shinozaki K (1996). Molecular responses to drought and cold stress. Current opinion in biotechnol. 7: 161-167.

Somers DA, Samac DA, Olhoft PM (2003). Recent advances in legume transformation. Plant Physiol. 131: 892-899.

Van Handel E (1968). Direct microdetermination of sucrose. Anal. Biochem. 22: 280-283.

Wolkers WF, Oldenhof H, Alberda M, Hoekstra FA (1998). A Fourier transform infrared study of sugar glasses: application to anhydrobiotic higher plant cells. Biochim Biophys Acta. 1379: 83-96.

Xu ZZ, Zhou GS (2006). Combined effects of water stress and high temperature on photosynthesis, nitrogen metabolism and lipid peroxidation of a perennial grass Leymus chinensis. Planta, 24: 1080-1090.

Zhu JK (2001).Cell signaling under salt, water and cold stresses. Curr. Opin. Plant Biol. 4: 401-406.