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Osmoregulation as a key factor in drought hardening-induced drought tolerance in *Jatropha curcas*

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Abstract

Jatropha curcas L. is a plant with various commercial uses, and drought is an important limiting factor for its distribution and production. In this study, we investigated the role of drought hardening in an increased drought tolerance in *J. curcas*, and the involvement of osmoregulation and biochemical pathways in this enhanced tolerance. Results show that a drought hardening treatment with 10 % (m/v) polyethylene glycol 6000 for two days significantly increased a survival rate, decreased the content of malondialdehyde, and alleviated electrolyte leakage in the *J. curcas* seedlings under the drought stress. Measurements of leaf water potential, osmotic potential, and pressure potential show that this drought hardening treatment can improve the water status of *J. curcas* seedlings during the early phase of drought stress. In addition, the drought hardening treatment gradually increased the concentrations of compatible solutes proline, glycinebetaine, and soluble sugars during drought hardening and subsequent drought stress. It also clearly raised the activity of betaine aldehyde dehydrogenase, a key enzyme for the glycinebataine biosynhthesis as well as the activity of enzymes Δ^1-pyrroline-5-carboxylate synthetase (P5CS), glutamate dehydrogenase, arginase, and ornithine aminotransferase, all key enzymes in the proline biosynthesis. The expression of *P5CS* gene in the *J. curcas* seedlings also increased during drought hardening and subsequent drought stress, but the activity of proline dehydrogenase decreased. These results show that the drought hardening treatment can enhance drought tolerance in *J. curcas*, and osmoregulation is a key factor in this increased drought tolerance.

Additional key words: compatible solutes, proline, glycinebetaine, Δ^1-pyrroline-5-carboxylate synthetase, betaine aldehyde dehydrogenase.

Introduction

Drought is an important abiotic stress factor for both natural plant populations and agricultural crops. Plants cope with drought stress by adapting key physiological processes like photosynthesis, respiration, osmoregulation, and antioxidant and hormonal metabolism (Hessine *et al*. 2009, Thapa *et al*. 2011, Bhargava and Sawant 2013, Hameed *et al*. 2013). An osmotic adjustment, the active accumulation of compatible osmolytes in response to drought has been reported to be an important drought adaptation mechanism in several plants (Babita *et al*. 2010, Silva *et al*. 2010). A variety of compatible osmolytes can contribute to the osmotic adjustment, but soluble sugars (*e.g*., glucose and sucrose), proline, and glycinebetaine predominate in most plant species (Villar-Salvador *et al*. 2004, Silva *et al*. 2010).

Proline is a structural component of proteins and may

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Abbreviations: BADH - betaine aldehyde dehydrogenase; GDH - glutamate dehydrogenase; MDA - malondialdehyde; OAT - ornithine aminotransferase; P5CS - Δ^1-pyrroline-5-carboxylate synthetase; PEG 6000 - polyethylene glycol 6000; ProDH - proline dehydrogenase.
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function as osmoticum, sink of energy and reducing power, nitrogen-storage compound, hydroxyl-radical scavenger, and compatible solute that protects enzymes (Trovato *et al*. 2008, Szabados and Savoure 2010). In higher plants, proline can be synthesized from either glutamate or ornithine. Key enzymes involved in the proline biosynthesis are glutamate dehydrogenase (GDH) and Δ^1-pyrroline-5-carboxylate synthetase (P5CS) in the glutamate pathway, and arginase and ornithine aminotransferase (OAT) in the ornithine pathway (Stines *et al*. 1999, Zhao *et al*. 2001, Yang *et al*. 2009). The onset of stress-induced proline accumulation is correlated with a transcriptional activation of *P5CS* gene (Yang *et al*. 2009, Szabados and Savoure 2010). Metabolism and accumulation of proline also depends on its degradation which is catalyzed by a mitochondrial enzyme proline dehydrogenase (ProDH) (Sánchez *et al*. 2001, Szabados and Savoure 2010).

Glycinebetaine, a quaternary ammonium compound, is regarded as one of the most effective osmoprotectants. Some plants therefore can protect themselves against drought stress by an enhanced synthesis and accumulation of glycinebetaine (Ashraf and Foolad 2007). In plants, glycinebetaine is synthesized by a two-step oxidation of choline. Betaine aldehyde dehydrogenase (BADH) is a key enzyme in the glycinebataine biosynthesis (Moghaieb *et al*. 2006).

Jatropha curcas L. belonging to the family *Euphorbiaceae*, is native to South America and is widely distributed in South and Central America, Africa, and Asia (Divakara *et al*. 2010, Silva *et al*. 2012). *J. curcas* has multiple commercial values: oil from its seeds is an ideal raw material for biodiesel (Makkar and Becker 2009, Koh *et al*. 2011) and its metabolites show anticancer properties along with antibacterial and insecticidal features (Debnath *et al*. 2008, Silva *et al*. 2012). In addition, these plants can also be used for the restoration of ecosystems affected by desertification processes (Makkar and Becker 2009). It is able to grow under a wide range of rainfall regimes, from 200 to over 1500 mm per annum (Basha *et al*. 2009). Previously, *Jatropha* was thought to have a high drought resistance (Debnath *et al*. 2008, Divakara *et al*. 2010); however, drought is the main factor that affects distribution and production of this plant (Maes *et al.* 2009).

Many studies have shown that drought hardening can enhance drought tolerance of plants (Banon *et al*. 2006, Coopman *et al*. 2008, Thomas 2009). Water restrictions applied during cultivation of seedlings can induce an osmotic adjustment, and increase cell membrane stability to dehydration; these factors could be essential for plant survival and growth during drought (Villar-Salvador *et al*. 2004, Silva *et al*. 2010). Our previous study showed that drought hardening with 10 % (m/v) polyethylene glycol (PEG) 6000 for two days significantly increases the drought tolerance of *J. curcas*, and that osmoregulation may play an important role in this enhanced drought tolerance (data not presented). However, information available on the mechanism of osmoregulation in drought tolerance of *J. curcas* is unconfirmed. In the present study, the objective was to investigate the effect of drought-hardening focused on osmoregulation and biochemical pathways involved in enhancing drought tolerance in *J. curcas*.

Materials and methods

Plants: Seeds of *Jatropha curcas* L. collected from Yuanmou, Yunnan Province, China, were surface sterilized in 1 % (m/v) $CuSO_4$ for 30 min and pre-soaked for imbibition in a distilled water for 24 h. The soaked seeds were sowed on six layers of filter paper wetted with a distilled water in covered trays (200 seeds per tray) and germinated in the dark at 25 °C for 7 d. A surface water layer was maintained by adding 50 cm^3 of water per day. Seedlings of a uniform size were then selected and transferred into pots (20 ×18 ×15 cm, 20 seedlings per pot) containing silica sand wetted with a Hoagland's nutrient solution. The pots were placed in a climate chamber (day/night temperatures: 25/20 °C, a relative humidity: 75 %, a photoperiod: 16 h, and a photon flux density: 250 μmol m^{-2} s^{-1}) and sequentially grown for 8 d. A Hoagland's solution (100 cm^3 per pot) was added every day. Then, the seedlings were harvested for subsequent experiments. All experiments were repeated at least three times and two replications in each time.

Drought stress and survival: Seedlings of a uniform size were transferred to trays (60 seedlings per tray) containing six layers of dry filter paper and placed in a climate chamber for 5 d. The treated seedlings were then transferred into pots containing silica sand and wetted with a Hoagland's solution for recovery in the climate chamber and their survival percentage was calculated.

Drought hardening: Seedlings of a uniform size (15 d after sowing, approximately 8.0 cm in height, 60 seedlings per tray) were gently transferred to a Hoagland's solution supplemented with 5 to 15 % PEG 6000 (m/v, an osmotic potential from -0.25 to -0.75 MPa) for 1 to 2 d, and a Hoagland's solution (150 cm^3 per tray) was added each day to maintain a suitable osmotic potential. Control seedlings (60 seedlings per tray) continued to be cultured in the Hoagland's solution. Following the treatment, all the seedlings were simultaneously subjected to a drought stress and their survival percentage was calculated.

Based on the results of the above preliminary drought hardening experiments, 15-d-old seedlings (60 seedlings per tray) were subjected to drought hardening at 10 %

PEG 6000 (an osmotic potential of -0.50 MPa) for 2 d. Control seedlings (60 seedlings per tray) continued to be cultured in the Hoagland's solution. At the end of drought hardening, the hardened and non-hardened (control) seedlings were exposed to a drought stress for 3 d. The following physiological and biochemical indexes were determined daily during the process of drought hardening and the drought stress.

Measurement of electrolyte leakage and malondialdehyde content: Electrolyte leakage in leaves was measured according to the method described by Gong *et al*. (2001). Lipid peroxidation was measured as the amount of malondialdehyde (MDA) determined by the thiobarbituric acid reaction as described by Bailly *et al*. (1996).

Measurement of water and osmotic potentials and main osmolytes: To assess the effect of drought hardening and the drought stress on water, osmotic, and pressure potentials in the seedlings, leaf discs were cut using a cork borer. Measurements were then taken at 25 °C with a thermocouple psychrometer (*HR-33T Dew point Microvoltmeter*, *Wescor*, Logan, USA) as described by Ao *et al*. (2013).

Content of soluble sugars was determined colorimetrically using the phenolsulfuric acid technique (Tissue *et al*. 1995). Proline content was estimated by the acid ninhydrin method (Bates *et al*. 1973), and glycinebetaine content was assayed according to the method of Nishimura *et al*. (2001).

Enzyme assays: BADH activity was assayed according to the method described by Weretilnyk and Hanson (1989). Activities of P5CS and GDH were assayed according to methods mentioned in our previous paper (Yang *et al*. 2009). The P5CS assay was carried out in 50 mM Tris-HCl (pH 7.2), 5 mM $MgCl_2$, 50 mM glutamate, 5 mM ATP, 0.4 mM NADPH, and 10 - 50 mg of crude extract at 25 °C. The rate of consumption of NADPH was monitored as decrease in absorption at 340 nm as a function of time. The GDH was assayed by determining the rate of NADH oxidation. The reaction mixture contained 50 mM NH_4Cl, 1 mM $CaCl_2$, 0.3 mM NADH, 25 mM 2-oxoglutarate, and 50 mM Tris-HCl (pH 8.2). Arginase and OAT were assayed according to the methods of Ruter (1983). Proline dehydrogenase (ProDH) was determined according to the method described by Sánchez *et al*. (2001).

RNA isolation and quantitative real time PCR (RT-qPCR): Seedlings were subjected to drought hardening (10 % PEG 6000 for 2 d) and the drought stress treatment as described in the above-mentioned procedure. Total RNA was isolated according to the recommendation of the manufacturer for polysaccharide-rich plant tissue (*RNAiso*, *TaKaRa Biotechnology*, Dalian, China) and used to generate a cDNA pool. RT-qPCR was performed using an *ABI 7500 Fast Real-Time PCR* (*TaKaRa*) instrument. The following gene-specific primers were designed using the *Primer Premier 5.0* software and used for RT-qPCR: for *P5CS* (GenBank accession No. GU358610) 5′-GGCAGATGGACTCCT GTTAGA-3′ and 5′-TTTCATTTGACCGCTTGGC-3′ (an amplicon size: 164 bp), and for the *actin* gene used as internal control (acc. No. HM044307) 5′-GTGTTATGG TTGGGATGGGT-3′ and 5′-AAGCACTGGGTGTTC CTCTG-3′ (an amplicon size: 188 bp). RT-qPCR was performed according to the recommendation of *One Step SYBR® PrimeScript™* RT-PCR *Kit II* (*TaKaRa*). The relative expression was analyzed by the comparative CT method using *Microsoft Excel 2010* as described by Livak and Schmittgen (2001).

Statistical analysis: Data were processed statistically using the analysis of variance (*ANOVA*) and the Duncan's multiple range test to determine the significance of differences among treatments at an $\alpha = 0.05$ level. Figures were drawn by *SigmaPlot 10.0* from at least three experiments.

Results

The drought stress significantly decreased the survival percentage of *J. curcas* seedlings (Fig. 1). On the third day of the drought stress treatment, all seedlings showed mortality higher than 50 %, whereas on the fifth day, the survival percentage was only 22.1 % ($P < 0.01$).

Drought hardening significantly increased the survival percentage of seedlings under the drought stress for three days (Fig. 2). When the seedlings were drought hardened for one day with 5 to 15 % PEG 6000, their survival percentage increased gradually (Fig. 2*A*). However, when a similar treatment was given on the second subsequent day, the survival percentage of *J. curcas* seedlings under 10 % PEG 6000 treatment was observed to be the highest. It increased about 80.3 % as compared to the seedlings without drought-hardening ($P < 0.001$; Fig. *2B*). Hence, the treatment with 10 % PEG 6000 for two days was considered as suitable drought hardening for *J. curcas*. The treatment with 10 % PEG 6000 for two days increased the electrolyte leakage and the MDA content of seedlings. Interestingly, after the first day of drought stress, the electrolyte leakage and the MDA content were significantly reduced in the drought-hardened seedlings compared to the control group (Fig. 3).

On the first day of drought stress, the water and osmotic potentials in the control and drought-hardened

seedlings were reduced sharply. However, the pressure potential in leaves of the drought-hardened seedlings showed a positive value, but it was almost zero in the control group. On the second day of drought stress, the water and osmotic potentials significantly declined as compared to those of the control seedlings, but the pressure potential showed only a small change ($P > 0.05$, Fig. 4). These results demonstrate that drought hardening could maintain a higher pressure potential *via* reduction of osmotic potential in leaves of *J. curcas* seedlings.

Drought hardening gradually increased the content of soluble sugars during the drought stress. On the third day of stress, the amount of soluble sugars in the treated seedlings was about 13.9 % higher than in the controls ($P < 0.01$; Fig. 5*A*). Drought hardening for two days also enhanced the glycinebetaine and proline content under the drought stress. On the third day of stress, the content of glycinebetaine and proline increased 17.8 and 27.0 %, respectively, in plants that had undergone drought hardening compared to those that had not ($P < 0.01$; Fig. 5*B*,*C*).

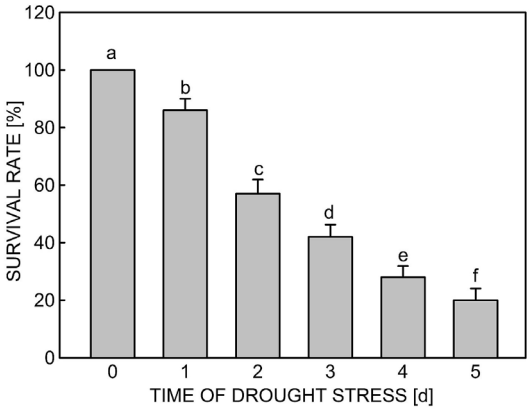

Fig. 1. Changes in the survival rate of *J. curcas* seedlings under drought stress. Means ± SE of at least six replicates of three independent experiments. Different letters indicate significant differences ($P < 0.05$) according to the Duncan's multiple test.

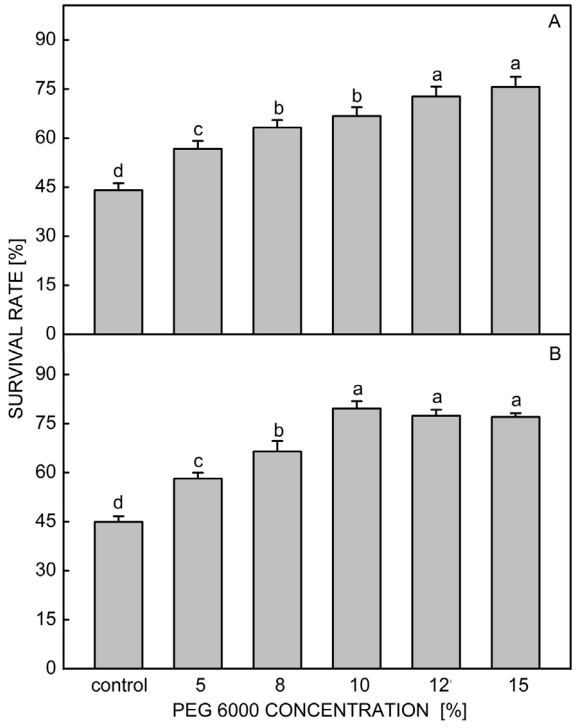

Fig. 2. Effects of drought hardening with PEG 6000 (5 - 15 %) for one day (*A*) or two days (*B*) on the survival rate of *J. curcas* seedlings under drought stress for 3 d. Means ± SE of at least six replicates of three independent experiments. Different letters indicate significant differences ($P < 0.05$) according to the Duncan's multiple test.

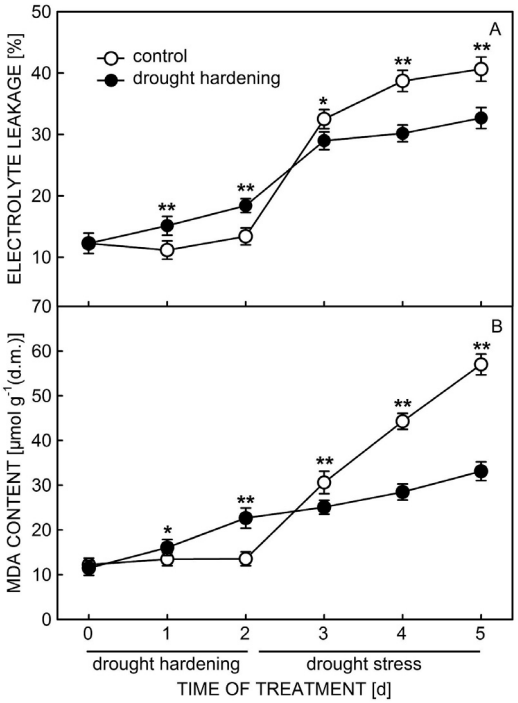

Fig. 3. Changes in electrolyte leakage (*A*) and MDA content (*B*) in leaves of *J. curcas* seedlings during drought hardening with 10 % PEG 6000 for 2 d and subsequent drought stress for 3 d. Means ± SE of at least six replicates of three independent experiments. *Asterisks* and *double asterisks* indicate significant differences ($P < 0.05$) and very significant differences ($P < 0.01$), respectively, between control and treated plants.

To further explore the effect of the drought hardening-induced accumulation of glycinebetaine and proline, the activities of enzymes BADH, P5CS, GDH, OAT, arginase, and ProDH were determined in the *J. curcas* seedlings. Results show that two days of drought hardening increased the activity of BADH which was also significantly enhanced by the drought stress (Fig. 6). Moreover, drought hardening significantly increased the activities of arginase and OAT in plants under the drought stress compared to the control (Fig. 7*A*,*B*). It also induced an almost immediate and rapid increase in the activity of GDH and P5CS (Fig. 7*C*,D), but decreased the

ProDH activity (Fig. 8).

The P5CS is a rate-limiting enzyme in the proline biosynthesis in plants (Stines *et al*. 1999, Szabados and Savoure 2010). In order to further determine the effects of drought hardening on the biosynthesis of proline under the drought stress, mRNA levels of *P5CS* gene were measured. Drought hardening induced a significant upregulation of *P5CS* gene expression and also enhanced the expression of *P5CS* gene under the drought stress (Fig. 9).

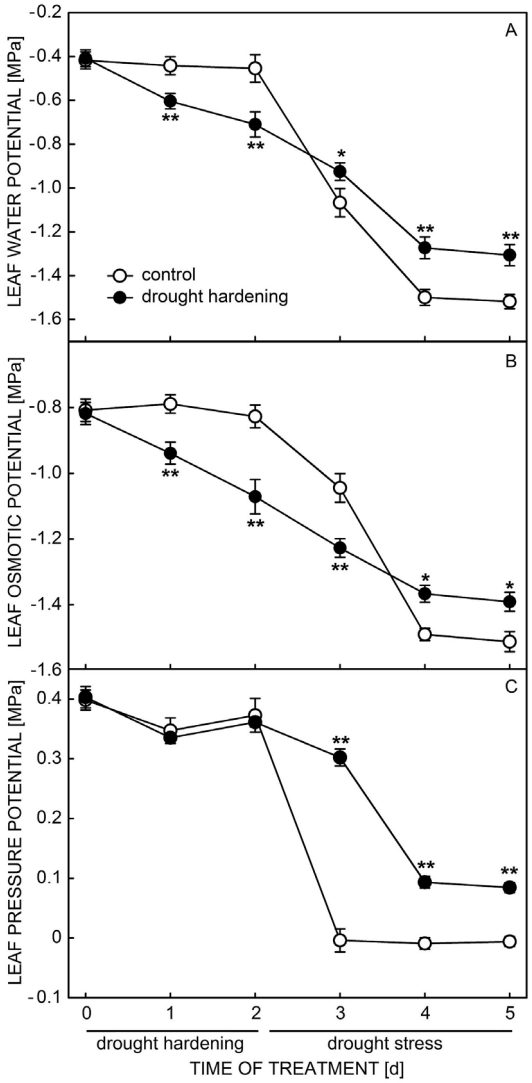

Fig. 4. Changes in leaf water potential (*A*), osmotic potential (*B*), and pressure potential (*C*) in *J. curcas* seedlings during drought hardening with 10 % PEG 6000 for 2 d and subsequent drought stress for 3 d. Means ± SE of at least six replicates of three independent experiments. *Asterisks* and *double asterisks* indicate significant differences ($P < 0.05$) and very significant differences ($P < 0.01$), respectively, between control and treated plants.

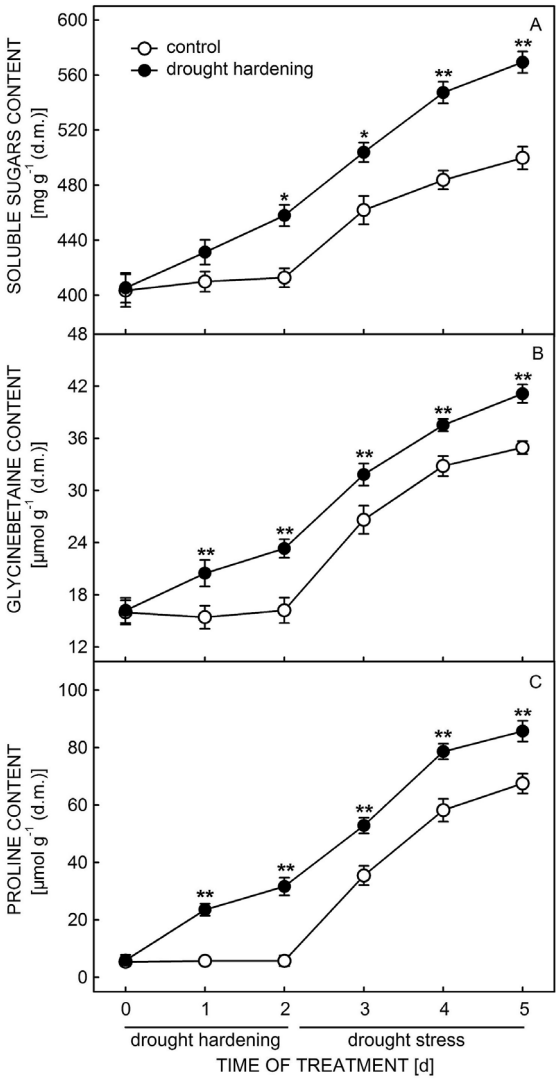

Fig. 5. Changes in content of soluble sugars (*A*), glycinebetaine (*B*), and proline (*C*) in *J. curcas* seedlings during drought hardening with 10 % PEG 6000 for 2 d and subsequent drought stress for 3 d. Means ± SE of at least six replicates of three independent experiments. *Asterisks* and *double asterisks* indicate significant differences ($P < 0.05$) and very significant differences ($P < 0.01$) between control and treated plants.

Discussion

Plants exposed to a sub-lethal water-stress exhibit less drought injury in a subsequent stress than non-stressed plants (Gong *et al*. 2001, Villar-Salvador *et al*. 2004, Silva *et al*. 2010). Drought hardening demonstrated an increase in drought tolerance and transplanting performance of *Quercus ilex* seedlings (Villar-Salvador *et al*. 2004). The survival rate of *Eucalyptus pilularis* seedlings after transplanting into controlled dry

conditions is enhanced by hardening treatments (Thomas 2009). In the present study, drought hardening with 10 % PEG 6000 for one or two days significantly increased the survival rate (Fig. 2), decreased the content of the lipid peroxidation product, MDA (Fig. 3*B*), and alleviated the electrolyte leakage (Fig. 3*A*) in the *J. curcas* seedlings under the severe drought stress indicating that drought hardening can enhance drought tolerance in *J. curcas* seedlings.

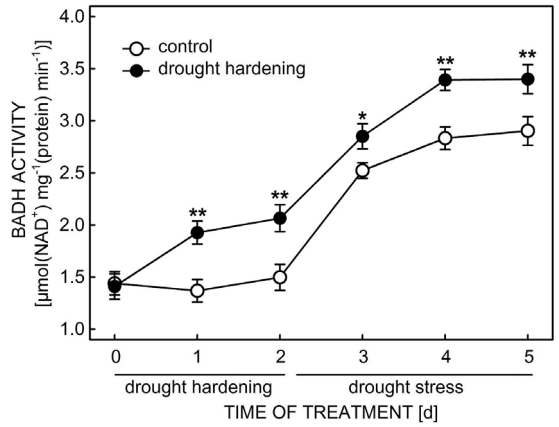

Fig. 6. Changes in BADH activity in *J. curcas* seedlings during drought hardening with 10 % PEG 6000 for 2 d and subsequent drought stress for 3 d. Means ± SE of at least six replicates of three independent experiments. *Asterisks* and *double asterisks* indicate significant differences ($P < 0.05$) and very significant differences ($P < 0.01$) between control and treated plants.

An osmotic adjustment has been considered as an important physiological adaptation to drought (Ashraf and Foolad 2007, Hessine *et al*. 2009, Yousifi *et al*. 2010). Coopman *et al*. (2008) reported that drought hardening treatments have no effect on water relation parameters in *Eucalyptus globulus*, such as the volumetric module of elasticity or the osmotic potential and the relative water content at zero pressure potential. Thomas (2009) found that drought hardened *Eucalyptus pilularis* seedlings have a lower stomatal conductance and leaf water potential when receive an insufficient irrigation in comparison with seedlings without drought hardening. In contrast, the measurements of leaf water potential, osmotic potential, and pressure potential in the present study (Fig. 4) indicate that drought-hardening (10 % PEG 6000 for two days) improved the water status of *J. curcas* seedlings in the early phase of drought stress. This suggests that the drought-hardened seedlings were more able to retain water than the non-hardened seedlings.

An osmotic adjustment involves the accumulation of compatible solutes, such as soluble sugars, proline, and

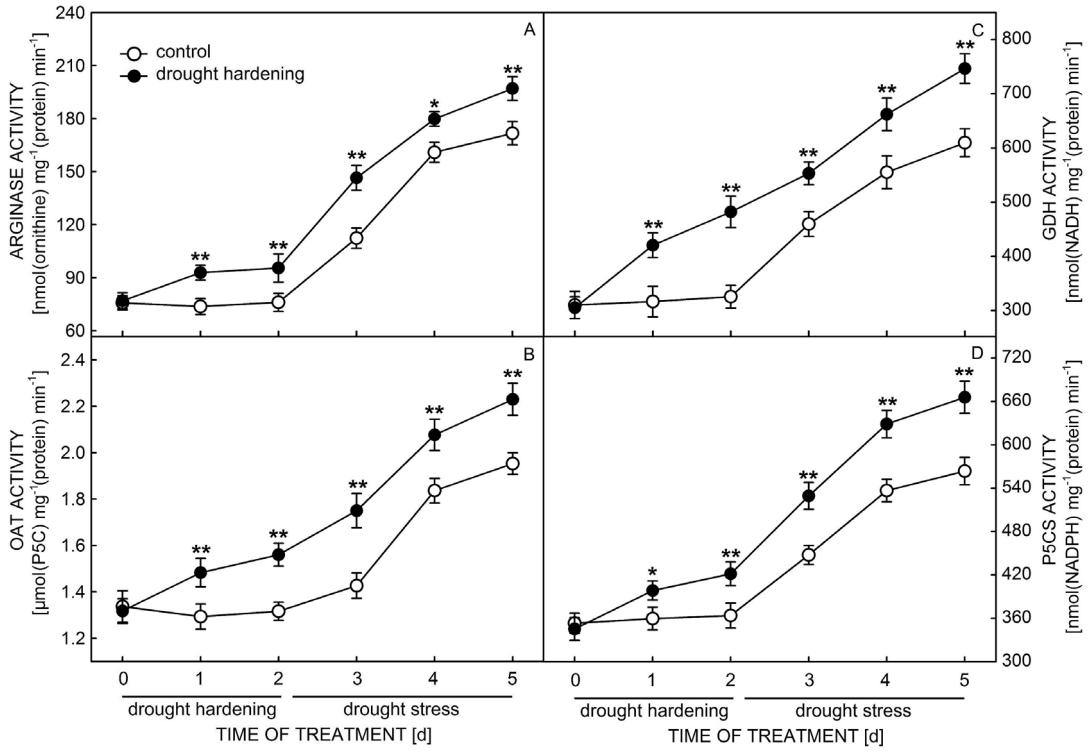

Fig. 7. Changes in activities of arginase (*A*), OAT (*B*), GDH (*C*), and P5CS (*D*) in *J. curcas* seedlings during drought hardening with 10 % PEG 6000 for 2 d and subsequent drought stress for 3 d. Means ± SE of at least six replicates of three independent experiments. *Asterisks* and *double asterisks* indicate significant differences ($P < 0.05$) and very significant differences ($P < 0.01$) between control and treated plants.

glycinebetaine in plant cells in response to water loss (Pérez-Pérez *et al*. 2009), and their active accumulation could enhance plant drought tolerance (Delauney and Verma 1993, Chen and Murata 2008, Trovato *et al* 2008, Szabados and Savoure 2010). In addition, our results show that the drought hardening treatment could induce a more rapid accumulation of all these osmolytes in the *J. curcas* seedlings during the hardening treatment and subsequent drought stress (Fig. 5).

(Fig. 9) was also observed in the *J. curcas* seedlings during drought hardening and subsequent drought stress, along with a decreased activity of ProDH, the key enzyme in proline degradation (Fig. 8). These findings imply that glycinebetaine and proline played an important role in drought hardening-induced drought tolerance in the *J. curcas* seedlings. Moreover, various researchers

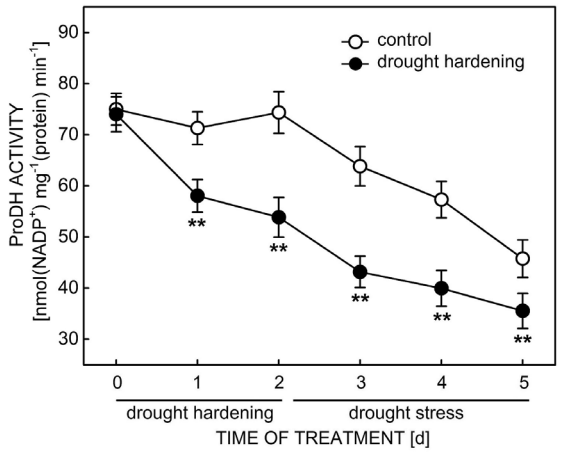

Fig. 8. Change in proline dehydrogenase (ProDH) activity in *J. curcas* seedlings during drought hardening with 10 % PEG 6000 for 2 d and subsequent drought stress for 3 d. Means ± SE of at least six replicates of three independent experiments. *Double asterisks* indicate very significant differences ($P < 0.01$) between control and treated plants.

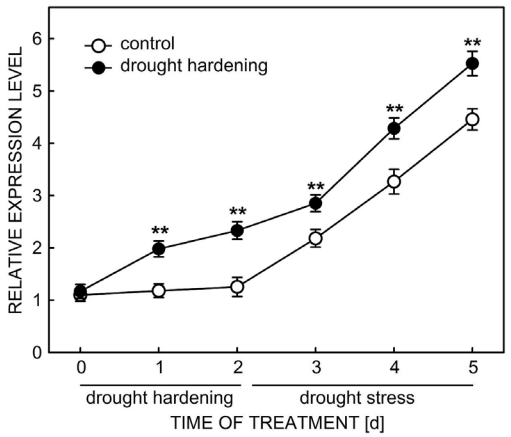

Fig. 9. Change in expression of *P5CS* gene (analyzed by RT-qPCR) in *J. curcas* seedlings during drought hardening with 10 % PEG 6000 for 2 d and subsequent drought stress for 3 d. Means ± SE of at least six replicates of three independent experiments. *Double asterisks* indicate very significant differences ($P < 0.01$) between control and treated plants.

The investigation of changes in the metabolic pathways of glycinebetaine and proline indicates that the drought-hardening treatment rapidly increased the activity of the key enzyme BADH (Fig. 6) involved in the glycinebetaine biosynthesis. It also enhanced the activities of the other important enzymes, arginase (Fig. 7*A*) and OAT (Fig. 7*B*) which participate in the ornithine pathway for proline biosynthesis. In addition, hardening induced an almost immediate increase in the activity of the crucial enzymes, GDH (Fig. 7*C*) and P5CS (Fig. 7*D*), involved in the glutamate pathway for proline biosynthesis. An upregulation of *P5CS* gene expression have found that compatible solutes not only display an osmotic adjustment function, but also exert a very important role in the stabilization of proteins, enzymes, and biomembranes (Chen and Murata 2008, Trovato *et al.* 2008, Szabados and Savoure 2010).

In conclusion, drought hardening with PEG 6000 could enhance drought tolerance of the *Jatropha curcas* seedlings, and osmoregulation was involved in this increased drought tolerance. The drought hardening-induced drought tolerance was partly due to the activation of the biosynthetic pathways of glycinebetaine and proline.

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