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ORIGINAL PAPER

Involvement of antioxidant defense system in chill hardening-induced chilling tolerance in *Jatropha curcas* seedlings

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Abstract *Jatropha curcas* L. is a sustainable energy plant with great potential for biodiesel production, and low temperature is an important limiting factor for its distribution and production. In this present work, chill hardening-induced chilling tolerance and involvement of antioxidant defense system were investigated in *J. curcas* seedlings. The results showed that chill hardening at 10 or 12 °C for 1 and 2 days greatly lowered death rate and alleviated electrolyte leakage as well as accumulation of the lipid peroxidation product malondialdehyde (MDA) of *J. curcas* seedlings under severe chilling stress at 1 °C for 1–7 days, indicating that the chill hardening significantly improved chilling tolerance of *J. curcas* seedlings. Measurement of activities of the antioxidant enzymes superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), peroxidase (POD), and glutathione reductase (GR), and the levels of the antioxidants ascorbic acid (AsA) and glutathione (GSH) showed the chill hardening at 12 °C for 2 days could obviously increase the activities of these antioxidant enzymes and AsA and GSH contents in the hardened seedlings. When the hardened and non-hardening (control) seedlings were subjected to severe chilling stress at 1 °C for 1–7 days, the chill-hardened seedlings generally maintained significantly higher activities of the antioxidant enzymes SOD, APX, CAT, POD, and GR, and content of the antioxidants AsA and GSH as well as ratio of the reduced antioxidants to total antioxidants [AsA/(AsA + DHA) and GSH/(GSH + GSSG)], when compared with the control without chill hardening. All above-mentioned results indicated that the chill hardening could enhance the chilling tolerance, and the antioxidant defense system plays an important role in the chill hardening-induced chilling tolerance in *J. curcas* seedlings.

Keywords Antioxidants · Antioxidant enzymes · Chilling hardening · Chilling tolerance · *Jatropha curcas* L.

Abbreviations

ANOVA	Analysis of variance
APX	Ascorbate peroxidase
AsA	Ascorbic acid
CAT	Catalase
DHA	Dehydroascorbate
DW	Dry weight
GR	Glutathione reductase
GSH	Glutathione
GSSG	Oxidized glutathione
MDA	Malondialdehyde
NBT	Nitroblue tetrazolium
POD	Peroxidase
ROS	Reactive oxygen species
SOD	Superoxide dismutase

Introduction

The *Jatropha curcas*, considered as an important energy plant due to the fact that its seed contains high oil content, belongs to the tribe *Jatropheae* in the *Euphorbiaceae*

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family (Carels 2009; King et al. 2009; Mukherjee et al. 2011). The seed contains 30–40 % oil with 21 % saturated fatty acids and 79 % unsaturated fatty acids, and recognition that *J. curcas* oil can yield a high quality biodiesel has led to a surge of interest in *J. curcas* across the globe (King et al. 2009; Mukherjee et al. 2011). The seed oil has a good oxidation stability compared to soybean oil, low viscosity compared to castor oil, and a low pour point (the temperature where it starts to become solid) compared to palm oil. The fuel properties of *J. curcas* biodiesel are close to those of fossil diesel fuel and match the American and European standards (Carels 2009; King et al. 2009; Mukherjee et al. 2011).

Low temperature presents a severe environmental challenge to plant growth and distribution in temperate and subtropical regions. All plants, as sessile and poikilothermic organisms, have optimal temperature ranges for their proper growth and development, as well as minimum and maximum temperatures for survival (Levitt 1980). Plants can be divided into three broad categories: chilling sensitive, chilling tolerant but freezing sensitive, and freezing tolerant according to their responses to low temperature. Chilling-sensitive plants from tropical and subtropical regions, such as rice and maize, can be irreparably damaged when the temperature drops below 10 °C, mainly due to the loss in both membrane integrity and compartmentalization of the intracellular organelles, and largely lack the capacity for cold acclimation (Heidarvand and Amiri 2010; Janska et al. 2010; Jan et al. 2009; Ruelland et al. 2009). In addition to these, chilling stress can induce the overproduction of reactive oxygen species (ROS) in plants, which then in turn negatively affects cellular structures and metabolism by oxidative stress. Higher plants have developed several strategies to cope with oxidative stress. One of the defense mechanisms is the antioxidation defense system, including antioxidant enzymes and low molecular weight antioxidants (Foyer and Noctor 2009; Jaleel et al. 2009; Prasad et al. 1994). The superoxide radical ($O_2^{\cdot -}$) is dismutated to hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD). Ascorbate peroxidase (APX), peroxidase (POD), and catalase (CAT) metabolize H_2O_2 into water. In the presence of $O_2^{\cdot -}$ and H_2O_2, trace amounts of transition metals can give rise to the highly toxic hydroxyl radical ($OH^{\cdot -}$). Rapid detoxification of both $O_2^{\cdot -}$ and H_2O_2 is essential for preventing oxidative damage (Foyer and Noctor 2009; Jaleel et al. 2009).

Exposure of plants to low-, non-freezing moderate temperature for several days can improve their resistance to subsequent low temperature, which is known as chill hardening (Levitt 1980; Prasad et al. 1994). Three-day-old maize seedlings were exposed to 4 °C for 7 days and did not survive chilling stress unless they were pre-exposed to 14 °C for 3 days (Prasad et al. 1994). Lange and Cameron (1997) have reported that sweet basil subjected to chill hardening at 10 °C for 4 h daily for 2 days could increase average shelf life at 5 °C. In maize seedlings, cold-shock pretreatment at 1 °C for 4 h, followed by a 6-h recovery at 26.5 °C significantly enhanced survival rate under severe chilling stress at 1 °C (Li et al. 2011). Our previous results also found that the chill hardening could maintain higher chlorophyll content, photosynthetic rate and lower electrolyte leakage at chilling temperature, which in turn improved resistance of chilling sensitive plant rice to chilling and freezing (Gong et al. 1989).

However, we hypothesized that chill hardening may improve chilling tolerance in *J. curcas* as chilling-sensitive plant and the acquisition of this chilling tolerance involved in antioxidant defense system, but detailed data is poorly known (Liang et al. 2007; Zheng et al. 2009). In this article, *J. curcas* seedlings were used as materials, effect of chill hardening on chilling tolerance, and involvement of antioxidant defense system was investigated, the results showed that chill hardening could improve chilling tolerance of *J. curcas* seedlings and this improvement involved in antioxidant defense system.

Materials and methods

Plant materials and treatments

Seeds of *J. curcas*, a mix of cultivars, were collected from Yuanmou, Yunnan Province, China. Seeds were surface sterilized in 1 % $CuSO_4$ for 15 min and rinsed thoroughly with sterilized distilled water according to our previous methods (Li and Gong 2011), and then pre-soaked for imbibition in distilled water for 24 h. The soaked seeds were sowed on six layers of wetted filter papers in trays (200 seeds per tray) with covers and germinated at 26 °C in the dark for 5 days. Then germinated seeds were selected and transferred to pot containing sterilized soil with perlite, peat and sand (1:2:1) as well as wetted 1/2 MS (Murashige and Skoog 1962) basal salts in climate chamber with 26/20 °C (day/night), 30 μmol m^{-2} s^{-1} and 16 h photoperiod, and sequentially grown for 7 days.

To explore effect of chill hardening on chilling tolerance, 2-week-old seedlings were subjected to chill hardening at 10 or 12 °C for 1 or 2 days (the control seedlings grown in the climate chamber with above-mentioned parameters). At the end of chill hardening, hardened and non-hardened seedlings were exposed to chilling stress at 1 °C for 1, 2, 3, 4, 5, 6, and 7 days, death rate, electrolyte leakage, malondialdehyde (MDA) content, antioxidant enzyme activities, and antioxidant contents were determined daily.

Determination of death rate, electrolyte leakage, and MDA content

Death rate of seedlings, electrolyte leakage, and MDA content in leaves of the seedlings were determined using

our previous methods (Gong et al. 2001) and death rate, electrolyte leakage as well as MDA content were expressed as %, %, and μmol g^{-1} DW, respectively.

Antioxidant enzyme activities assay

Antioxidant enzymes SOD, APX, CAT, POD, and GR in leaves and stems were extracted and measured according to our methods described previously (Li et al. 2002). SOD activity was determined by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) and one unit of enzyme activity was defined as the amount of the enzyme bringing about 50 % inhibition of the photochemical reduction of NBT. Activities of APX, CAT, POD, and GR were counted using the extinction coefficient 2.9, 0.04, 26.6, and 2.9 mM^{-1} cm^{-1} at 290, 240, 470, and 260 nm, respectively.

Measurement of water soluble antioxidant content

AsA and GSH contents were extracted and measured as our procedures described previously (Li et al. 2003), and AsA, DHA, AsA/(AsA + DHA), and GSH, GSSG as well as GSH/(GSH + GSSG) were expressed as μmol g^{-1}DW and %, respectively.

Statistics analysis

All experiments were repeated at least three times and two replications in each time. The results were processed statistically using one-way analysis of variance (ANOVA, variance test, LSD). Figures were drawn by SigmaPlot 10.0, error bars represent standard error and each data in figure represents the mean ± SE of at least three experiments.

Results

Effect of chill hardening on chilling tolerance of *J. curcas* seedlings

When 2-week-old seedlings of *J. curcas* were exposed to chilling stress at 1 °C, as shown in Fig. 1, death rate of the seedlings increased rapidly with prolongation of the stress time, and reached almost 100 % at 1 °C for 7 days. On the other hand, chill hardening at 10 or 12 °C for 1 or 2 days all obviously lowered death rate of the hardened seedling when compared with the control without the chill hardening, and this difference demonstrated especially significantly during the fourth and fifth days of the chilling stress at 1 °C ($P < 0.05$, $P < 0.01$; Fig. 1).

Environmental stresses primarily damage membrane systems and lead to leakage of electrolytes from plant cells,

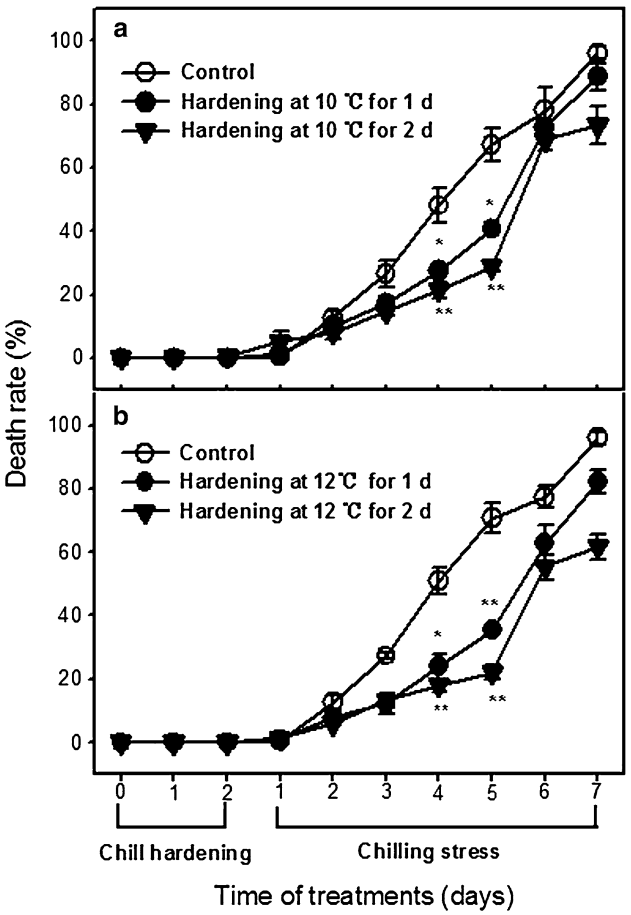

Fig. 1 Effect of chill hardening at 10 °C (**a**) or 12 °C (**b**) on death rate of *J. curcas* seedlings under chilling stress at 1 °C. 2-week-old seedlings of *J. curcas* were subjected to chill hardening at 10 or 12 °C for 1 or 2 days, respectively, and then were exposed to chilling stress at 1 °C for 1, 2, 3, 4, 5, 6, or 7 days. Death rate (%) of the seedlings was counted after recovery under normal growth conditions for 15 days, and about 150–200 seedlings were investigated each treatment. *Error bars* represent standard error and each data in the figures represents the mean ± SE of at least three experiments, *asterisk and double asterisks* indicate significant difference ($P < 0.05$) and very significant difference ($P < 0.01$) from the control without chill hardening, respectively

lipid peroxidation, and accumulation of MDA (Gong et al. 2001; Jambunathan 2010). To substantiate the above death rate data, we also measured leakage of electrolytes and MDA content in the chill hardening and non-chill hardening seedlings. As shown in Figs. 2 and 3, chilling stress at 1 °C led to a significant increase of leakage of electrolytes and MDA content in leaves of *J. curcas* seedlings, but the latter started to decline after fifth day of the chilling stress (Fig. 2b), and the chill-hardened seedlings significantly lowered the leakage of electrolytes and MDA content than the seedlings without chill hardening under the chilling stress at 1 °C. These results indicated that the chill hardening could improve obviously chilling tolerance of *J. curcas* seedlings. In addition, effect of the chill

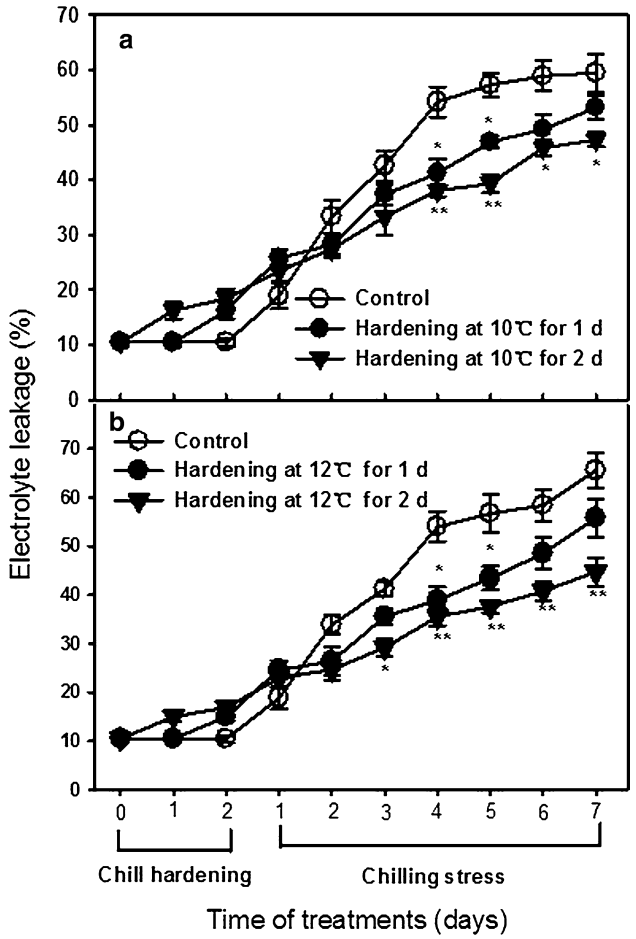

Fig. 2 Effect of chill hardening at 10 °C (**a**) or 12 °C (**b**) on electrolyte leakage content of *J. curcas* seedlings under chilling stress at 1 °C. 2-week-old seedlings of *J. curcas* were subjected to chill hardening at 10 or 12 °C for 1 or 2 days, respectively, and then were exposed to chilling stress at 1 °C for 1, 2, 3, 4, 5, 6, or 7 days. Electrolyte leakage in leaves of the seedlings was measured daily. *Error bars* represent standard error and each data in the figures represents the mean ± SE of at least three experiments, *asterisk and double asterisks* indicate significant difference ($P < 0.05$) and very significant difference ($P < 0.01$) from the control without chill hardening, respectively

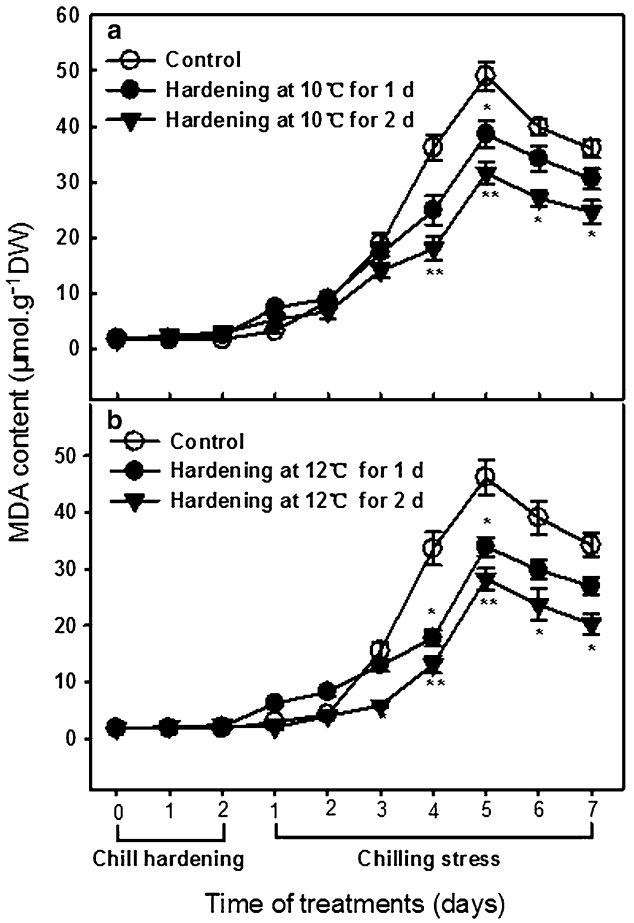

Fig. 3 Effect of chill hardening at 10 °C (**a**) or 12 °C (**b**) on MDA content of *J. curcas* seedlings under chilling stress at 1 °C. 2-week-old seedlings of *J. curcas* were subjected to chill hardening at 10 or 12 °C for 1 or 2 days, respectively, and then were exposed to chilling stress at 1 °C for 1, 2, 3, 4, 5, 6, or 7 days. MDA content in leaves of the seedlings was measured daily. *Error bars* represent standard error and each data in the figures represents the mean ± SE of at least three experiments, *asterisk and double asterisks* indicate significant difference ($P < 0.05$) and very significant difference ($P < 0.01$) from the control without chill hardening, respectively

hardening at 12 °C for 2 days on chilling tolerance of *J. curcas* seedlings was more efficient than that of other hardenings, therefore, chill hardening at 12 °C for 2 days was used for the following experiments.

Effect of chill hardening and chilling stress on antioxidant enzyme activities and antioxidant levels of *J. curcas* seedlings

To better understand the mechanism of the above-mentioned chill hardening-induced chilling tolerance, activities of the antioxidant enzymes SOD, APX, CAT, POD and GR as well as contents of the antioxidants AsA and GSH in leaves and stems of *J. curcas* seedlings were determined. During the process of the chill hardening at 12 °C for 2 days, the activities of antioxidant enzymes all raised with the extension of chill hardening time, particularly the activities of APX and POD showed more significant increase than those of other antioxidant enzymes (Fig. 4). At the same time, the chill hardening at 12 °C for 2 days also obviously enhanced levels of the antioxidants AsA and GSH in leaves and stems of *J. curcas* seedlings to some extent as compared to the non-hardening control (Figs. 5, 6).

When the chill hardening and non-hardening seedlings were transferred to 1 °C for severe chilling stress, the activities of their antioxidant enzymes showed different change tendencies: SOD, CAT, POD, and GR activities first increased differentially to some extent during the early

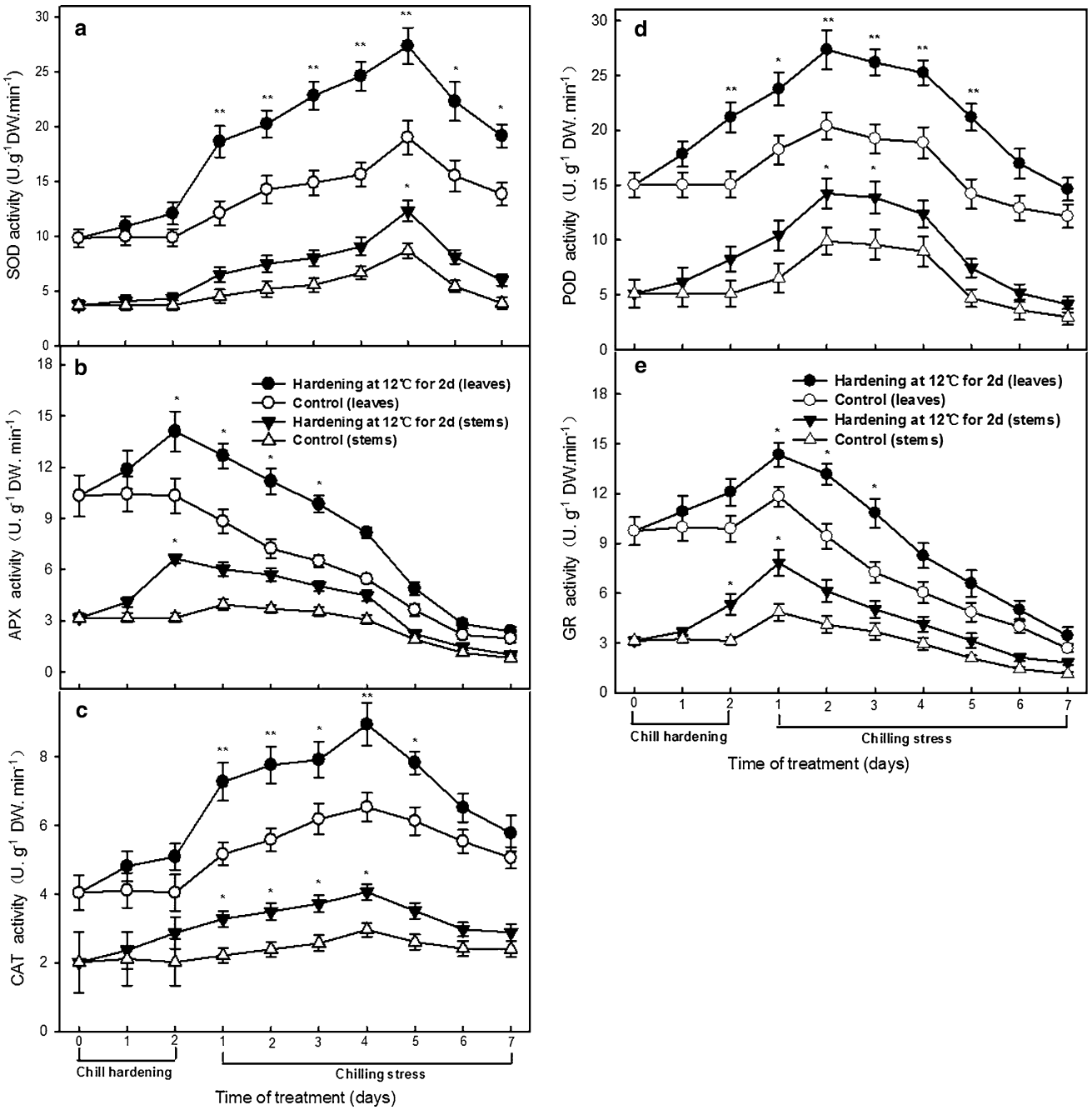

Fig. 4 Effect of chill hardening and chilling stress on activities of the antioxidant enzymes SOD (**a**), APX (**b**), CAT (**c**), POD (**d**), and GR (**e**) in leaves and stems of *J. curcas* seedlings. 2-week-old seedlings of *J. curcas* were subjected to chill hardening at 12 °C for 2 days, and then were exposed to chilling stress at 1 °C for 1, 2, 3, 4, 5, 6, or 7 days. Activities of the antioxidant enzymes in leaves or stems of the seedlings were assayed daily during the chill hardening and chilling stress. *Error bars* represent standard error and each data in the figures represents the mean ± SE of at least three experiments, *asterisk and double asterisks* indicate significant difference ($P < 0.05$) and very significant difference ($P < 0.01$) from the control without chill hardening, respectively

stage of the chilling stress, then declined gradually; while APX activity declined during the chilling stress (Fig. 4). However, in general, the chill-hardened seedlings retained significantly higher activities of the antioxidant enzymes in their leaves and stems than non-hardening seedlings did under the chilling stress at 1 °C for 7 days (Fig. 4).

Similarly, the chill hardening at 12 °C for 2 days also obviously raised contents of the antioxidant ASA and GSH in the leaves and stems of chill-hardened seedlings. When the seedlings were transferred to 1 °C for severe chilling stress, the contents of ASA and GSH showed a further increase during the early phase of the chilling stress, then

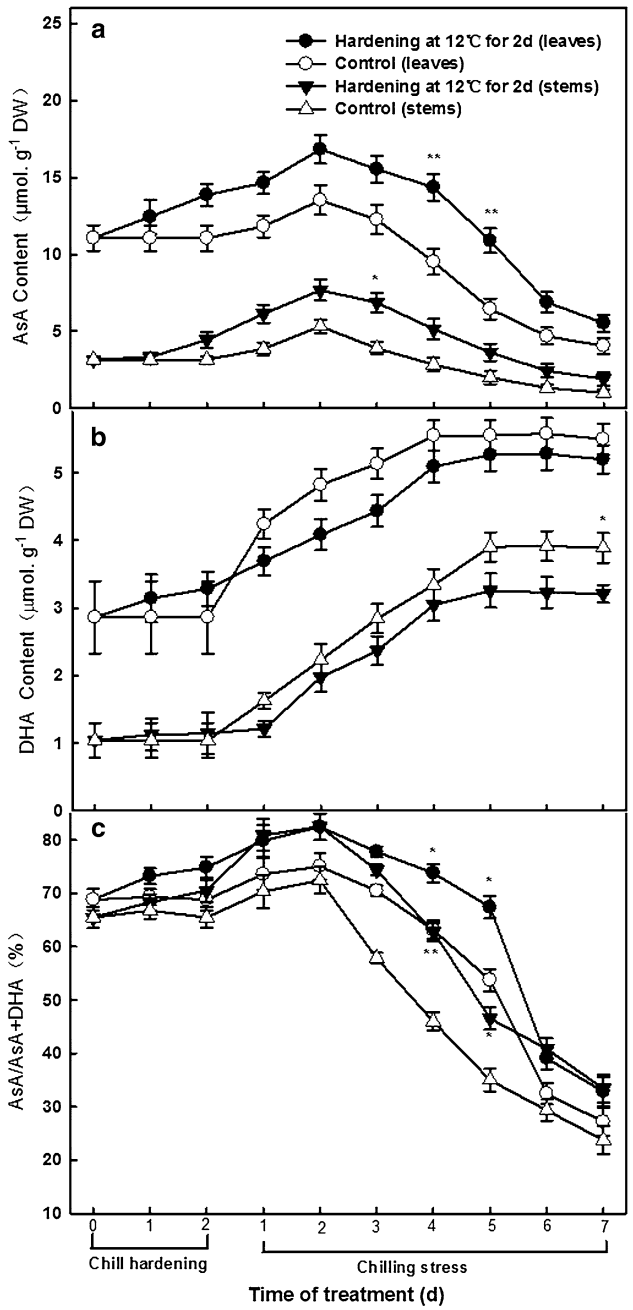

Fig. 5 Effect of chill hardening and chilling stress on levels of AsA (**a**), DHA (**b**), and AsA/(AsA + DHA) ratio (**c**) in leaves or stems of *J. curcas* seedlings. See the caption to Fig. 3 for details of these treatments. *Error bars* represent standard error and each data in the figures represents the mean ± SE of at least three experiments, *asterisk and double asterisks* indicate significant difference ($P < 0.05$) and very significant difference ($P < 0.01$) from the control without chill hardening, respectively

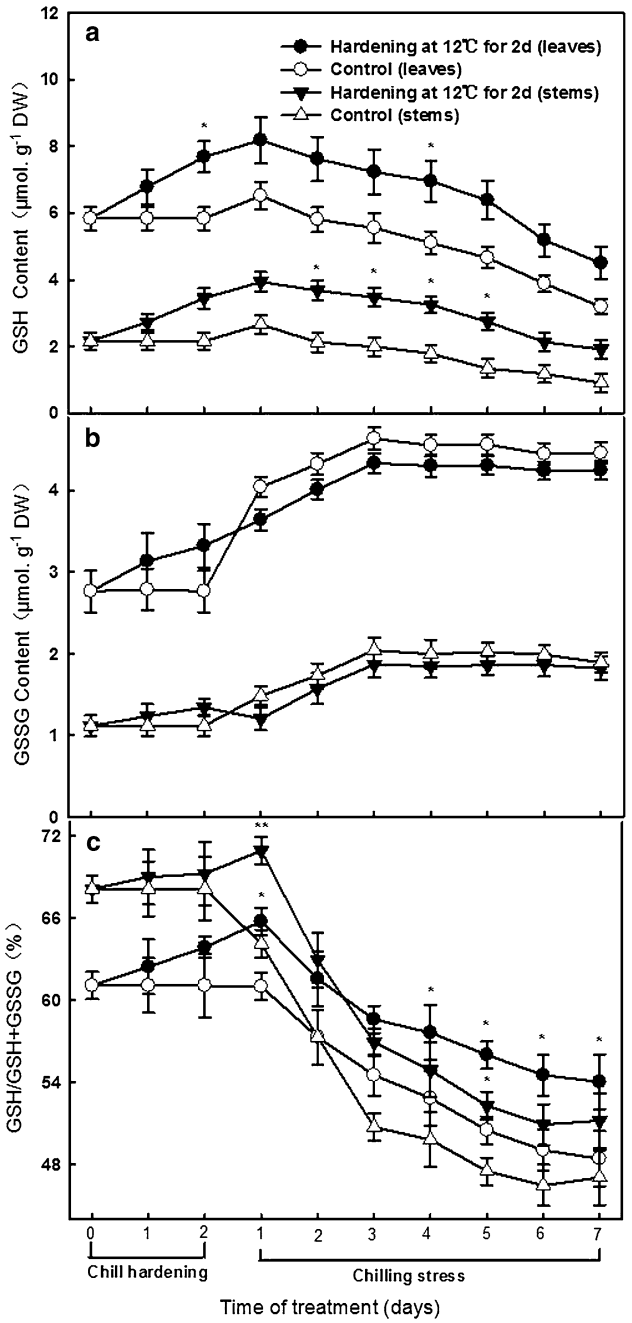

Fig. 6 Effect of chill hardening and chilling stress on levels of GSH (**a**), GSSG (**b**), and GSH/(GSH + GSSG) ratio (**c**) in leaves or stems of *J. curcas* seedlings. See the caption to Fig. 3 for details of these treatments. *Error bars* represent standard error and each data in the figures represents the mean ± SE of at least three experiments, *asterisk and double asterisks* indicate significant difference ($P < 0.05$) and very significant difference ($P < 0.01$) from the control without chill hardening, respectively

declined gradually (Figs. 5a, 6a). In contrast, the oxidized form of the antioxidant ASA and GSH, namely DHA and GSSG content increased continuously during the chill hardening and following chilling stress (Figs. 5b, 6b). As a result, the ratio of AsA/(AsA + DHA) and (GSH + GSSG), which represent intracellular redox status (Suzuki et al. 2012; Scheibe and Dietz 2012), declined sharply after 1 °C chilling stress for 2 days (Figs. 5c, 6c). In general, the chill-hardened seedlings maintained significantly higher level of the antioxidant ASA and GSH and the

ratio of AsA/(AsA + DHA) and GSH/(GSH + GSSG), and lower level of the oxidized antioxidant DHA and GSSG than those of non-hardening seedlings did.

Discussion

A number of studies showed that chill hardening of plants can allow plants to withstand subsequent and more severe stress (Guy 1999; Chinnusamy et al. 2007; Bafeel and Ibrahimi 2008; Survila et al. 2010; Ciarmiello et al. 2011; Catala et al. 2012). Three-day-old maize seedlings were pre-exposed to 14 °C for 3 days, which can improve survival percentage under chilling stress at 4 °C for 7 days (Prasad et al. 1994). Similarly, Lange and Cameron (1997) also found that chill hardening at 10 °C for 4 h daily for 2 days could increase average shelf life at 5 °C in sweet basil. Here, 2-week-old seedlings of *J. curcas* were exposed to chilling stress at 1 °C after chill hardening at 10 or 12 °C for 1 or 2 days, which all obviously lowered death rate of the hardened seedling when compared with the control without the chill hardening (Fig. 1), but the mechanism of chill hardening-induced chilling tolerance in plants is not all clear.

Also, chill hardening appears to bring about changes in their metabolism, including an increase in unsaturation of membrane lipids, enhancement in activities of antioxidant enzymes and levels of antioxidants, especially antioxidant defense system (Guy 1999; Chinnusamy et al. 2007; Bafeel and Ibrahimi 2008; Survila et al. 2010; Ciarmiello et al. 2011; Catala et al. 2012). Cellular redox homeostasis is considered to be an ''integrator'' of information from metabolism and the environment controlling plant growth and acclimation responses, as well as cell suicide events (Foyer and Noctor 2009; Jaleel et al. 2009; Jan et al. 2009).

The loss of metabolic homeostasis due to adverse environmental factors results in a greater production of ROS, but plants possess non-enzymatic and enzymatic scavenging systems, which should keep ROS at a level that is not harmful. Scavenging enzymes operate as ROS scavengers or, rather, they are involved in recycling antioxidants (Leipner and Stamp 2009). Low molecular antioxidants (e.g., ascorbate, glutathione) serve not only to limit the lifetime of the ROS signals but also to participate in an extensive range of other redox signaling and regulatory functions (Foyer and Noctor 2009; Jaleel et al. 2009; Jan et al. 2009; Leipner and Stamp 2009). The involvement of ROS in chilling acclimation has been shown in plants, where a very rapid transient increase in H_2O_2 content was observed in response to chilling (Prasad et al. 1994; Xu et al. 2008, 2010; Li et al. 2011; Leipner and Stamp 2009). This change in the H_2O_2 concentration then affects components of the antioxidant ascorbate-glutathione cycle, which remove excess H_2O_2 (Foyer and Noctor 2009; Leipner and Stamp 2009). Activities of antioxidant enzymes under chilling stress have been correlated with tolerance to the stress. In tobacco seedlings, chilling stress increased POD activity and reduced SOD activity in shoots, and CAT activity was little affected (Xu et al. 2010; Leipner and Stamp 2009). In alfalfa, activity of SOD increased straight away the dark chilling stress, whereas CAT, APX, and GR activities were slightly increased after chilling treatment (Wang et al. 2009). Our previous results also showed that the cold-shock pretreatment at 1 °C for 4 days brought out an endogenous H_2O_2 peak, which enhanced the activities of five antioxidant enzymes POD, CAT, APX, GR, and SOD in maize mesocotyls and remained significantly higher activities of these five antioxidant enzymes after chilling stress (Li et al. 2011). In this present work, chill hardening at 10 or 12 °C for 1 or 2 days could alleviate increase in death rate of *J. curcas* seedlings under chilling stress, trigger increase in activities of antioxidant enzymes, especially in APX and POD (Fig. 4). In addition, change in levels of antioxidants AsA and GSH showed the same trend (Figs. 5, 6). Under chilling stress at 1 °C, SOD, CAT, and POD activities gradually increased with the extension of stress time, but the later stage of chilling stress started to decline, while APX and GR activities declined with the prolongation of chilling stress time (Fig. 4). Also, change in AsA content and AsA/(AsA + DHA) ratio was similar to SOD, CAT, and POD activities, whereas GSH content and GSH/(GSH + GSSG) ratio consisted with activities of APX and GR under chilling stress at 1 °C (Figs. 5, 6).

In conclusion, it is clearly shown that 2-week-old seedlings of *J. curcas* were subjected to chill hardening could alleviate decrease in death rate of seedling, increase in electrolyte leakage, and accumulation of MDA compared with the control without chill hardening, implying that chill hardening could improve chilling tolerance of *J. curcas* seedlings. In addition, chill hardening also could improve activities of antioxidant enzymes and levels of antioxidants, which maintained higher antioxidant enzyme activities and antioxidant levels from beginning to end compared with control under chilling stress, suggested that antioxidant defense system plays a very important role in chill hardening and chilling tolerance of *J. curcas* seedlings.

Author contribution In this study, Zhong-Guang Li and Ming Gong carried out conception, design, and writing the article, Ping-Xing Ao coordinated the study and carried out data analysis and interpretation, Dong-Mei Fan participated in the design of the study. All authors have read and approved the final manuscript and have no conflicts of interest in regard to this research or its funding.

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