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ORIGINAL PAPER

Combined action of an antioxidant defence system and osmolytes on drought tolerance and post-drought recovery of *Phoebe zhennan* S. Lee saplings

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Abstract Nanmu (*Phoebe zhennan* S. Lee) is a well-known rare tree species in China that is valued as an ornamental garden plant and for its high timber quality. Recently, the cultivation of nanmu has gained attention for use in tree resource conservation and ecological restoration projects. Drought is a major environmental factor that affects the growth and development of plants. In this study, the drought tolerance and post-drought recovery of nanmu, which is associated with antioxidative enzymes and osmotic adjustment, were examined by exposing nanmu saplings to drought for 30 days followed by 10 days of re-watering in a greenhouse. Drought stress resulted in increased levels of osmolytes, sugars and proteins in nanmu leaves compared with the well-watered controls as well as higher concentrations of superoxide radicals and hydrogen peroxide, leading to lipid peroxidation and significantly higher activities of superoxide dismutase, catalase and guaiacol peroxidase and higher levels of ascorbic acid. After re-watering for 5 days, most of the antioxidant enzymes and ascorbic acid were restored to their original levels, whereas the activity of guaiacol peroxidase and the levels of soluble sugar and soluble protein remained markedly high. Moreover, nanmu saplings maintained normal turgor pressure under mild and moderate drought conditions, indicating the presence of a mechanism that affects osmotic adjustment and growth restriction, thus enabling the plant to adjust to drought stress. These results suggested that an antioxidant defence system and osmolytes play important roles in nanmu during drought stress and recovery.

Keywords *Phoebe zhennan* S. Lee · Drought stress · Re-watering · Antioxidant enzymes · Antioxidant · Osmolytes

Abbreviations

ROS	Reactive oxygen species
OA	Osmotic adjustment
SWC	Soil water content
LRWC	Leaf relative water content
O_2^-	Superoxide radical
H_2O_2	Hydrogen peroxide
MDA	Malondialdehyde
SOD	Superoxide dismutase
CAT	Catalase
POD	Guaiacol peroxidase
APX	Ascorbate peroxidase
AsA	Ascorbic acid
Pro	Proline
SS	Soluble sugar
SP	Soluble protein

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Introduction

Drought and water stress are among the main environmental factors that limit crop growth and yields worldwide (Panozzo and Eagles 1999). Drought stress can be constant in regions with low water availability or in regions with

random and unpredictable water availability due to changes in weather conditions during plant growth (Niu et al. 2013). For example, the upper and middle reaches of the Yangtze River usually experience seasonal droughts during spring and autumn. Furthermore, the long-term sustainability of these ecosystems may be further threatened by the regional effects of global warming, which are expected to result in droughts of extended length, severity and frequency (David et al. 2007). Thus, plants, especially those used for afforestation, must adapt and evolve survival strategies to increase their tolerance to rapid environmental changes. Furthermore, an understanding of the mechanisms of plant tolerance to short-term drought is needed to provide a theoretical reference for the forestry water management of individual species.

Drought stress induces a series of morphological, physiological and biochemical changes that result in the disturbance of normal growth and development in many plant species. Several studies have suggested that plants have developed various strategies to survive water deficits and to improve their drought tolerance (Bohnert and Jensen 1996; Chaves et al. 2002). Drought stress can also lead to oxidative stress by increasing the concentrations of reactive oxygen species (ROS), including singlet oxygen ($^1O_2$), superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH·), all of which are highly reactive and cause cellular damage through the oxidation of lipids, proteins, and nucleic acids (Apel and Hirt 2004; Gill and Tuteja 2010). Plants can only survive under such stressful conditions if they are able to perceive the causative stimulus, generate and transmit signals, and initiate various physiological and biochemical changes (Bohnert and Jensen 1996); in other words, the rapidity and efficiency of responses might determine the viability of individual species. Previous studies have indicated that to counteract the phenomenon of oxidative stress, plants have evolved an antioxidant defence system involving both enzymatic and nonenzymatic elements, which are present in plant cells (Impa et al. 2012; Li et al. 2013a); these elements include superoxide dismutase (SOD), guaiacol peroxidase (POD), catalase (CAT) and ascorbic acid (AsA). SOD is part of the first line of defence against oxidative stress in plants and converts O_2^- to H_2O_2 through the metal-catalysed Haber–Weiss-type reaction (Smirnoff 1993). POD and CAT remove H_2O_2 (Arafat 2011), whereas AsA reduces H_2O_2 through an oxidising reaction in cooperation with other enzymes, such as ascorbate peroxidase (APX) (Foyer and Noctor 2011). In general, drought stress can trigger an oxidative burst, accelerate the degradation of cell membranes, induce the expression of an array of antioxidant enzymes, and elicit membrane lipid accumulation.

Osmotic adjustment (OA) is a cellular adaptation mechanism that is vital for tolerance to drought stress in plants, enabling them to withstand drought stress or grow in areas of limited water availability (Ackerson 1981; Silva et al. 2010). This mechanism allows water uptake, cell enlargement and plant growth during water stress and is associated with partial stomata opening, thus enabling CO_2 assimilation at otherwise inhibitory low water potentials (Alves and Setter 2004). Although the accumulation of solutes facilitates OA in many species, the involved compounds differ widely. Previous studies indicated that the accumulation of proline (Pro) (Wang et al. 2004) and sugars (Patakas et al. 2002) commonly occurs as a metabolic response of higher-order plants to a water deficit. Furthermore, these compounds are compatible solutes that absorb water into plant cells by maintaining turgor (Yancey 2001). According to Szabados and Savouré (2010), Pro also acts as an effective quencher of reactive oxygen species, thus protecting plants against singlet oxygen and free radical damage inducers. Many studies have indicated that drought induces metabolic changes related to protein turnover, such as increasing protein synthesis, aggregation, denaturation, or degradation (Zhang et al. 2011; Demirevska et al. 2009). Furthermore, these drought-induced or drought-inhibited proteins have been shown to be hydrophilic, and they are frequently used to evaluate the drought tolerance of plants (Kang et al. 2005).

Nanmu (*Phoebe zhennan* S. Lee) is an evergreen tree that produces high-quality timber and is widely distributed in southern China. This tree is also favoured as an ornamental plant, especially for landscaping. A programme for the protection and development of rare and precious tree species resources has been launched in Sichuan province. Nanmu is one of the rare species on the list of protected plants in China, and a project based on nanmu plantations has been underway for 5 years. Nevertheless, additional experience in nanmu cultivation would be beneficial for this project. Improving our understanding of the physiological mechanisms of stress tolerance in nanmu would benefit its breeding and cultivation. Understanding the physiological effects of post-drought recovery in nanmu is of equal importance and will provide better insights into the mechanisms of the drought response and recovery potential. According to studies of forest plantations, nanmu rarely reach their full growth potential due to abiotic (particularly seasonal drought) and biotic restraints (Wu 2009). However, little information exists about the tolerance of nanmu to drought stress and post-drought recovery or about the mechanisms of antioxidant and osmotic adjustment. In this article, we hypothesise that antioxidant systems and the accumulation of compatible solutes play important roles by supporting nanmu saplings during drought and post-drought recovery. To evaluate the possible roles of the antioxidant defence system and osmolytes during drought and post-drought recovery in nanmu

saplings, we studied the activities of several antioxidant enzymes (SOD, POD, and CAT) as well as antioxidant (AsA) concentrations, lipid peroxidation, and the accumulation of major solutes, including Pro, soluble sugar (SS) and soluble protein (SP).

Materials and methods

Plant material and site description

Nanmu seeds were collected during November 2011 at Yaan, Sichuan Province, China. The seeds were surface-sterilised in 1 % $CuSO_4$ for 15 min, rinsed thoroughly with sterile distilled water, and then stored in sand with a volumetric water content of 10–15 % for 6 months. After this time (in late March), germinated seeds were selected and cultivated in the forestry nursery at the Sichuan Agricultural University. After an additional 12 months, seedlings with an average height of 20 cm were transferred to plastic pots (inner diameter, 30 cm; height, 35 cm) containing 15 kg of soil per pot on April 10, 2012; one sapling was planted in each of forty pots, which were watered every 3 days. The cultivation substrate materials was sandy loam soil with the following characteristics: field capacity, 25 % (volumetric water content); bulk density, 1.32 g cm^{-3}; total Nitrogen content, 1.53 g kg^{-1}; total Phosphorus content, 1.34 g kg^{-1}; and total Kalium content, 27.12 g kg^{-1}.

Twenty-eight saplings, with an average height of 50 cm, were selected and evenly distributed in the greenhouse at Sichuan Agricultural University. The area has a subtropical humid climate with an annual average temperature of 16.2 °C and monthly temperatures of 3.7 °C (January) to 29.9 °C (July). The average annual sunshine period is 1039.6 h; the average frost-free period is 298 days; and the average annual rainfall is 1774.3 mm.

Stress treatment

The 28 saplings were subjected to various water deficit treatments, in which watering was halted for 30, 25, 20, 15, 10, 5 or 0 days (denoted D30, D25, D20, D15, D10, D5 and D0, respectively). All plants were measured at the same time (after having been exposed to a variable number of days of irrigation cessation). The soil water content (SWC) of each pot was measured using an HH2 soil moisture analyser (DELTA-T ML2x, GBR). Fresh leaves were collected from every treatment to determine the antioxidant defence status and solute accumulation indicators. All pots were then immediately watered, and fresh leaves were collected to determine the same indicators on the 5th and 10th days after watering.

Growth characteristics and leaf relative water content (LRWC)

Sapling height and diameter were measured using a tape measurer and an electronic Vernier calliper, respectively. The fresh weights (FW) of the leaves under drought stress and following re-watering were determined, and the leaves were then soaked in distilled water for several hours until they reached a constant weight. Absorbent paper was used to remove water from the surface of the leaves, and their saturation fresh weight (TW) was measured. These leaves were first dried at 105 °C for 15 min and then at 80 °C to a constant weight, which was recorded as the dry weight (DW). The following equation was used to calculate LRWC:

$$\mathrm{LRWC}\ (\%) = (\mathrm{FW} - \mathrm{DW})/(\mathrm{TW} - \mathrm{DW}) \times 100.$$

Determination of reactive oxygen species and lipid peroxidation

Fully expanded nanmu leaves were randomly selected, and the O_2^-, H_2O_2 and malondialdehyde (MDA) contents were determined. Superoxide radicals (O_2^-) were measured using the method described in Chen et al. (2011). Samples of nanmu leaf (0.5 g) were allowed to react with 1 ml of hydroxylamine hydrochloride for 1 h; then, 1 ml of *p*-aminobenzene sulphonic acid and 1 ml of α-naphthylamine were added, and the solution was maintained at 25 °C for 20 min. The absorbance of the mixture was measured at 530 nm, and $NaNO_2$ was used to prepare a standard curve. The H_2O_2 content was determined as described by Brennan and Frekel (1977). Briefly, nanmu leaf samples (0.5 g) were centrifuged at 10,000*g* for 10 min after being homogenised in 5 ml of ice-cold acetone. The collected supernatant (1 ml) was subsequently added to a concentrated HCl solution containing 0.1 ml of 20 % $TiCl_4$ and 0.2 ml of concentrated ammonia, and the mixture was re-centrifuged at 8000*g* for 15 min. The obtained pellets were then added to 3 ml of 1 mol/L H_2SO_4, and the absorbance was observed at 410 nm.

Oxidative damage to leaf lipids was expressed as equivalents of MDA content following the description in Hodges et al. (1999), with some modifications. Fresh leaves (0.3 g) were homogenised in 10 ml of 10 % trichloroacetic acid (TCA) and centrifuged at 12,000*g* for 10 min. A 2-ml aliquot of 0.6 % thiobarbituric acid (TBA) in 10 % TCA was added to 2 ml of the supernatant. Test tubes filled with the mixture were heated in boiling water for 20 min and rapidly cooled in an ice bath. The mixture was then centrifuged at 12,000*g* for 10 min. The absorbance of the supernatant was measured at 532, 600 and 450 nm. The MDA concentration was calculated by

subtracting the OD_{600} value from the sum of the OD_{532} and OD_{450} values.

Antioxidant enzyme activity assay

The same leaf samples that were used to measure the reactive oxygen species content and lipid peroxidation were used to determine the activity of the antioxidant enzymes. SOD (EC 1.15.1.1) was measured according to the method described by Li et al. (2013a): fresh leaves (0.1 g) were ground in a mortar containing 5 ml of extraction buffer (50 mM Tris–HCl (pH 7.0), 0.1 mM EDTA, 1 mM AsA, 1 mM dithiothreitol and 5 mM $MgCl_2$). The homogenates were centrifuged at 10,000*g* for 15 min at 4 °C, and the supernatants were assayed for antioxidant enzyme activity. Detailed protocols are described below. Enzyme activity was expressed as the specific enzyme activity (U) g^{-1} DW.

SOD activity was determined by measuring the inhibition of the photochemical reduction of nitroblue tetrazolium (NBT). The reaction mixture (3 ml) contained 50 mM Tris–HCl (pH 7.8), 13.37 mM methionine, 0.1 mM NBT, 0.1 mM riboflavin, 0.1 mM EDTA and 0.1 ml of enzyme extract. One unit of enzyme activity was defined as the amount of enzyme resulting in 50 % inhibition of the photochemical reduction of NBT.

CAT (EC 1.11.1.6) activity was determined by measuring the decrease in the absorbance of H_2O_2 at 240 nm. The reaction mixture (3 ml) consisted of 50 mM Tris–HCl (pH 7.0), 0.1 mM EDTA, 12.5 mM H_2O_2 and 0.1 ml of enzyme extract. CAT activity was calculated based on an extinction coefficient of 0.04 mM^{-1} at 240 nm. One unit of enzyme activity was defined as a variation of 0.01 in the absorbance at 240 nm per min.

POD (EC 1.11.1.7) activity was estimated by measuring the increase in absorbance at 470 nm due to guaiacol oxidation. The reaction mixture contained 50 mM Tris–HCl (pH 7.0), 10 mM guaiacol, 5 mM H_2O_2 and 0.1 ml of enzyme extract. One unit of enzyme activity was defined as a variation of 0.1 in the absorbance at 470 nm per min.

Water-soluble antioxidant content determination

In addition to the antioxidant enzyme activities, the ascorbic acid (AsA) content was measured; fresh nanmu leaves were extracted and AsA content was measured as described by Foyer et al. (1983). Briefly, fresh leaves (0.3 g) were ground in a mortar containing 3 ml of 5 % (v/v) sulphosalicylic acid. The homogenates were centrifuged at 10,000*g* for 15 min at 4 °C, and the resulting supernatants were assayed for AsA content using a method based on the formation of a red chelate between ferrous ions and 2, 2′- dipyridyl, which absorbs at 525 nm; AsA content was expressed as μmol g^{-1} DW.

Determination of soluble sugar (SS), soluble protein (SP) and proline (Pro)

SS were extracted and analysed according to the method of Wang et al. (2006). Leaf samples (0.2 g) were homogenised in 2 ml of 80 % (v/v) alcohol, and the mortar was washed three times with 3 ml of 80 % alcohol. The homogenates were allowed to stand at room temperature for 30 min and were then centrifuged at 4000*g* for 10 min. The resulting supernatants were stored at 4 °C until analysis. The supernatants (0.5 ml) were then mixed with 3 ml of anthrone, and the resulting mixtures were incubated at 100 °C for 10 min, after which the absorbance was measured at 620 m.

SP content was quantified using the method described by Bradford (1976). Random samples of fresh leaves (0.1 g) were homogenised in 0.05 M sodium phosphate buffer (pH 7.8) containing 1 % PVP; the resulting suspensions were centrifuged at 12,000*g* for 10 min, and the supernatants were mixed with 5 ml of Coomassie brilliant blue. The absorbance was then measured at 595 nm; bovine serum albumin was used as the standard.

Pro was specifically quantified according to the method described by Wu et al. (2012), with some modifications. Fresh samples (0.3 g) were transferred to hermetically sealed tubes containing distilled water, which were then placed in a water bath at 100 °C for 1 h. The reaction mixture was subsequently extracted with toluene (5 ml), and the absorbance was recorded at 520 nm, using toluene as a blank. The Pro concentration was determined based on a standard curve.

Statistical analysis

All measurements and determinations were repeated three times, and the results were subjected to one-way analysis of variance (ANOVA), followed by post hoc multiple comparisons. The least significant difference (LSD) test was used to compare the differences between means at $P < 0.05$. The figures were prepared using Sigmaplot 10.0; error bars represent standard errors, and all data in the figures represent the mean ± SE.

Results

The effects of drought stress on the soil water content, leaf relative water content, and levels of reactive oxygen species in nanmu saplings

Figure 1a reveals that the soil water content (SWC) under drought stress was significantly lower ($P < 0.01$) than that of the control (D0). To conveniently analyse the SWC, we

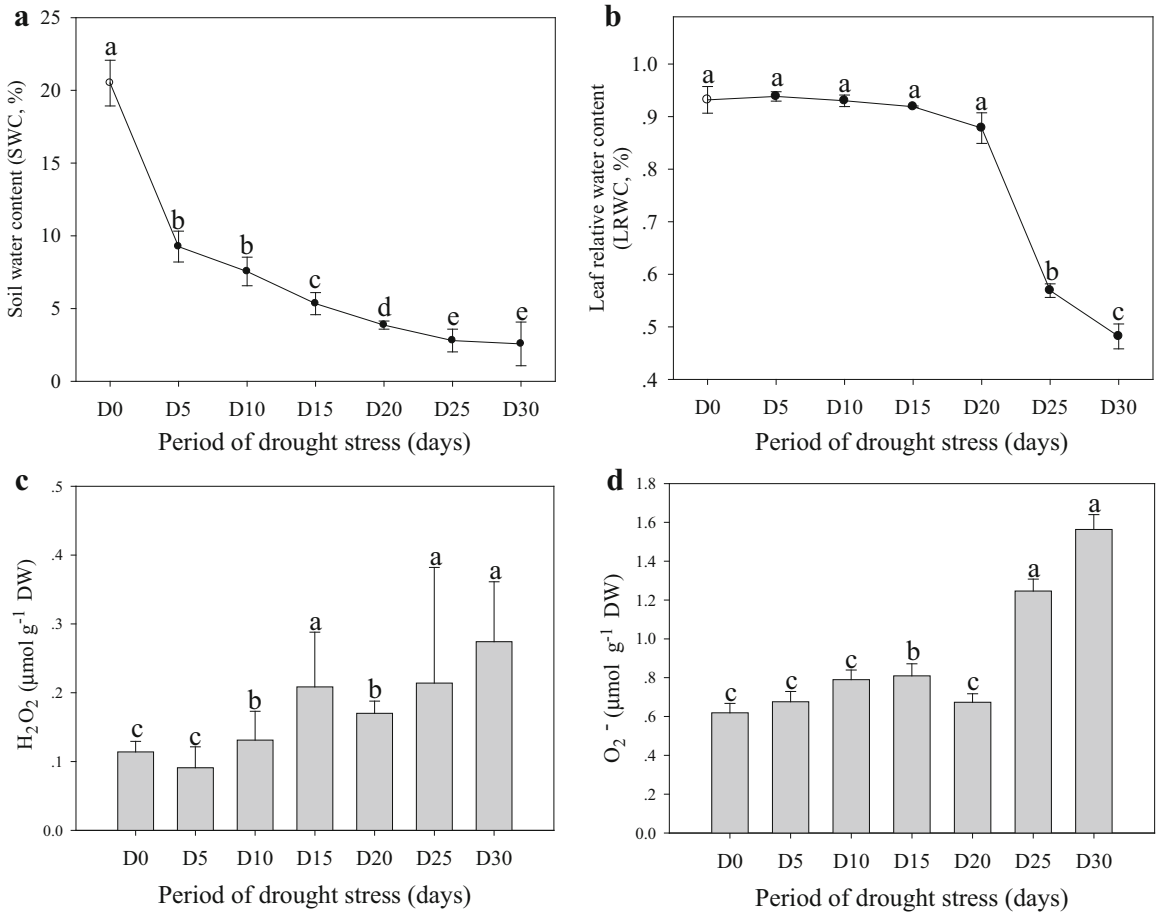

Fig. 1 Soil water content (**a**), leaf relative water content (**b**), H_2O_2 content (**c**) and O_2^- content (**d**) in nanmu saplings subjected to drought stress for 0–30 days. *Error bars* represent standard errors, and all data in the figures represent the mean ± SE of three replicates. *Different letters* indicate significant difference between treatments at 0.05 probability level (one-way ANOVA followed Fisher's least significant different test)

grouped the seven treatments (including the control) into four drought levels according to SWC as follows: control (D0; SWC >20 %), mild drought (D5, D10; SWC 7–10 %), moderate drought (D15, D20; SWC 3–7 %), and severe drought (D25, D30; SWC <3 %). LRWC was significantly inhibited by drought (Fig. 1b). No significant differences were found among the control, mild drought and moderate drought treatments, whereas 36–45 % decreases in LRWC were observed for the severe drought treatment.

As a strong oxidant, together with superoxide radicals (O_2^-), hydrogen peroxide (H_2O_2) exerts toxic effects on enzymes and the membrane. Compared with the control (D0), drought stress increased the levels of both H_2O_2 and O_2^- in the leaves of nanmu saplings (Fig. 1c, d). At the beginning of drought stress, the O_2^- and H_2O_2 levels were significantly different ($P < 0.05$) from those of the control (D0). However, the H_2O_2 content gradually increased from day 10, and the O_2^- content increased significantly from day 15. Accordingly, under severe drought conditions, a 68 % increase in the H_2O_2 content and a 118 % increase in the O_2^- content were observed.

Effects of drought stress and post-drought recovery on antioxidant enzyme activities and antioxidant levels of nanmu saplings

To better understand the possible mechanisms underlying the physiological response of nanmu to drought stress, the activities of the antioxidant enzymes SOD, POD, and CAT and the AsA content in the leaves of nanmu saplings were determined. During drought stress, the activities of SOD, CAT and POD and the AsA content were elevated and this increase depended on the duration of drought stress (Figs. 2, 3). The SOD activity increased from day 0 to day 20 of drought and decreased thereafter (Fig. 2a). Similar trends were observed for POD and CAT activities, which increased at the beginning of the drought, reaching

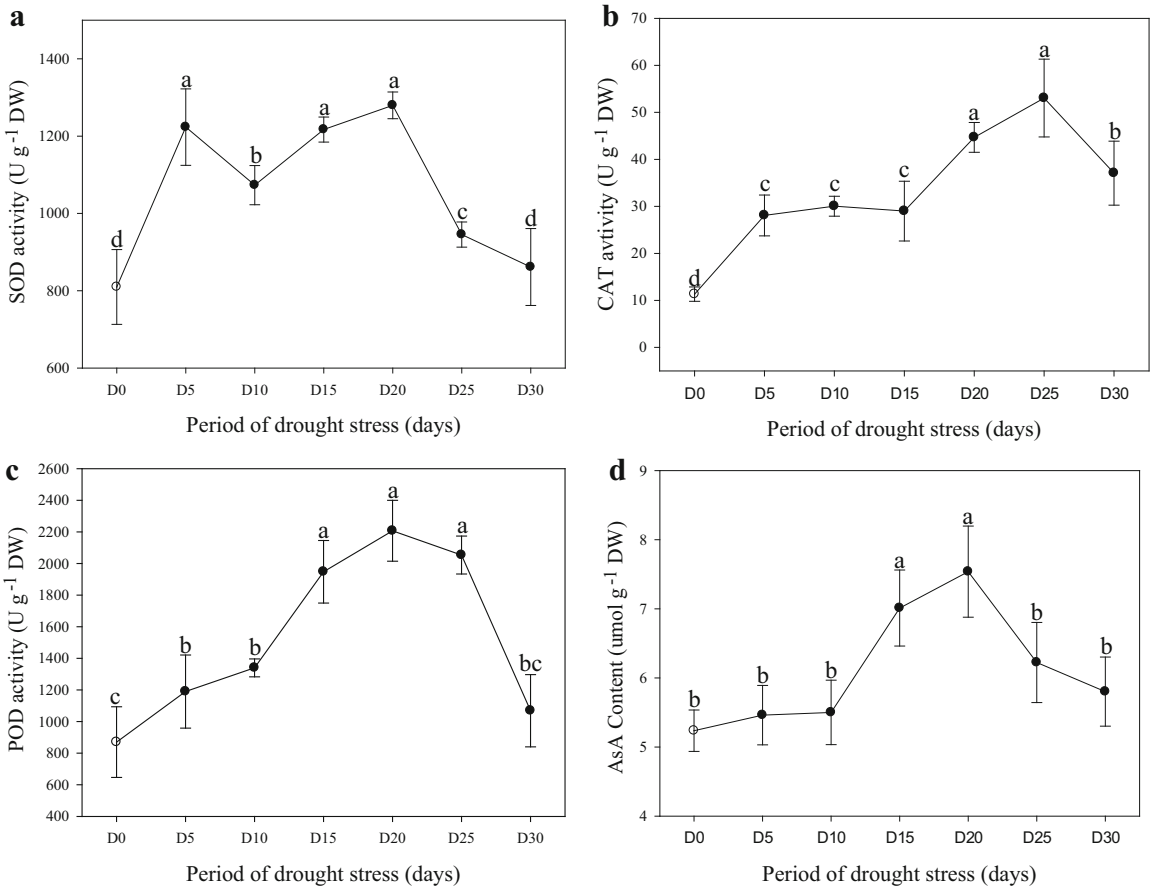

Fig. 2 SOD activity (**a**), CAT activity (**b**), POD activity (**c**), and AsA content (**d**) in leaves of nanmu saplings subjected to drought stress for 0–30 days. *Error bars* represent standard errors, and all data in the figures represent the mean ± SE of three replicates. *Different letters* indicate significant difference between treatments at 0.05 probability level (one-way ANOVA followed Fisher's least significant different test)

maximal levels at day 20 and day 25, respectively, and decreased thereafter (Fig. 2b, c). The AsA content did not differ significantly ($P > 0.05$) under mild drought (D5, D10) conditions but increased dramatically under moderate drought (D15, D20) conditions, gradually increasing further under severe drought (D25, D30) conditions (Fig. 2d). During re-watering, SOD and CAT activities and AsA levels decreased, and no significant difference from the control was apparent after re-watering for 5 days; however, after mild drought (Fig. 3c), the activity of POD remained significantly different ($P > 0.05$) from the control. After 10 days of re-watering, all antioxidant enzyme activities and AsA contents were restored to the control level (D0).

Effects of drought stress and post-drought recovery on lipid peroxidation in nanmu saplings

MDA, a product of membrane peroxidation, is widely used as an indicator of oxidative damage (Li et al. 2013b). Early in the drought treatment (0–10 days), the MDA content was significantly increased compared with the control (Fig. 4a). Under mild and moderate drought conditions, the MDA content remained stable. However, during severe drought, the MDA content was 65–70 % higher than in the control (D0). After re-watering for 5 days, lipid peroxidation decreased but did not recover to the control level. Furthermore, after re-watering for 10 days, lipid peroxidation continued (Fig. 5a). This finding indicates that after severe drought (D30), the plants did not recover after re-watering. Furthermore, these results show that nanmu maintained considerable ROS contents under mild and moderate drought conditions, and a significant increase in ROS caused lipid peroxidation (Figs. 1c, d, 4a).

Effects of drought stress and post-drought recovery on solute accumulation in nanmu saplings

Drought also led to higher soluble sugar (SS), soluble protein (SP) and proline (Pro) contents in nanmu leaves; however, some differences were apparent (Fig. 4b–d).

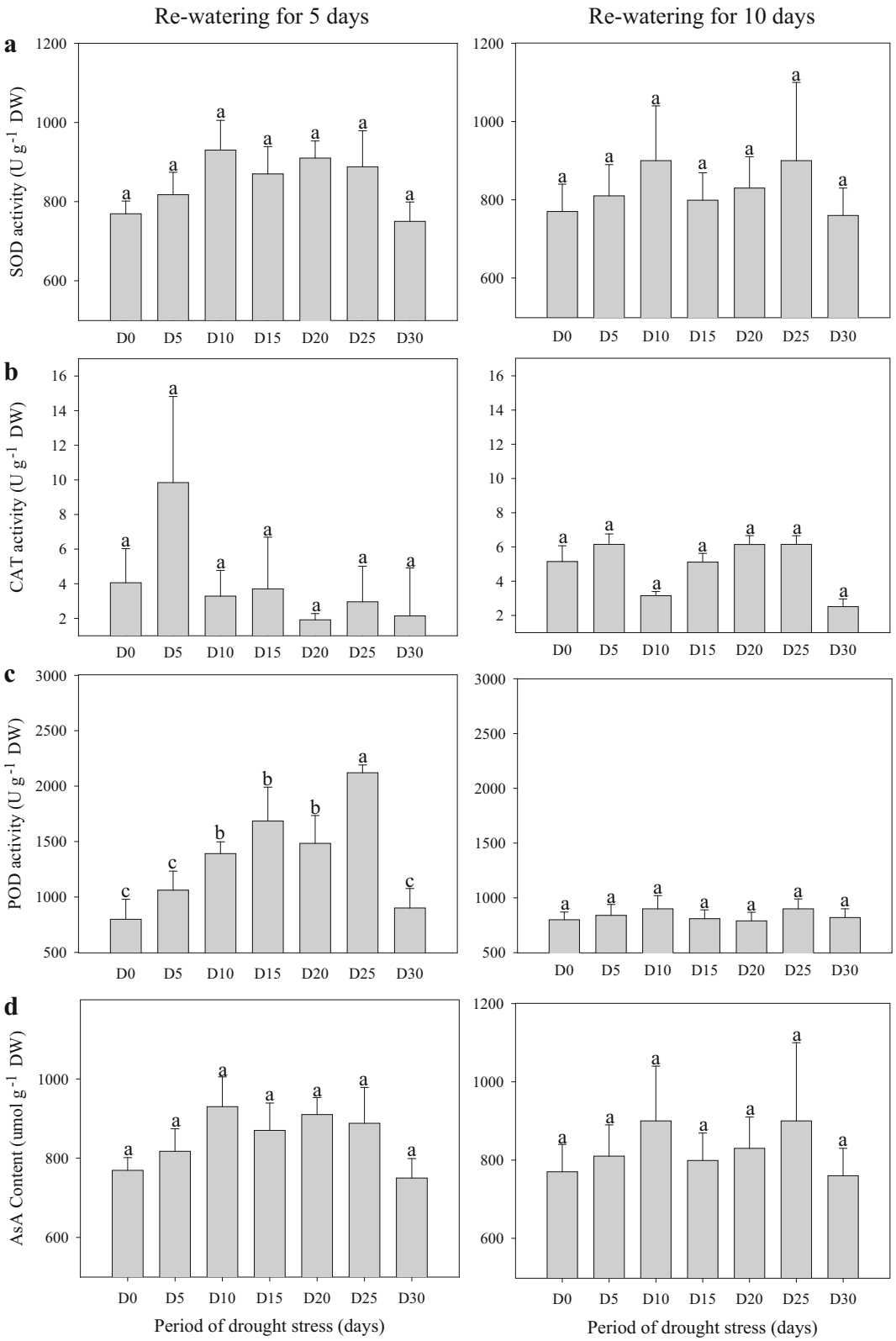

Fig. 3 Effects of post-drought recovery on SOD activity (**a**), CAT activity (**b**), POD activity (**c**), and AsA content (**d**) in the leaves of nanmu saplings. *Error bars* represent standard errors, and all data in the figures represent the mean ± SE of three replicates. *Different letters* indicate significant difference between treatments at 0.05 probability level (one-way ANOVA followed Fisher's least significant different test)

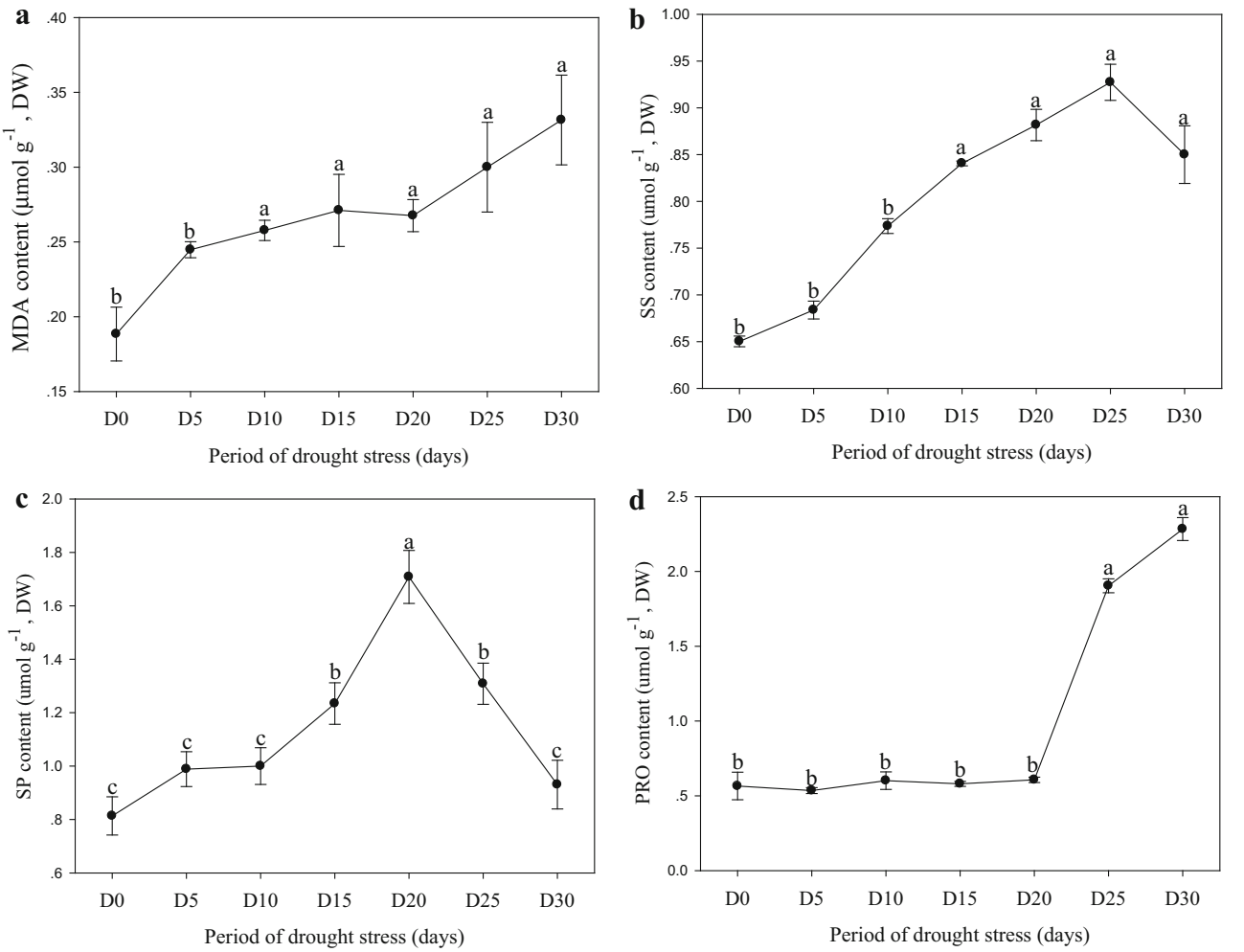

Fig. 4 MDA content (**a**), SS content (**b**), SP content (**c**), and Pro content (**d**) in leaves of nanmu saplings subjected to drought stress for 0–30 days. *Error bars* represent standard errors, and all data in the figures represent the mean ± SE of three replicates. *Different letters* indicate significant difference between treatments at 0.05 probability level (one-way ANOVA followed Fisher's least significant different test)

From the beginning of the drought, the SS content increased continuously; SS content became significantly higher ($P < 0.05$) after 15 days of drought but then sharply decreased after reaching maximum levels at 25 days (Fig. 4b). Similar changes in SP content were observed; SP levels were significantly higher ($P < 0.05$) under moderate drought (D15, D20) conditions and gradually decreased under severe drought (D25, D30) condition (Fig. 4c). After re-watering for 5 days, the nanmu leaves maintained high SS and SP contents (Fig. 5b, c). Importantly, mild and moderate drought treatments led to significant increases ($P < 0.05$) in SP content, indicating on-going post-drought recovery.

During drought stress, no significant differences in Pro content were observed among the control, mild drought and moderate drought treatments; however, the values increased exponentially under severe drought condition (Fig. 4d). These increases were slightly reversed upon re-watering 5 days later, but the Pro contents remained significantly higher than those in the control (Fig. 5d). No obvious correlation ($r = 0.04$, $P = 0.33$) was found between Pro and SP during mild and moderate drought (SWC >3 %, time ≤20 days), whereas a significant negative correlation ($r = -1.07$, $P = 0.7$) between Pro and SP was observed during severe drought (SWC <3 %, time ≥20 days). These results indicated that protein degradation was the main source of Pro accumulation in nanmu leaves during severe drought.

Effects of drought stress and post-drought recovery on the net growth of nanmu saplings

The net growth of nanmu was significantly lower ($P < 0.05$) under conditions of drought stress than in the

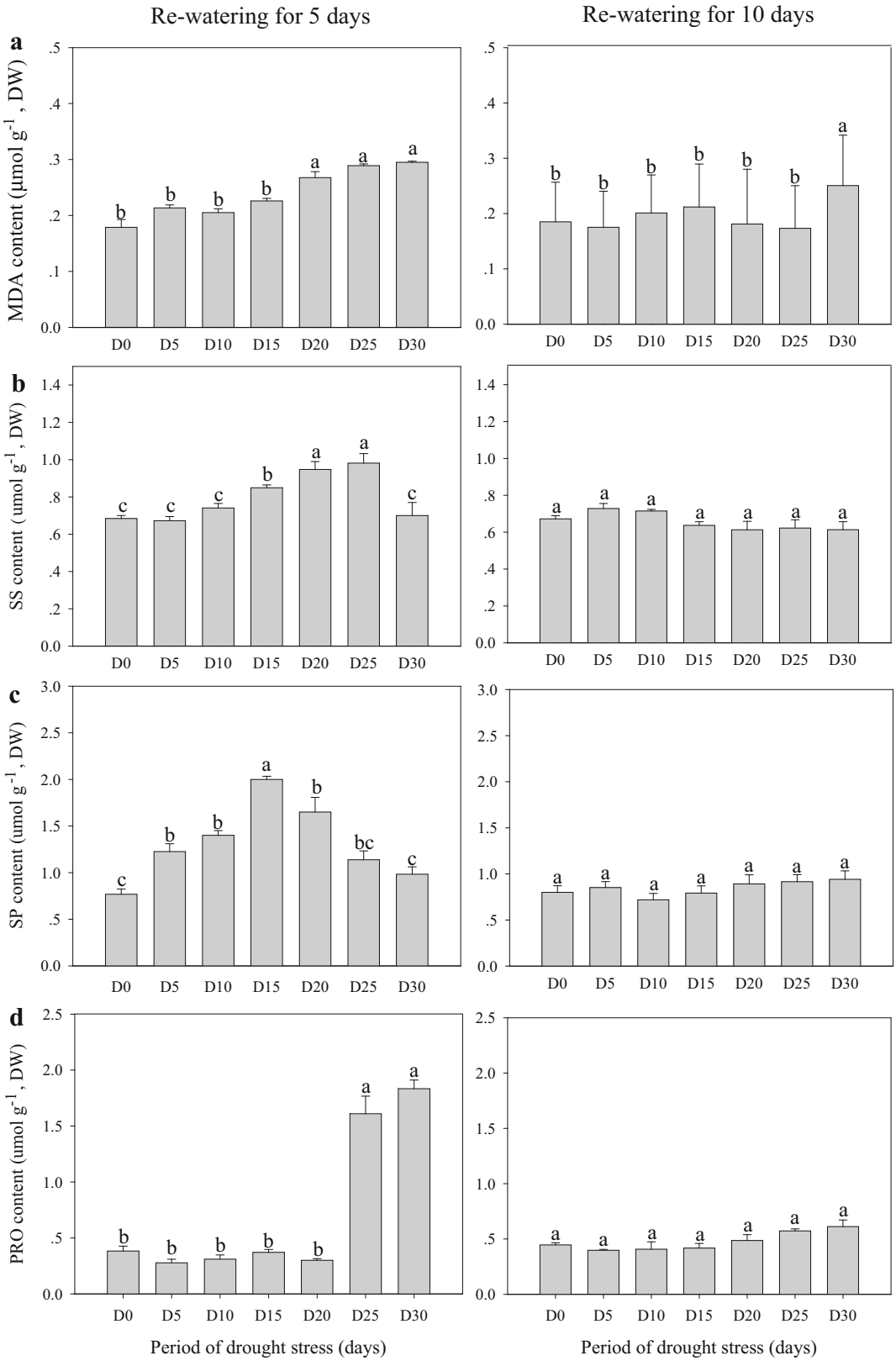

Fig. 5 Effects of post-drought recovery on MDA (**a**), SS (**b**), SP (**c**), and Proline content (**d**) in the leaves of nanmu saplings. *Error bars* represent standard errors, and all data in the figures represent the mean ± SE of three replicates. *Different letters* indicate significant difference between treatments at 0.05 probability level (one-way ANOVA followed Fisher's least significant different test)

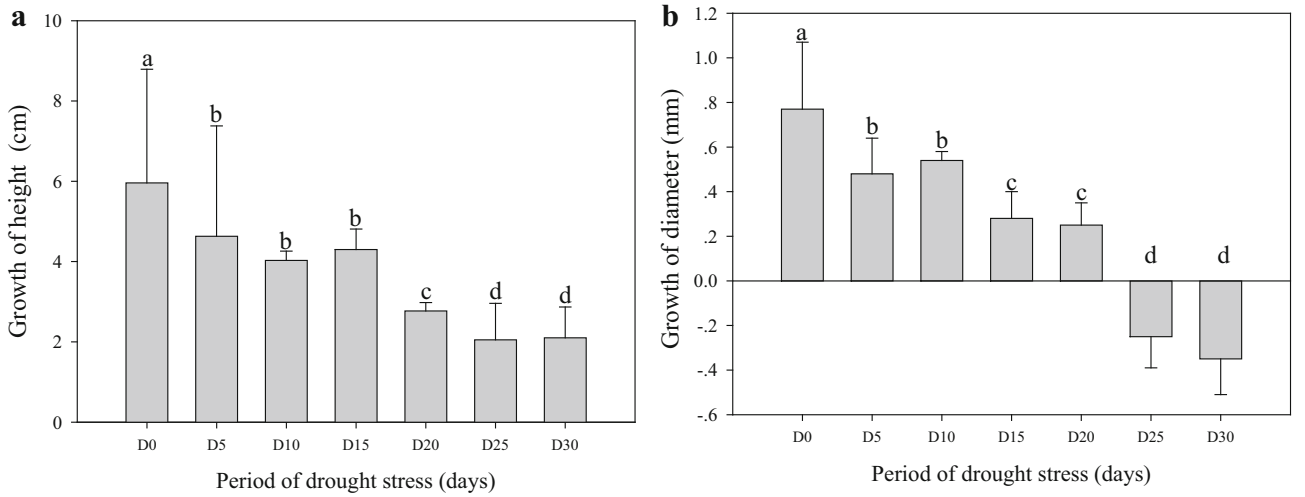

Fig. 6 Net growth of height (**a**) and diameter (**b**) in nanmu saplings subjected to drought stress for 0–30 days. *Error bars* represent standard errors, and all data in the figures represent the mean ± SE of three replicates. *Different letters* indicate significant difference between treatments at 0.05 probability level (one-way ANOVA followed Fisher's least significant different test)

control (D0) (Fig. 6). The net increases in height and diameter were similar under drought stress treatments; the net increases in height were reduced by 27, 40 and 65 % under mild, moderate and severe drought conditions, respectively, whereas the net increases in diameter were reduced by 33, 65 and 138 %, respectively. It is worth noting that under severe drought conditions, the net diameter decreased, indicating that severe drought caused substantial stem desiccation in nanmu plants.

Discussion

Abiotic stresses (drought, high temperature, salt, etc.) heavily disturb the balance between ROS production and the antioxidant defence mechanism, leading to a sudden increase in intracellular ROS levels. When maintained at lower levels, ROS act as components of the stress signalling pathway; however, when present in excess, they are deleterious and can lead to cell death (Reddy et al. 2004). Previous studies have revealed that plants have evolved efficient ROS scavenging mechanisms to control ROS toxicity (Li et al. 2013a; Niu et al. 2013); in other words, when plants are subjected to drought stress, increased levels of ROS facilitate the expression of antioxidant genes and antioxidant enzymes, such as SOD, POD and CAT, and increase the levels of nonenzymatic antioxidants, such as AsA (Moller 2001). SOD acts as the key enzyme in the active oxygen scavenger system because it catalyses the dismutation of superoxide free radicals into H_2O_2 and O_2; POD and CAT further convert H_2O_2 into H_2O and O_2, and the damage caused by ROS is eliminated from plants (Wu et al. 2012). In summary, the concentration of ROS can indicate oxidative stress levels, and the status of the ROS scavenging system reflects the response to drought stress.

In this study, antioxidant enzymes and metabolites were measured to understand whether nanmu avoids toxic ROS formation by increasing antioxidant enzyme activity and antioxidant levels or whether it uses ROS as signalling molecules. Shortly after beginning the water deficit treatment (0–20 days), O_2^- levels were not significantly changed ($P > 0.05$) except in D15 (Fig. 3b); however, H_2O_2 levels were increased significantly ($P < 0.05$), which induced the increases in antioxidant levels and antioxidant enzyme activities (Fig. 2). SOD activity increased during mild and moderate drought (SWC >3 %, time ≤20 days), which might represent an adaptation to alleviate oxidative stress. Subsequently, SOD activity decreased sharply during severe drought (SWC <3 %, time ≥20 days). CAT is important for overcoming oxidative stress caused by drought (Hrishikesh et al. 2008). Our experiments demonstrated that CAT activity increased immediately when nanmu saplings were subjected to water deficit and increased exponentially during the later period of severe drought (SWC <5 %, time >15 days). POD activity increased dramatically as the drought time lengthened, reached a maximum at D20, and then sharply decreased. The AsA-GSH cycle involves four enzymes, APX, DR, MR, and GR, and is an effective detoxification mechanism against oxidative stress (Asada 1992). In this study, nanmu leaf AsA content increased significantly ($P < 0.05$) when the plants were subjected to moderate drought (SWC <6 %, time >10 days) and reached maximum levels at D20. After D20, the levels declined gradually, possibly due to the excessive ROS content. Interestingly, after re-watering for 5 days, the AsA content and most of the

antioxidant enzymes (except POD) were gradually restored, implying that the ability of POD to scavenge singlet oxygen and H_2O_2 was not weakened. After re-watering for 10 days, all antioxidant enzymes and antioxidants were restored to the levels found in saplings under normal watering conditions. Furthermore, elevated H_2O_2 levels led to significant membrane damage (MDA) in nanmu leaves (Fig. 3c), and the MDA content remained steady during that period (10–20 days). Following a longer period of drought (20–30 days), ROS levels increased sharply. Additionally, the MDA content increased further, indicating further oxidative damage to nanmu leaves. After re-watering for 5 days, the MDA contents of the saplings subjected to short-term drought (5–15 days) were restored to the levels observed under normal watering; however, in the plants subjected to long-term drought (20–30 days), the MDA content remained significantly higher ($P < 0.05$) than that of the control. After re-watering for 10 days, the MDA levels in all treatments were restored to those observed in the control, except for D30. These results suggest that ROS act as signalling molecules under mild and moderate drought, inducing the expression of antioxidant enzymes to cope with the oxidative pressure. However, under severe drought, the excessive accumulation of ROS resulted in the down-regulation of antioxidant enzyme activities and caused cellular membrane damage. The lipid peroxidation observed in plants subjected to short-term drought was restored to normal levels 5 days after re-watering, whereas the lipid peroxidation observed in plants subjected to the moderate and severe drought treatments was restored 10 days later; however, after more severe drought (time $\geq$30 days, SWC <3 %), the nanmu saplings did not recover.

Osmotic adjustment affects the plant drought response primarily in two ways: first, the osmotic potential is reduced, thereby increasing water absorption during moderate drought stress conditions; second, osmotic protection stabilises plant membranes and maintains the conformation of proteins at low leaf water potentials (Lambers et al. 2006). Additionally, clear evidence has emerged that the accumulation of substances such as soluble sugars (SS), proline (Pro) and soluble protein (SP) is one of the most important responses of plants to water deficit (Silva et al. 2010; Li et al. 2014; Kang et al. 2005). In this study, drought stress promoted the accumulation of SS, SP, and Pro. The SS and SP contents followed similar trends during the drought period, increasing significantly ($P < 0.05$) during moderate drought, reaching their maximum at D25 and then decreasing under more severe drought conditions (SWC <3 %, time $\geq$30 days). After re-watering for 5 days, both SS and SP contents increased, although some differences were apparent: the SS content increased from 15 to 25 days of drought, and the SP content increased during mild and moderate drought (5–20 days). These results are consistent with previous findings (Reddy et al. 2004), indicating that SS and SP play a key role in drought tolerance and post-drought recovery. Pro has been suggested to stabilise the structures of enzymes and proteins, maintain membrane integrity and scavenge reactive oxygen species (Verslues and Sharma 2010; Verbruggen and Hermans 2008). Pro also acts as a source of energy, carbon and nitrogen during post-drought recovery (Szabados and Savouré 2010). Our data indicated that Pro did not contribute to plant osmotic adjustment during mild and moderate drought (SWC >3 %, time $\leq$20 days). However, it accumulated to a much higher level under severe drought (SWC <3 %, time $\geq$30 days) and acted as an osmoprotectant. After re-watering for 5 days, the Pro content under severe drought was maintained at a higher level, but it declined sharply after re-watering for 10 days, with the regrowth of new buds. These results are consistent with previous studies (Hayat et al. 2012; An et al. 2013), indicating an important role of Pro in plant recovery from severe drought.

Furthermore, our data reinforce the suggestion that plants have two mechanisms to cope with drought stress that act simultaneously (Silva et al. 2010). Under less than severe drought conditions, the LRWC of nanmu leaves remained at levels found in normally watered plants (Fig. 1b). This strategy is associated with rapid growth restriction (Fig. 5) and the impairment of photosynthesis resulting from effective stomatal closure (data not shown). Water management in the leaf tissues prevents water loss by transpiration, and this is also accomplished through an effective osmotic adjustment mechanism.

Conclusion

These results suggest that the function of the active scavenging system in nanmu for reducing ROS accumulation is limited to very severe drought. High activities of SOD, CAT, and POD and high levels of AsA might be important means of preventing membrane damage, decreasing lipid peroxidation and postponing senescence in nanmu plants. The findings also suggest that nanmu uses an effective osmotic adjustment mechanism to respond to a wide range of drought stress; this mechanism might involve the participation of organic solutes (SS, SP). The results obtained here will improve our understanding of the physiological mechanisms used by nanmu during drought stress and post-drought recovery.

Author contribution statement Prof. Tingxing Hu, the corresponding author, designed the research plan, supervised the data collection and analysis, revised the first draft,

reviewed the final one, and made the decision for the submission of manuscript. Mr. Yi Hu and Mr. Bin Wang prepared the experimental material, conducted the experiments and prepared the first draft of the manuscript. Dr. Hong Chen, Dr. Han Li, Dr. Wei Zhang, Dr. Yu Zhong and Dr. Hongling Hu conducted the pre-cultivation and helped to revise the draft of the manuscript.

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References

Ackerson RC (1981) Osmoregulation in cotton in response to water stress II. Leaf carbohydrate status in relation to osmotic adjustment. Plant Physiol 67:489–493

Alves AAC, Setter TL (2004) Response of cassava leaf area expansion to water deficit: cell proliferation, cell expansion and delayed development. Ann Bot 94:605–613

An Y, Zhang M, Liu G et al (2013) Proline accumulation in leaves of *Periploca sepium* via both biosynthesis up-regulation and transport during recovery from severe drought. PLoS One. doi:10.1371/journalpone.0069942

Apel K, Hirt H (2004) Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Ann Rev Plant Biol 55:373–399

Arafat AHAL, He XC (2011) Effect of arbuscular mycorrhizal fungi on growth, mineral nutrition, antioxidant enzymes activity and fruit yield of tomato grown under salinity stress. Sci Hortic 127:228–233

Asada K (1992) Ascorbate peroxidase-A hydrogen peroxide-scavenging enzyme in plants. Physiol Plant 85:235–241

Bohnert HJ, Jensen RG (1996) Strategies for engineering water-stress tolerance in plants. Trends Biotechnol 14:89–97

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Brennan T, Frenkel C (1977) Involvement of hydrogen peroxide in the regulation of senescence in pear. Plant Physiol 59:411–416

Chaves MM, Pereira JS, Maroco J, Rodrigues ML et al (2002) How plants cope with water stress in the field? Photosynthesis and growth. Ann Bot 89:907–916

Chen LH, Han Y, Jiang H, Korpelainen H, Li CY (2011) Nitrogen nutrient status induces sexual differences in responses to cadmium in *Populus yunnanensis*. J Exp Bot 62:5037–5050

David TS, Henriques MO, Kurz-Besson C et al (2007) Water-use strategies in two co-occurring mediterranean evergreen oaks: surviving the summer drought. Tree Physiol 27:793–803

Demirevska K, Zasheva D, Dimitrov R et al (2009) Drought stress effects on Rubisco in wheat: changes in the Rubisco large subunit. Acta Physiol Plant 31:1129–1138

Foyer CH, Noctor G (2011) Ascorbate and glutathione: the heart of the redox hub. Plant Physiol 155:2–18

Foyer C, Rowell J, Walker D (1983) Measurement of the ascorbate content of spinach leaf protoplasts and chloroplasts during illumination. Planta 157:239–244

Gill SS, Tuteja N (2010) Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiol Biochem 48:909–930

Hayat S, Hayat Q, Alyemeni MN et al (2012) Role of proline under changing environments. Plant Signal Behavior 7:1–11

Hodges DM, DeLong JM, Forney CF, Robert K, Prange (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

Hrishikesh U, Sanjib KP, Biman KD (2008) Variation of physiological and antioxidative responses in tea cultivars subjected to elevated water stress followed by rehydration recovery. Acta Physiol Plant 30:457–468

Impa SM, Nadaradjan S, Jagadish SVK (2012) Drought stress induced reactive oxygen species and anti-oxidants. Abiotic stress responses in plants. Springer, New York, pp 131–147

Kang JM, Yang QC, Fan FC (2005) Effects of drought stress on induced protein in the different drought resistance Alfalfa leaf. Acta Agrestia Sinca 13:199–202

Lambers H, Shane MW, Cramer MD, Pearse SJ, Veneklaas EJ (2006) Root structure and functioning for efficient acquisition of phosphorus: matching morphological and physiological traits. Ann Bot 98:693–713

Li ZG, Yuan LX, Wang QL, Ding ZL, Dong CY (2013a) Combined action of antioxidant defence system and osmolytes in chilling shock-induced chilling tolerance in *Jatropha curcas* seedlings. Acta Physiol Plant 35:2127–2136

Li Z, Peng Y, Ma X (2013b) Different response on drought tolerance and post-drought recovery between the small-leafed and the large-leafed white clover (*Trifolium repens* L.) associated with antioxidative enzyme protection and lignin metabolism. Acta Physiol Plant 35:213–222

Li Z, Peng Y, Pan MH, Ma HP, Wang XJ (2014) Effects of spermidine on the accumulation of osmoregulation matter in leaves of white clover under PEG stress. Chin J Grassland 36:31–36

Moller IM (2001) Plant mitochondrial and oxidative stress: electron transport, NADPH turnover, and metabolism of reactive oxygen species. Ann Rev Plant Physiol Plant Mol 52:561–591

Niu Y, Wang Y, Li P, Zhang F, Liu H, Zheng GC (2013) Drought stress induces oxidative stress and the antioxidant defence system in ascorbate-deficient vtc1 mutants of *Arabidopsis thaliana*. Acta Physiol Plant 35:1189–1200

Panozzo JF, Eagles HA (1999) Rate and duration of grain filling and grain nitrogen accumulation of wheat cultivars grown in different environments. Crop Pasture Sci 50:1007–1016

Patakas A, Nikolaou N, Zioziou E, Radoglou K, Noitsakis B (2002) The role of organic solute and ion accumulation in osmotic adjustment in drought-stressed grapevines. Plant Sci 163:361–367

Reddy AR, Chaitanya KV, Vivekanandan M (2004) Drought-induced responses of photosynthesis and antioxidant metabolism in higher plants. J Plant Physiol 161:1189–1202

Silva EN, Ferreira SL, Viegas RA, Silveira JAG (2010) The role of organic and inorganic solutes in the osmotic adjustment of drought-stressed *Jatropha curcas* plants. Environ Exp Bot 69:279–285

Smirnoff N (1993) The role of active oxygen in the response of plants to water deficit and desiccation. New Phytol 125:27–58

Szabados L, Savouré A (2010) Proline: a multifunctional amino acid. Trends Plant Sci 15:89–97

Verbruggen N, Hermans C (2008) Proline accumulation in plants: a review. Amino Acids 35:753–759

Verslues PE, Sharma S (2010) Proline metabolism and its implications for plant-environment interaction. The Arabidopsis Book. doi: 10.1199/tab.0140

Wang S, Wan C, Wang Y et al (2004) The characteristics of Na^+, K^+ and free proline distribution in several drought-resistant plants of the Alxa Desert, China. J Arid Environ 56:525–539

Wang XK, Zhang WH, Hao ZB (2006) The principle and technology of plant physiology and biochemistry. Higher Eduacation Press, Beijing

Wu WM (2009) Study on stand structure and growth law of near-mature *Phoebe bourmei* plantation. China Forestry Sci Technol 23:75–78

Wu H, Wu X, Li Z, Duan LS, Zhang M (2012) Physiological evaluation of drought stress tolerance and recovery in cauliflower (*Brassica oleracea* L.) seedlings treated with methyl jasmonate and coronatine. J Plant Growth Regul 31:113–123

Yancey PH (2001) Water stress, osmolytes and proteins. Am Zool 41:699–709

Zhang XF, Kong HY, Li PF, Li JN, Xiong JL, Wang SM, Xiong CY (2011) Recent advances in research on drought-induced proteins and the related genes in wheat (*Triticum aestivu* L.). Acta Ecologica Sinica 31:2641–2653