



Full Length Article

Effects of drought on Physiological Responses of *Bretschneidera sinensis*

Sha Zhang^{1,2}, Hongfeng Chen^{1*}, Juxiu Liu¹, Dongming Liu¹, Huiling Yan¹ and Faguo Wang¹

¹Key Laboratory of Plant Resources Conservation Sustainable Utilization, South China Botanical Garden, Chinese Academy of Sciences, Guangzhou, Guangdong, 510650

²University of Chinese Academy of Sciences, Beijing, 10049, China

*For correspondence: h.f.chen@scbg.ac.cn

Abstract

Bretschneidera sinensis Hemsl. (*B. sinensis*) is a rare and endangered species in China. Understanding the effects of drought on its physiological responses is required for the introduction and protection of this species with future climate change. In order to know the drought effects on plasma membrane permeability (PMP), malondialdehyde, proline, superoxide dismutase (SOD) and chlorophyll (*chl*) in *B. sinensis*, 100 biennial seedlings of *B. sinensis* were divided into five groups and subjected to different treatments, *i.e.*, a reduction in the amount of water applied on a daily basis. The soluble sugar, soluble starch and nonstructural carbohydrate (NSC) contents were also measured prior to the death of *B. sinensis*. The results indicated that, as the drought continued and intensified, the proline and SOD contents changed slightly, while the PMP and the malondialdehyde content increased constantly, especially under severe drought. The *chl* content also showed a decreasing trend. Consequently, *B. sinensis* became highly sensitive to the drought because the repair mechanisms could not keep pace with the damage. When close to death, the *B. sinensis* seedlings did not use high levels of NSCs. Although, NSCs did not decline, but gradually transferred from leaf to stem and then to roots. Meanwhile, the composition of NSCs changed *i.e.*, soluble starch was degraded to soluble sugar, which indicates an adaptive mechanism in a long-term drought environment. It was concluded that *B. sinensis* is highly sensitive to the drought because of several physiological changes in *B. sinensis*, however, carbon starvation would not occur in *B. sinensis* during drought. © 2019 Friends Science Publishers

Keywords: Endangered species; Drought; Plasma membrane permeability; Malondialdehyde; Superoxide dismutase

Abbreviations: PMP: plasma membrane permeability; SOD: superoxide dismutase; NSC: nonstructural carbohydrate. *chl*: chlorophyll

Introduction

Over the past 100 years, the global average temperature has increased by $0.74 \pm 0.18^\circ\text{C}$ (I.P.C.C., 2014). Even though changes in climate cannot cause drought directly, may increase the frequency, intensity and duration of drought resulting in new challenges for plant survival (Field *et al.*, 2014; Trenberth *et al.*, 2014). The amplification in drought intensity and duration would have a profound impact on plant persistence if plants do not acclimate to these novel conditions (Nicotra *et al.*, 2010). Hence, it is necessary to understand plant response under drought condition.

Drought often causes physiological changes in plants. For example, the plasma membrane can be damaged, increasing permeability and resulting in electrolyte leakage (Sofa *et al.*, 2004; Farooq *et al.*, 2009). Drought can also accelerate reactive oxygen species (ROS) accumulation to such an extent that macromolecular substances in cells undergo oxidation (Reddy *et al.*, 2004; Farooq *et al.*, 2017). As a membrane lipid peroxidation product, malondialdehyde

can inactivate enzyme in the membrane system and further damage the structure and function of the membrane (Sofa *et al.*, 2004). To prevent this type of damage, plants modulate osmotic adjustment substances to maintain the stability of the membrane system and to eliminate active oxygen through the antioxidant defense system (Hernandez *et al.*, 2000; Watanabe *et al.*, 2000; Sofa *et al.*, 2004; Farooq *et al.*, 2018). Proline and soluble sugar, two effective osmotic regulation substances, can maintain cell turgor pressure by reducing the osmotic potential. Their accumulation helps to maintain the structural and functional stability of the membrane system (Hernandez *et al.*, 2000; Watanabe *et al.*, 2000). In addition, the antioxidant defense system includes a protective enzyme system and antioxidants. In the protective enzyme system, superoxide dismutase (SOD) transforms the superoxide anion into H_2O_2 ; then, peroxidase and catalase transform H_2O_2 into H_2O (Mittler, 2002), resulting in detoxification. Antioxidants, *e.g.*, glutathione, proline and ascorbic acid, can effectively inhibit the formation of ROS. However, drought stress can decrease the photosynthetic rate of plants through stomatal closure or chloroplast structural damage

(Hu *et al.*, 2018). Some previous reports have showed that photosynthetic pigment content significantly decreased under drought stress (Zhang *et al.*, 2015; Hu *et al.*, 2018).

During drought, the contents of nonstructural carbohydrate (NSC) in plants may gradually decrease, resulting in “carbon starvation” (McDowell *et al.*, 2008). In recent years, many works have tried to test this hypothesis (Anderegg *et al.*, 2012; Adams *et al.*, 2013; Nardini *et al.*, 2013; Will *et al.*, 2013; Duan *et al.*, 2014), but the results were inconsistent. Changes in the NSC content are complicated under drought conditions. Previous studies that examined NSC changes in plants were confined to limited areas and only a few species. Therefore, more scientific data are needed for reference.

Bretschneidera sinensis Hemsl., an endemic and monotypic species, belongs to Bretschneideraceae. This species is only distributed in areas with abundant rainfall, e.g., south of the Yangtze River, China (Liang *et al.*, 2012). Previous study showed although the annual amount of rainfall in some area of south China did not show any significant changes, the annual number of no rain days has significantly increased in the past five decades (Zhou *et al.*, 2013). *B. sinensis* was listed in the China National Key Protected Wild Plants (First Group) and was documented as a rare species in the China Plant Red Data Book (Fu, 1992). This species has no root hair but vessels with small diameters and a thin cuticular membrane, which makes it difficult to absorb, transport and retain water (Lin *et al.*, 2011). In the context of global climate change, endangered *B. sinensis* is likely to face a survival crisis. Previous studies on *B. sinensis* mostly focused on phylogenetic systematics (Doweld, 1996), morphological anatomy (Qiao *et al.*, 2010), pollination biology (Qiao *et al.*, 2012), population genetics (Xu *et al.*, 2013) and propagation techniques (Qiao *et al.*, 2009). Few studies have been conducted on ecological adaptation, although it is essential for plant development under stress. It was hypothesized that drought would cause physiological changes (such as the changes of PMP, SOC and NSC etc.) in *B. sinensis*; physiological responses of *B. sinensis* may be highly sensitive to the drought treatment and carbon starvation may occur in *B. sinensis* during drought. Research on the changes in *B. sinensis* metabolism during drought will provide a theoretical basis for effective protection in the future.

Materials and Methods

Experimental Setup

The experiment started in July 2015 and was conducted in a plastic greenhouse in South China Botanical Garden, Guangdong province. One hundred biennial seedlings, with a similar age and height, were used in this experiment. Accordingly, one hundred 200-mm diameter planting bags were numbered, and one plant was placed in each bag. The planting bags were filled with mixed soil. The average

organic carbon of the soil was 2.98%. The average total nitrogen of the soil was 1.50 g/kg. The available phosphorus was 29.185 mg/kg with total K, Ca and Mg of 0.23 mg/g, 1.24 mg/g and 0.16 mg/g, respectively. All cultivated soil was wetted to field capacity before the drought stress imposed.

The plants were fully watered in the first week and the drought stress treatments started from the second week. Previous drought stress experiments typically used fixed water quantities along different gradients, but this trial gradually reduced the amount of water applied, similar to the method used by Mitchell *et al.* (2013). This was done to truly simulate the natural state, allowing for the continuous observation of plant physiological indexes. One hundred potted plants were randomly divided into five groups, *i.e.*, 20 pots per group. One of the five groups was the control treatment, group A. This group was maintained at field capacity; the amount of water applied accounted for 100% of the water lost *via* evapotranspiration during the previous 24 h. The four other groups were subjected to a gradual reduction in the amount of water applied on a daily basis, but the reduction varied among the different groups, *i.e.*, 80%, 60%, 40% and 20% of water lost the previous day was applied to group B, C, D and E, respectively; all other conditions were the same. All plants were watered using artificial and manual methods. Three plants per treatment were selected as representative samples for calculating the daily watering regime; thus, the amount of water added to a particular treatment was based on the average of the three plants. As plants lose water mainly through evapotranspiration (including water lost from the soil), the watering amount was estimated using a weighing method, *i.e.*, the pot mass difference between the previous day (half an hour after watering) and the current day (before watering), weighed using an electronic scale with accuracy of one gram. To minimize environmental effects, the pot positions were randomly exchanged in each group. During the experiment, the plants were weighed between 16:00 h and 18:00 h. In our experiment, the exact amounts of added water in each treatment were shown in Fig. 1. Once water supply to the plants < 10 mL for three consecutive days, no additional water was added, and the pots allowed to dry until symptoms of plant death noted. In present study, plants were considered to be dead when all leaf material turned brown and the stems were brown. To confirm this, the plants were re-watered and observed whether recovered after one month (Mitchell *et al.*, 2013). When the plant mortality rate exceeded 90% (18 pots) in a group, it was considered that all plants in the group dead. We did not add any nutrient in each pot during the whole experiment in our study.

Sample Collection and Measurement

Since July 26, 2015, fresh current-year leaves were selected randomly, collected mature leaves from the upper part of the canopy every day between 7 and 8 a.m. The fresh leaves were

immediately wrapped in a wet cloth, placed in hermetic bags and transported to the laboratory as soon as possible. The leaves were initially cleaned using tap water, followed by washing with distilled water 1~2 times and then gently dried using clean gauze. Plasma membrane permeability (PMP), malondialdehyde, proline, SOD and *chlorophyll* were determined every week to observe metabolic changes under continuous drought. Determination of the former three followed Li (2000); the latter two were tested as suggested by Chen and Wang (2006).

When plants in one of the five groups were almost dead, all plants were harvested. Before harvest, bag soils were soaked for one full day, and the high pressure water was used to spray the soil until all soil particles removed. The plants were divided into roots, stems and leaves. After washing for some times, the plants were gently dried using clean gauze. For some times, the plants were gently dried using clean gauze. Whole plants were used for NSC determination. The roots, stems and leaves were dried in an oven at 110°C for 15 min and then left overnight at 70°C. The oven-dried plant samples were ground to a fine powder in a ball mill. Samples (50 mg) were extracted with 4 mL of 80% aqueous ethanol in a polyethylene tube. The mixture was decolorized in a water bath at 80°C for 30 min and then centrifuged at 3000 rpm for 5 min. The supernatant was collected and the pellet was re-extracted with 4 mL of 80% alcohol solution, followed by boiling and centrifugation. The supernatant and lower sediment were reserved for soluble sugar and soluble starch content determination, respectively. The concentrations of total soluble sugar and soluble starch were determined following the revised anthrone method (Ebell, 1969). NSC concentration was calculated as the sum of the soluble starch (mg g^{-1}) and soluble sugar (mg g^{-1}) concentrations.

Statistical Analysis

One-way analyses of variance (ANOVA) and significance analysis were performed to test for treatment effects in cell PMP, malondialdehyde, proline, SOD and chlorophyll using SPSS13.0 ($P < 0.05$). Histograms (mean \pm SE) were also constructed using Excel 2013. Differences between treatments with respect to cell PMP, malondialdehyde, proline, SOD and *chl* under different drought durations were tested using two-way ANOVA considering drought duration and treatment as variables ($P < 0.05$). Tukey's test was used to test for treatment differences in NSC, soluble sugar and soluble starch among plant organs (leaf, stem and root).

Results

Plant Mortality

The plants grew well for the first week. The number of days where less than 10 mL of water was added to groups B, C, D and E was 56, 34, 30 and 15, respectively (Fig. 1). Group A was fully watered and grew well throughout the experiment.

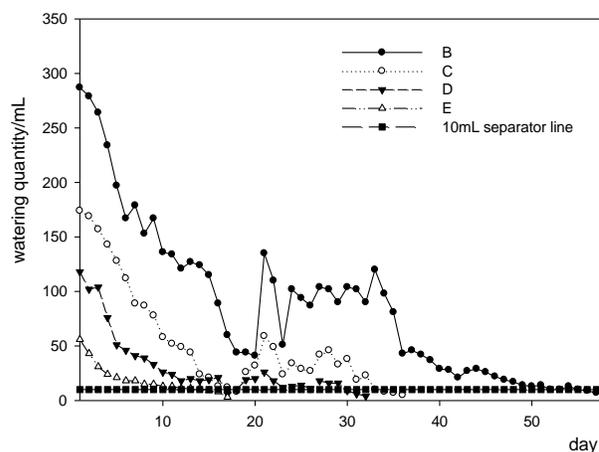


Fig. 1: Amount of water added to the different treatments. B, C, D and E represent the different treatments, *i.e.*, where 80%, 60%, 40% and 20%, respectively, of water lost the previous day was added

At the beginning of the drought stress, the leaves of group E turned yellow and withered. The damage observed in group E became increasingly serious as the drought continued, but the other treatments continued to grow well. A number of leaves in group E started to wither and fall in the third week, indicating a tendency to die. In the fourth week, some leaves of group D turned yellow; at this time, a number of plants in group E almost died and all leaves had fallen off. In the fifth week, a high number of leaves in group D dropped and the plant mortality rate of group E approached 80%. As the amount of water was reduced, the remaining groups also died. After death, the plants were re-watered to each group for one month; however, all plants failed to recover.

Damage and Regulation of Plasma Membrane

The PMP of each group was unstable, increasing in parallel with the duration and severity of the drought (Fig. 2a). The PMP continuously increased and reached a maximum value in the fifth week. Starting from the second week, PMP of groups D and E were significantly higher than the control group, which suggested that severe drought could rapidly damage the plasma membrane of the *B. sinensis* seedlings. In the fourth week, group C started to be significantly higher than the control group. As the drought progressed, the PMP of each group reached the maximum in the fifth week. Compared with the control group, PMP of groups B, C, D and E increased by 2.51%, 5.38%, 12.86% and 23.66%, respectively, but group B was not significantly different from the control group at any time.

For proline, there was no significant difference between the groups which received sufficient water in the first week. From the second week to the third week, proline slightly increased under mild drought (groups B and C) but it exhibited significantly decreased (groups D and E) under severe drought in the fifth week (Fig. 2b).

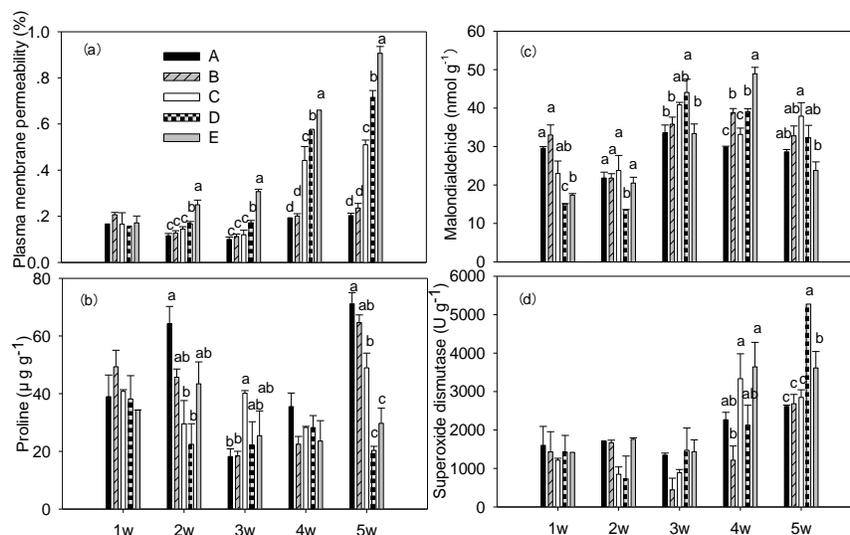


Fig. 2: Changes in plasma membrane permeability, malondialdehyde, proline and superoxide dismutase contents in the different treatments in response to drought (mean \pm SD). Different letters (a, b, c and d) above the error bars indicate significant differences among groups A, B, C, D and E ($P < 0.05$). A, B, C, D and E represent different watering treatments, i.e., a reduction in the amount of water applied on a daily basis, corresponding to 100%, 80%, 60%, 40% and 20% of water lost on the previous day. 1 w to 5 w represent different weeks from the first week to the fifth week

Changes in Malondialdehyde and SOD

Under the continuous drought, the malondialdehyde content of each group generally decreased at first followed by an increase (Fig. 2c). In the first week, the malondialdehyde contents in groups D and E was significantly lower than in the control group. Each group was fully watered in the first week, but significant differences were observed. This indicated that *B. sinensis* is extremely sensitive to drought. Since the active oxygen elimination rate was far lower than the productive rate, active oxygen constantly accumulated. Thus, malondialdehyde was released and increased gradually. Subsequently, the malondialdehyde content of some groups decreased, especially that of group E, which exhibited a rapid reduction and was the lowest among the groups in the fifth week. This was not due to SOD, but occurred because of the severe water deficit. At later stages of drought, the *B. sinensis* seedlings were unable to synthesize malondialdehyde, but the degradation continued and showed a downward trend.

Both drought intensity and duration treatments significantly affected the SOD contents in the *B. sinensis* seedlings ($P < 0.05$). The SOD content did not differ significantly between the groups in the first three weeks (Fig. 2d). However, in the fifth week, the SOD contents in the groups D and E significantly increased when compared to those in the control. Active oxygen accumulated readily in the *B. sinensis* seedlings under drought, but the defensive system initiated late and was only slightly effective. As a result, membrane structure and function were further damaged and due to membrane lipid peroxidation, the zymoprotein in the membrane system was inactivated.

Changes in Chlorophyll Content

The total *chl* content generally exhibited a descending trend, the rate of which increased with drought intensity (Table 1). In the first week with sufficient water, there was no significant difference among *chl*-a, *chl*-b and the total *chl* content of each group. After one week, the *chl* content of all groups except the control decreased rapidly and groups C and E were significantly lower than the control group. Plants started to synthesize *chl* in the third week; thus, the decline in the *chl* content in some groups was slight and some even increased. As the drought continued, the *chl* synthesis rate was far lower than the rate at which the *chl* content decreased. In the fifth week, the *chl* content of all groups except the control decreased obviously.

Changes in Non-structural Carbohydrate Content

Compared with the control group, the NSC content in the roots of the treatment groups basically did not show a reduction and of group B even increased (Fig. 3a). Although the NSC concentrations in the roots of the *B. sinensis* changed slightly, differences of drought effect were observed between the root, stem and leaf (Fig. 3a). For example, the average NSC concentrations of the root, stem and leaf of group E was 138.31, 92.00 and 29.43 $\text{mg}\cdot\text{g}^{-1}$, respectively and significantly different from the control group, i.e., 108.52, 73.41 and 73.65 $\text{mg}\cdot\text{g}^{-1}$. More specifically, NSC concentrations increased in the root when *B. sinensis* seedlings were under drought stress.

Table 1: Changes of *chl-a*, *chl-b* and total contents (mg g^{-1} , mean \pm SD) in leaves of *Bretschneidera sinensis* under different drought treatments

week		A	B	C	D	E
1	<i>chl-a</i>	1.66 \pm 0.23a	1.95 \pm 0.14a	1.64 \pm 0.07a	2.14 \pm 0.10a	2.15 \pm 0.35a
	<i>chl-b</i>	0.63 \pm 0.04a	0.73 \pm 0.10a	0.69 \pm 0.11a	0.81 \pm 0.04a	0.99 \pm 0.28a
	<i>chl-a+b</i>	2.30 \pm 0.27a	2.68 \pm 0.24a	2.33 \pm 0.18a	2.94 \pm 0.14a	3.13 \pm 0.62a
2	<i>chl-a</i>	1.67 \pm 0.05a	1.67 \pm 0.13a	0.72 \pm 0.06b	1.57 \pm 0.01a	0.83 \pm 0.04b
	<i>chl-b</i>	0.70 \pm 0.04a	0.67 \pm 0.06a	0.32 \pm 0.04b	0.71 \pm 0.01a	0.41 \pm 0.02b
	<i>chl-a+b</i>	2.37 \pm 0.04a	2.34 \pm 0.06a	1.03 \pm 0.04b	2.28 \pm 0.01a	1.25 \pm 0.02b
3	<i>chl-a</i>	1.64 \pm 0.04a	1.54 \pm 0.07a	1.15 \pm 0.07ab	1.84 \pm 0.46a	0.69 \pm 0.11b
	<i>chl-b</i>	0.67 \pm 0.02a	0.58 \pm 0.02ab	0.56 \pm 0.11ab	0.36 \pm 0.15ab	0.26 \pm 0.08b
	<i>chl-a+b</i>	2.31 \pm 0.06a	2.12 \pm 0.08a	1.71 \pm 0.15a	2.20 \pm 0.31a	0.95 \pm 0.19b
4	<i>chl-a</i>	1.63 \pm 0.06a	1.62 \pm 0.07a	1.45 \pm 0.07ab	1.32 \pm 0.06b	1.00 \pm 0.10c
	<i>chl-b</i>	0.63 \pm 0.02a	0.60 \pm 0.04a	0.57 \pm 0.04a	0.67 \pm 0.13a	0.45 \pm 0.04a
	<i>chl-a+b</i>	2.26 \pm 0.08a	2.22 \pm 0.10a	2.02 \pm 0.11a	1.99 \pm 0.19a	1.46 \pm 0.14b
5	<i>chl-a</i>	1.78 \pm 0.01a	0.71 \pm 0.06b	0.68 \pm 0.02b	0.05 \pm 0.01c	0.10 \pm 0.05c
	<i>chl-b</i>	0.32 \pm 0.02a	0.28 \pm 0.03a	0.32 \pm 0.06a	0.05 \pm 0.01b	0.14 \pm 0.05b
	<i>chl-a+b</i>	2.09 \pm 0.03a	0.88 \pm 0.09b	1.00 \pm 0.04b	0.11 \pm 0.02c	0.24 \pm 0.05c

Different numbers (1, 2, 3, 4, 5) represent treatment duration, *i.e.*, one week, two weeks, three weeks, four weeks and five weeks. Different letters (a, b, c) indicate significant differences among groups A, B, C, D and E ($P < 0.05$). A, B, C, D, and E represent different watering treatments, *i.e.*, the application of 100%, 80%, 60%, 40% and 20% of water lost during the previous day, respectively

Table 2: Soluble sugar and soluble starch (expressed as a percentage of the NSC content) in different treatments (%)

	A	B	C	D	E
Soluble sugar	16.32%	12.23%	24.77%	38.20%	22.66%
Soluble starch	83.68%	87.77%	75.23%	61.80%	77.34%

A, B, C, D and E represent different watering treatments, *i.e.*, the application of 100%, 80%, 60%, 40% and 20%, respectively, of water lost the previous day

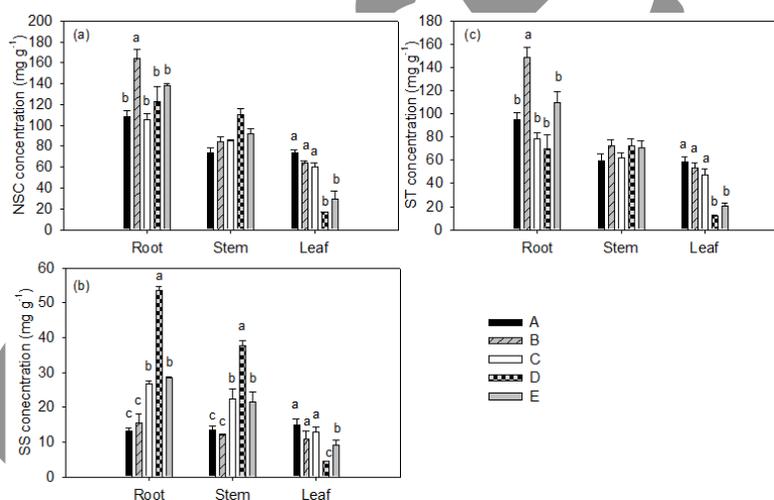


Fig. 3: The concentrations of soluble sugar (SS), soluble starch (ST) and total nonstructural carbohydrate (NSC) in the root, stem and leaf of *B. sinensis* seedlings subjected to different treatments (mg g^{-1} , mean \pm SD). Different letters (a, b and c) above the error bars indicate significant differences among groups A, B, C, D and E ($P < 0.05$). A, B, C, D and E represent different watering treatments, *i.e.*, the application of 100%, 80%, 60%, 40% and 20%, respectively, of water lost the previous day

Similar responses were observed for soluble sugar and soluble starch (Fig. 3b and c). During the drought, the concentrations of soluble sugar and soluble starch were ranked in the order: leaf<stem<root. Compared with the control, NSC concentrations in the root and stem in the treatment groups did not decrease, however, NSC concentrations in the leaf of group D and E significantly decreased ($P < 0.05$). In addition, the contribution of soluble sugar and soluble starch concentrations to the total NSC concentrations changed. The percentages of soluble sugar concentrations in groups C, D and E were higher than in the

control group, whereas the percentages of soluble starch concentrations in groups C, D and E were lower than in the control group (Table 2).

Discussions

Interplay between drought Intensity and Secondary Metabolites

The *B. sinensis* seedlings suffered serious damage from the drought, showing a weak resistance to drought. Substantial

proline accumulation is a common plant response to drought stress (Hernandez *et al.*, 2000; Watanabe *et al.*, 2000). When drought damages the plasma membrane of plants, proline can accumulate as an osmoprotectant but does not damage cellular macromolecules (Sofa *et al.*, 2004). In this study, under early drought stress, the plasma membranes of the seedlings were damaged, but the proline content did not rise rapidly. The repair mechanisms could not keep pace with the damage; consequently, the plasma membrane suffered irreversible damage (Fig. 2). As an osmotic regulator, proline can reduce the osmotic potential in tissues (Nanjo *et al.*, 1999), enabling leaves to withstand strong evaporative demand without a loss of turgor (Holbrook and Putz, 1996). The phenomenon that plants adjust their osmotic potential *via* solute accumulation to resist drought stress has been observed in some studies (Wang *et al.*, 1995). Proline accumulation differs among species, for example, proline accumulation in two Mediterranean shrubs (*Halimium halimifolium* L. and *Pistacia lentiscus* L.) was twofold higher than in an olive tree under increasing water deficit (Ain-Lhout *et al.*, 2001). As *B. sinensis* exhibited a low proline content, we speculated that this species probably lacks the mechanisms to synthesis proline under a water deficit.

Malondialdehyde is one of the final products of lipid peroxidation and reflects the severity of oxidative damage caused to membrane lipids (Ozkur *et al.*, 2009). Our study showed that an increase in ROS accumulation occurred in the early phases of drought, resulting in the production and gradual increase of malondialdehyde. This result is similar to studies concerning other species (Khan and Naqvi, 2010). Under water deficit conditions, the over-accumulation of active oxygen species induced by drought stress could give rise to lipid peroxidation (Reddy *et al.*, 2004), which is the formation process of malondialdehyde. There is evidence that tolerance ability to drought differ in their MDA levels, and sensitive species generally accumulate this compound more than tolerant species (Devi and Giridhar, 2015). In this respect, the significant increase of MDA content under drought was observed in *B. sinensis*, which suggest that this species is sensitive to drought. Plants possess both enzymatic and non-enzymatic defense systems to scavenge active oxygen. The enzymatic defense system mainly includes SOD, which is the first line of cellular defense against ROS by scavenging O_2^- to produce H_2O_2 (Bowler *et al.*, 1992); Peroxidase and catalase transform H_2O_2 into H_2O for detoxification (Mittler, 2002). Secondary antioxidants include glutathione, proline and ascorbic acid. The balance between the accumulation and elimination of active oxygen determines whether oxidative signaling and damage will occur (Møller *et al.*, 2007). In this study, there was no significant change in the SOD content initially and a slight increase occurred later, indicating that antioxidant enzymes may be partly responsible for the protection from drought stress. The rate of ROS scavenging was far less than accumulation. As a result, the malondialdehyde content increased continuously rather than declined.

In general, stomatal limitations are mainly responsible for a decline in photosynthesis under mild to moderate drought conditions, while under severe drought, nonstomatal limitations dominate (Flexas *et al.*, 2006; Liu *et al.*, 2010). This may explain the observed incomplete recovery from severe drought events when damage to chloroplasts is involved (Ennahli and Earl, 2005). Our results suggested that photosynthetic pigments degraded rapidly under severe drought, but the *chl* content did not decline at first under the mild to moderate drought, which agrees with previous studies. However, the *chl* content of the plants in all groups decreased as the drought persisted.

During the long period of natural selection, plants formed a defense system to adapt to adverse conditions and to reduce damage. *B. sinensis* is extremely vulnerable to drought, shown by its weak resistance to drought. It can be inferred that water may be one of the key factors that limits its growth. Guo *et al.* (2010) found that nitrogen supply decreased the malondialdehyde content and enhanced the activities of some antioxidant enzymes, especially peroxidase, which provided a feasible method to protect *B. sinensis* from drought.

Carbohydrate Starvation did not Occur in *B. sinensis*

Under drought, mild or severe, the NSC concentrations in the roots and stems did not decrease in our study. Anderegg *et al.* (2012) studied aspen (*Populus tremuloides*) and also indicated that drought did not lead to the decline in the tree NSC concentrations, and they believed that hydraulic failure was the determining factor that led to aspen death. Duan *et al.* (2014) reached the same conclusion by studying *Eucalyptus globulus* and *E. radiata* in Australia. However, some researchers argued that when trees die, the NSC concentrations decreased (Hartmann *et al.*, 2013; Sevanto *et al.*, 2013). For example, Mitchell *et al.* (2013) reported that the NSC concentrations of whole-tree North American Monterey pine (*Pinus radiata*) decreased by 50% when the plants died in a greenhouse study. In our experiments, compared with the control, NSC concentrations in the root and stem in the treatment groups did not decrease, however, NSC concentrations in the leaf of group D and E also significantly decreased ($P < 0.05$).

Higher concentrations of NSC were found in roots than other tissues under drought. The NSC concentrations of whole plants were gradually transferred to different organs during drought. NSC has a positive effect on drought resistance (O'Brien *et al.*, 2014). We hypothesized that embolisms first appeared between the leaves and stems, and then between the stems and roots. This prevented water transport to the stems and then to the leaves, resulting in leaf, stem and root death due to "moisture failure" in our study. To conserve NSC to resist drought, plants had to constantly shift carbohydrates from the leaves to the stems and then to the roots. The order of transfer from the aboveground parts to the root had a buffering effect on the drought. Death of the

aboveground part of *B. sinensis* reduced the consumption of water and nutrients, whereas roots are the important organs that absorb water and nutrients (Gargallo-Garriga *et al.*, 2014). Carbohydrate allocation to roots also appears to be an important adaptation for regeneration when plants face adverse conditions (Arndt *et al.*, 2001). As reported for palm seedlings, belowground NSC reserves allow for recovery from defoliation (McPherson and Williams, 1998). Due to NSC allocation, roots were unable to utilize carbohydrates due to low water potential for an extended period of time, which resulted in death through eventual loss of water.

B. sinensis, an indigenous plant in China, can provide more scientific data to reveal the mechanism responsible for plant death under extreme drought. Complex changes in NSCs were observed in response to extreme drought. Recently, Gaylord *et al.* (2015) compared 49 mature piñon pine (*Pinus edulis* Engelm.) trees that survived a severe drought and 49 trees that died during the drought across three sites in Arizona and New Mexico. These authors found that hydraulic failure, carbon starvation and biotic agents had interactive effect on plant death (Gaylord *et al.*, 2015). The mechanism underlying the death of *B. sinensis* under drought remains unclear. Future research is needed to understand the interaction between hydration failure and carbohydrate starvation. Besides the NSC content, physiological responses may differ among different tissues under drought.

Conclusion

B. sinensis is highly sensitive to moisture variation because the repair mechanisms could not keep pace with the damage. When close to death, the *B. sinensis* seedlings did not use excessive amounts of NSC. Although the NSC concentrations did not decline, NSC were gradually transferred in the order "leaf-stem-root," and the composition changed due to the degradation of soluble starch to soluble sugar, which is an adaptation mechanism in a long-term drought environment.

Acknowledgements

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References

Adams, H.D., M.J. Germino, D.D. Breshears, G.A. Barron-Gafford, M. Guardiola-Claramonte, C.B. Zou and T.E. Huxman, 2013. Nonstructural leaf carbohydrate dynamics of *Pinus edulis* during drought-induced tree mortality reveal role for carbon metabolism in mortality mechanism. *New Phytol.*, 197: 1142–1151

Ain-Lhout, F., F.A. Zunzunegui, M.C.D. Barradas, R. Tirado, A. Clavijio and F. Novo, 2001. Comparison of proline accumulation in two Mediterranean shrubs subjected to natural and experimental water deficit. *Plant Soil*, 230: 175–183

Anderegg, W.R., J.A. Berry, D.D. Smith, J.S. Sperry, L.D. Anderegg and C.B. Field, 2012. The roles of hydraulic and carbon stress in a wide-spread climate-induced forest die-off. *Proc. Natl. Acad. Sci. U.S.A.*, 109: 233–237

Arndt, S.K., S.C. Clifford, W. Wanek, H.G. Jones and M. Popp, 2001. Physiological and morphological adaptations of the fruit tree *Ziziphus roundifolia* in response to progressive drought stress. *Tree Physiol.*, 21: 705–715

Bowler, C., M. Montagu and D. Inze, 1992. Superoxide dismutase and stress tolerance. *Annu. Rev. Plant Biol.*, 43: 83–116

Chen, J.X. and X.F. Wang, 2006. *Plant Physiology Experiment Instruction*. South China University of Technology Press, Guangzhou (In Chinese)

Devi, M.K.A. and P. Giridhar, 2015. Variations in physiological response, lipid peroxidation, antioxidant enzyme activities, proline and isoflavones content in soybean varieties subjected to drought stress. *Proc. Natl. Acad. Sci. Ind. Sect. B: Biol. Sci.*, 85: 35–44

Doweld, A.B., 1996. The carpology and taxonomic relationships of "*Bretschneidera* (Bretschneideraceae)". *Acta Bot. Malacit.*, 21: 79–90

Duan, H., R.A. Duursma, G. Huang, R.A. Smith, B. Choat, A.P. O'Grady and D.T. Tissue, 2014. Elevated [CO₂] did not ameliorate the negative effects of elevated temperature on drought-induced mortality in *Eucalyptus radiata* seedlings. *Plant Cell Environ.*, 37: 1598–1613

Ebell, L.F., 1969. Variation in total soluble sugars of conifer tissues with method of analysis. *Phytochemistry*, 8: 227–233

Ennahli, S. and H.J. Earl, 2005. Physiological limitations to photosynthetic carbon assimilation in cotton under water stress. *Crop Sci.*, 45: 2374–2382

Farooq, M., F. Nadeem, M.Y. Arafat, M. Nabeel, S. Musadaq, S.A. Cheema and A. Nawaz, 2018. Exogenous application of allelopathic water extracts helps improving tolerance against terminal heat and drought stresses in bread wheat (*Triticum aestivum* L. Em. Thell.). *J. Agron. Crop Sci.*, 204:298-312

Farooq, M., N. Gogoi, S. Barthakur, B. Baroowa, N. Bharadwaj, S. S. Alghamdi and K.H. M. Siddique, 2017. Drought stress in grain legumes during reproduction and grain filling. *J. Agron. Crop Sci* 203:81–102

Farooq, M., A. Wahid, N. Kobayashi, D. Fujita and S.M.A. Basra, 2009. Plant drought stress: effects, mechanisms and management. *Agron. Sustain. Dev.*, 29: 185–212.

Field, C.B., V.R. Barros, D.J. Dokken, K.J. Mach, M.D. Mastrandrea, T.E. Bilir, M. Chatterjee, K.L. Ebi, Y.O. Estrada, R.C. Genova, B. Girma, E.S. Kissel, A.N. Levy, S. MacCracken, P.R. Mastrandrea and L.L. White, 2014. Climate Change 2014: Impacts, Adaptation and Vulnerability. In: *Working Group II Contribution to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change*, pp: 247–250. Cambridge University Press, University of Cambridge

Flexas, J., J. Bota, J. Galmes, H. Medrano and O.M. Ribas-Carbo, 2006. Keeping a positive carbon balance under adverse conditions: responses of photosynthesis and respiration to water stress. *Physiol. Plant*, 127: 343–352

Fu, L.G., 1992. *China Plant Red Data Book*, pp: 194–195. Science Press, Beijing (In Chinese)

Gargallo-Garriga, A., J. Sardans, M. Pérez-Trujillo, A. Rivas-Ubach, M. Oravec, K. Vecerova, O. Urban, A. Jentsch, J. Kreyling, C. Beierkuhnlein, T. Parella and J. Peñuelas, 2014. Opposite metabolic responses of shoots and roots to drought. *Sci. Rep.*, 4: 1–7

Gaylord, M.L., T.E. Kolb and N.G. McDowell, 2015. Mechanisms of piñon pine mortality after severe drought: a retrospective study of mature trees. *Tree Physiol.*, 35: 806–816

Guo, J.Y., Y. Yang, G.X. Wang, L.D. Yang and X.Y. Sun, 2010. Ecophysiological responses of *Abies fabri* seedlings to drought stress and nitrogen supply. *Physiol. Plant.*, 139: 335–347

Hu, W., S.B. Tian, Q. Di, S.H. Duan and K. Dai, 2018. Effects of exogenous calcium on mesophyll cell ultrastructure, gas exchange, and photosystem II in tobacco (*Nicotiana tabacum* Linn.) under drought stress. *Photosynthetica*, 56: 1204–1211

Hartmann, H., W. Ziegler, O. Kolle and S. Trumbore, 2013. Thirst beats hunger—declining hydration during drought prevents carbon starvation

- in Norway spruce saplings. *New Phytol.*, 200: 340–349
- Hernandez, S., C. Deleu and F. Larher, 2000. Accumulation de proline dans les tissus foliaires de tomate en réponse à la salinité. *Compt. Rend. Acad. Sci. -Ser. III-Sci. Vie*, 323: 551–557 (In French)
- Holbrook, N.M. and F.E. Putz, 1996. From epiphyte to tree: differences in leaf structure and leaf water relation associated with the transition in growth form in eight species of hemiepiphytes. *Plant Cell Environ.*, 19: 631–642
- I.P.C.C., 2014. *Synthesis Report*. Contribution of three working groups to the fifth assessment report of the Intergovernmental Panel on Climate Change
- Khan, N. and F.N. Naqvi, 2010. Effect of water stress on lipid peroxidation and antioxidant enzymes in local bread wheat hexaploids. *J. Food Agric. Environ.*, 88: 521–526
- Li, H.S., 2000. *Principles and Techniques of Plant Physiological Biochemical Experiment*. Higher Education Press, Beijing
- Liang, Y., G.B. Xu, H.P. Zhang, X.Q. Wu, X.B. Shen and A.Y. Wang, 2012. Genetic Diversity of Natural and Planted Populations of *B. sinensis* from Nanling Region. *Sci. Silv. Sin.*, 48: 45–52
- Lin, P., Y.M. Yun, H.H. Huang, Z.K. Tong, W.J. Luo and C.M. Chen, 2011. Study on morphological properties of *B. sinensis* Wood Fiber and Its Radial Variation. *J. Zhej. Sci. Technol.*, 31: 49–54
- Liu, C.C., Y.G. Liu, K. Guo, Y.R. Zheng, G.Q. Li, L.F. Yu and R. Yang, 2010. Influence of drought intensity on the response of six woody karst species subjected to successive cycles of drought and rewatering. *Physiol. Plant.*, 139: 39–54
- McDowell, N., W.T. Pockman, C.D. Allen, D.D. Breshears, N. Cobb, T. Kolb, J. Plaut, J. Sperry, A. West and D.G. Williams, 2008. Mechanisms of plant survival and mortality during drought: Why do some plants survive while others succumb to drought? *New Phytol.*, 178: 719–739
- McPherson, K. and K. Williams, 1998. The role of carbohydrate reserves in the growth, resilience, and persistence of cabbage palm seedlings (*Sabal palmetto*). *Oecologia*, 117: 460–468
- Mitchell, P.J., A.P. O'Grady, D.T. Tissue, D.A. White, M.L.O. Chlaeger and E.A. Pinkard, 2013. Drought response strategies define the relative contributions of hydraulic dysfunction and carbohydrate depletion during tree mortality. *New Phytol.*, 197: 862–872
- Mittler, R., 2002. Oxidative stress, antioxidants and stress tolerance. *Trends Plant Sci.*, 7: 405–410
- Møller, I.M., P.E. Jensen and A. Hansson, 2007. Oxidative modifications to cellular components in plants. *Annu. Rev. Plant Biol.*, 58: 459–481
- Nanjo, T., K. Kobayashi, Y. Yoshida, Y. Sanada, K. Wada, H. Tsukaya, Y. Kakubari, K. Yamaguchi-Shinozaki and K. Shinozaki, 1999. Biological functions of proline in morphogenesis and osmo tolerance revealed in antisense transgenic *Arabidopsis thaliana*. *Plant J.*, 18: 185–193
- Nardini, A., M. Battistuzzo and T. Savi, 2013. Shoot desiccation and hydraulic failure in temperate woody angiosperms during an extreme summer drought. *New Phytol.*, 200: 322–329
- Nicotra, A.B., O.K. Atkin, S.P. Bonser, A.M. Davidson, E.J. Finnegan, U. Mathesius, P. Poot, M.D. Purugganan, C.L. Richards, F. Valladares and M.V. Kleunen, 2010. Plant phenotypic plasticity in a changing climate. *Trends Plant Sci.*, 15: 684–692
- O'Brien, M.J., S. Leuzinger, C.D. Philipson, J. Tay and A. Hector, 2014. Drought survival of tropical tree seedlings enhanced by non-structural carbohydrate levels. *Nat. Clim. Change*, 4: 710–714
- Ozkur, O., F. Ozdemir, M. Bor and I. Turkan, 2009. Physicochemical and antioxidant responses of the perennial xerophyte *Capparis ovata* Desf. to drought. *Environ. Exp. Bot.*, 66: 487–492
- Qiao, Q., H.F. Chen, F.W. Xing, F.G. Wang, W.C. Zhong, X.Y. Wen and X.G. Hou, 2012. Pollination ecology of *Bretschneidera sinensis* (Hemsley), a rare and endangered tree in China. *Pak. J. Bot.*, 44: 1897–1903
- Qiao, Q., F.W. Xing, H.F. Chen and L. Fu, 2010. Leaf structural characteristics in *Bretschneidera sinensis*, a rare endemic plant in China. *J. Wuhan Bot. Res.*, 28: 229–233
- Qiao, Q., H.F. Chen and F.W. Xing, 2009. Seed germination protocol for the threatened plant species, *Bretschneidera sinensis* Hemsl. *Seed Sci. Technol.*, 37: 70–78
- Reddy, A.R., K.V. Chaitanya and M. Vivekanandan, 2004. Drought-induced responses of photosynthesis and antioxidant metabolism in higher plants. *J. Plant Physiol.*, 161: 1189–1202
- Sevanto, S., N.G. McDowell, T. Dickman, R. Pangle and W.T. Pockman, 2013. How do trees die? A test of the hydraulic failure and carbon starvation. *Plant Cell Environ.*, 37: 153–161
- Sofa, A., B. Dichio, C. Xiloyannis and A. Masia, 2004. Lipoxygenase activity and proline accumulation in leaves and roots of olive trees in response to drought stress. *Physiol. Plant.*, 121: 58–65
- Trenberth, K.E., A. Dai, G.V.D. Schrier, P.D. Jones, J. Barichivich, R. Briffa and J. Sheffield, 2014. Global warming and changes in drought. *Nat. Clim. Change*, 4: 17–22
- Wang, Z., B. Quebedeaux and G.W. Stutte, 1995. Osmotic adjustment: effect of water stress on carbohydrates in leaves, stems and roots of apple. *Aust. J. Plant Physiol.*, 22: 747–754
- Watanabe, S., K. Kojima, Y. Ide and S. Sasaki, 2000. Effects of saline and osmotic stress on proline and sugar accumulation in *Populus euphratica* in vitro. *Plant Cell Tiss. Org.*, 63: 199–206
- Will, R.E., S.M. Wilson, C.B. Zou and T.C. Hennessey, 2013. Increased vapor pressure deficit due to higher temperature leads to greater transpiration and faster mortality during drought for tree seedlings common to the forest-grassland ecotone. *New Phytol.*, 200: 366–374
- Xu, G.B., Y. Liang, Y. Jiang, X.S. Liu, S.L. Hu, Y.F. Xiao and B.B. Hao, 2013. Genetic diversity and population structure of *Bretschneidera sinensis*, an endangered species. *Biodivers. Sci.*, 21: 723–731
- Zhang, Y.J., F. Yan, H. Gao, Y.Z. Xu, Y.Y. Guo, E.J. Wang, Y.H. Li and Z.K. Xie, 2015. Chlorophyll content, leaf gas exchange and growth of oriental lily as affected by shading. *Russ. J. Plant Physiol.*, 62: 334–339
- Zhou, G.Y., C.H. Peng, Y.L. Li, S.Z. Liu, Q.M. Zhang, X.L. Tang, J.X. Liu, J.H. Yan, D.Q. Zhang and G.W. Chu, 2013. A climate change-induced threat to the ecological resilience of a subtropical monsoon evergreen broad-leaved forest in Southern China. *Glob. Change Biol.*, 19: 1197–1210

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