

A TEST OF THE CARBON STARVATION HYPOTHESIS IN SHRUBS DURING DROUGHT-INDUCED MORTALITY

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Abstract

The carbon starvation hypothesis is one of the current leading hypotheses in the mechanism of plant mortality, although it has not been verified due to lack of evidence. To provide a basis for the verification of carbon starvation hypothesis in plant mortality, we tested the role of the non-structural carbohydrate (NSC) reserves of three shrub species (*Sarcozygium xanthoxylon*, *Berberis diaphana* and *Sophora moorcroftiana*) during drought-induced mortality. NSC concentration ([NSC]) and content in the seedlings of three shrub species were determined during the cessation of photosynthesis and death and in well-watered controls. Our results show that drought induces NSC loss in three shrub species at death, and carbon starvation appears to occur after the cessation of photosynthesis. Differences among species exist during water stress regarding [NSC] dynamics in roots, stems and leaves, which appear to be caused by differences in drought resistance and NSC allocation strategy (root, stem, or leaf). Our data also show that survival time correlates with the size (biomass yield) and priority of NSC supply (growth or metabolism and defence), specifically, the shrub which had the biggest size and prioritized growth during drought was observed to have shortest survival time. Overall, our findings demonstrate that drought may cause a loss of NSC reserves leading to carbon starvation, and eventually death.

Key words: Carbon starvation, Drought, Mortality, *Sarcozygium xanthoxylon*, *Berberis diaphana*

Introduction

Worldwide massive tree mortality events have aroused wide public concern. These events may be associated with climate change-induced drought and have been observed in a variety of forests in different geologic and climatic provinces (Liang *et al.*, 2003; Bréda *et al.*, 2006; Adams *et al.*, 2009; van Mantgem *et al.*, 2009; Allen *et al.*, 2010; Niu *et al.*, 2014), including arid regions in China (Liang *et al.*, 2003; Zhang *et al.*, 2014). Compared to temperature variation, light and palaeo-CO₂ concentrations, drought is the predominant climatic characteristic associated with mortality (Rebetez & Dobbertin, 2004; Allen *et al.*, 2010). The mechanisms involved in drought-induced tree mortality remain unclear (McDowell *et al.*, 2008; Sala *et al.*, 2010), but the hydraulic failure and carbon starvation hypotheses are the current leading postulates in the mechanism of tree mortality. Numerous studies have revealed that trees can die of both hydraulic failure and carbon starvation (Sevanto *et al.*, 2014; McDowell, 2011). Overall, the hydraulic failure hypothesis is well accepted, but the carbon starvation hypothesis remains controversial. With the latest findings showing that hydraulic failure during drought-induced mortality is persistent across species, whereas carbon starvation is not universal (Adams *et al.*, 2017). Nonetheless, carbon starvation seems to be closely related to tree mortality due to its potential role in diminishing hydraulic function. Thus, loss of carbohydrate reserves should be experimentally observed and verified to better understand the mechanism of tree mortality.

As described by McDowell (2011), carbon starvation occurs when carbon supply from photosynthesis, the mobilization of non-structural carbohydrates (NSCs) and autophagy (vacuolar proteolysis) are lower than carbon use through respiration, growth and defence. Hence, plants succumb to drought when plant NSCs are unavailable for metabolism and regeneration (Sala *et al.*, 2010). The

carbon starvation hypothesis was formulated to explain tree mortality during long-term drought periods, but no direct evidence currently exists that supports its explaining drought-induced mortality in trees (Körner, 2003; Ward *et al.*, 2005; Sala & Hoch, 2009; Gruber *et al.*, 2012). Therefore, the mechanisms of carbon starvation, or if it even occurs, warrants further discussion (McDowell & Sevanto, 2010). To clarify the role of carbon starvation, most experts believe that NSCs (mainly including soluble sugars and starches) should be quantified in plants that are in the process of dying or have already died (Piper *et al.*, 2009; McDowell *et al.*, 2010; McDowell, 2011). In particular, the minimum required NSCs for the maintenance of metabolism and defense must be determined, and the threshold NSC concentration ([NSC]; per unit of dry matter (DW), mg/g DW) and content (per individual, mg/plant) should be quantified. Thus, future research on carbon starvation should focus on these tipping points of mortality (McDowell, 2011). In addition, during long drought periods, differential NSC allocation to plant organs should be also observed, which seems to differ among tree species and be related to distinct drought resistance abilities, strategies and to drought stress duration and severity.

In recent years, numerous studies have focused on NSC dynamics and the processes by which plants succumb to drought to examine the mechanism of carbon starvation, but they have tended to focus on trees (Adams *et al.*, 2017; Wiley *et al.*, 2017; Qiu *et al.*, 2018). In shrubs, a near 100% loss of hydraulic conductivity can induce mortality during an intense drought (Vilagrosa *et al.*, 2003). Paddock *et al.*, (2013) also stated that high resistance to xylem cavitation did not prevent mortality in adult shrubs. However, related studies on NSC metabolism and carbon starvation mechanisms in shrubs are rare. Large areas of shrubs yield to climatic drought in north-western China and the exact mechanism of mortality is of great concern. Predicting

survival time of different tree species is quite inconclusive due to lack of tests provided for understanding the mortality mechanism. In previous studies, Sevanto *et al.*, (2014) stated that hydraulic constraints on plant carbohydrate use determined survival time, further, Doughty *et al.*, (2015) observed that trees survival time was determined by the carbon utilization strategy during drought (i.e. prior to growth or metabolism and defense). In general, it remains uncertain that what indices are determined as the key parameters for predicting tree survival time during droughts of different duration and severity. Consequently, any study on predicting tree survival time is valuable and welcome.

Based on the studies mentioned above, NSC dynamics and the carbon starvation mechanisms associated with mortality are the foci of our study. We used two treatments, drought and control, on three kinds of common xerophilous shrubs (*Sarcozygium xanthoxylon*, *Berberis diaphana* and *Sophora moorcroftiana*) native to north-west China having different drought tolerances to determine [NSC] and content during drought-induced mortality. Our objectives were to (1) identify the changes in NSC reserves and allocation in three shrub species at two time points (zero net photosynthetic rate and death) due to drought, (2) observe whether carbon starvation occurs in shrubs at death and (3) examine the relationship between survival time, and the size (biomass yield) and growth of shrubs during drought. Our study provides a basis to test the carbon starvation hypothesis in the mechanism of plant mortality.

Materials and Methods

Plant material and growth conditions: *Sarcozygium xanthoxylon*, *Berberis diaphana* and *Sophora moorcroftiana* are deciduous broad-leaved shrubs. *S. xanthoxylon* is found mainly in desert and semi-desert areas; *B. diaphana* occurs mainly in arid regions in Qinghai and Gansu provinces; and *S. moorcroftiana* is an endemic species distributed on the Tibetan Plateau. According to the evaluation results of drought tolerance of the three shrubs based on their leaf anatomical structures and physiological responses to drought, their drought tolerance could be ranked as follows: *S. xanthoxylon* < *B. diaphana* < *S. moorcroftiana* (these observations have been reported by Pan *et al.*, 2014; 2015). Seedlings of each shrub species were collected from north-western China and transplanted into flower pots in early March 2012. The flower pots measured 35 cm × 35 cm × 30 cm, and plastic pellets were placed under the flower pots to avoid water and soil loss. The original soil around the roots was preserved and any empty space was filled with potting soil. Soil nutrition contents were determined as described by Lu (2000); the soil organic matter content was 31.64 g/kg, that of total nitrogen (N) was 1.57 g/kg, total phosphorus (P) content was 0.83 g/kg, total potassium (K) content was 20.11 g/kg, available N content was 154.03 mg/kg, available P content was 50.11 mg/kg and available K content was 104.22 mg/kg. The seedlings were planted and allowed to acclimate for 5 months in a plastic film greenhouse measuring 60.0 m × 8.0 m × 1.6 m with an arch height of 3.0 m, arch space of 1.0 m and a total area of 480.0 m². During acclimation, seedlings were well watered daily and protected against insects and disease. Our study site is

located at the Xiaolongshan Forestry Science and Technology Research Institution, Tianshui, Gansu Province (34°29' N, 105°48' E) at an elevation of 1160 m. It is in the temperate zone in a semi-humid monsoon climatic region. The average annual rainfall and evaporation capacity are ~600–800 mm and 1290 mm, respectively. The average annual temperature is 11°C and the frost-free period lasts ~180 days. During the experiment, the daily average temperature in the greenhouse ranged between 20 and 38°C with a daily average humidity of 40–65%. Before treatment, the average heights of *S. xanthoxylon*, *B. diaphana* and *S. moorcroftiana* were 0.60 m, 0.35 m and 0.36 m, respectively, and the corresponding ground diameters were 7.29 mm, 8.96 mm and 6.11 mm.

Experimental design: Seedlings were divided into two treatments: control (CK) and drought (D) (10 seedlings per treatment). CK seedlings were well watered during the experimental period. The soil water content (SWC, water volume/soil volume) was measured using a handheld time domain reflectometer (TDR, FOM/mts type; Easy Test Ltd., Lublin, Poland) every 5 days. SWC values averaged 27.16%, 25.95% and 26.31% for CK seedlings of *S. xanthoxylon*, *B. diaphana* and *S. moorcroftiana*, respectively. D seedlings had water withheld from early July 2012. CK and D seedlings were each divided into two groups: CK1 ($n = 5$) and CK2 ($n = 5$) and D1 ($n = 5$) and D2 ($n = 5$), respectively. D1 is defined as the group in which photosynthesis ceased (zero net photosynthetic rate), and D2 is defined as the group in which seedlings succumbed to drought. Once leaves discolored to orange and defoliation was apparent, death was confirmed by staining twig samples with 0.1% neutral red. CK1 and CK2 represented the respective controls versus D1 and D2. The instantaneous net photosynthetic rate (Pn) in leaves was determined at 9:00–10:30 h every 5 days under constant light (photosynthetically active photon flux density, PPFD; 1200 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) under the ambient CO₂ concentration (400 ppm) and leaf temperature (30°C) (Li-6400; Li-Cor Inc., Lincoln, NE).

Harvesting and sample preparation: Two D seedling harvests were obtained. When seedlings were determined to have reached the D1 stage, the first harvest, composed of CK1 and D1 plants, was obtained. The second harvest (CK2 and D2) followed the death of the drought-treated plants. Note that seedlings were harvested asynchronously due to differences in photosynthesis response and survival time during the drought period. After the two harvests, seedlings were divided into root, stem and leaf samples. The samples were oven-dried for 72 h at 80°C, and the biomass yield of roots (BY_{root}), stems (BY_{stem}), and leaves (BY_{leaf}) were determined and expressed as dry matter (DW, g). After biomass yield measurements, these samples were obtained by crushing and sieving through a 100-mesh screen and collection in sample bags and prepared for NSC analysis. As drought progressed, defoliation occurred, and the fallen leaves were collected as leaf samples at death (D2).

NSC analysis: NSC concentrations in roots ([NSC]_{root}), stems ([NSC]_{stem}), and leaves ([NSC]_{leaf}) were here calculated as the sum of total soluble sugars concentrations ([TSS]) and starch concentrations ([Starch]). [TSS] and [Starch] were measured using the

anthrone–sulphuric acid colourimetric method (Zou, 1995) and expressed as mg/g DW. NSC content, total NSC of the dry matter per plant, was calculated as $NSC\ content = [NSC]_{root} \times BY_{root} + [NSC]_{stem} \times BY_{stem} + [NSC]_{leaf} \times BY_{leaf}$ and expressed as mg/plant.

Statistical analysis: Data were expressed as the mean \pm standard error (SE). Figures were prepared using Sigma Plot 10.0 (Systat, San Jose, CA) and OriginPro 7.5 (Originlab, Northampton, MA). One-way ANOVA analysis and Duncan's multiple range test, and two-way ANOVA analysis were performed using SPSS 19.0 (SPSS Inc., Chicago, IL). $p < 0.05$ was considered to indicate statistical significance.

Results

As shown in Fig. 1, Pn rapidly declined after drought treatment. The Pn of *S. xanthoxylon*, *B. diaphana* and *Sophora moorcroftiana* respectively decreased to 0 at 20, 25, and 25 days after the start of the experiment, and the three shrub species died at 35, 40, and 45 days, respectively, after the start of the experiment (Fig. 1). Obviously, interspecific differences in survival time existed during long-term drought.

According to our two-way ANOVA results (Table 1), $[NSC]_{root}$, $[NSC]_{stem}$, and $[NSC]_{leaf}$ values were all significantly different in the three species, and our data also showed that seedlings under different treatments (CK1and D1; CK2 and D2; D1 and D2) generally showed significant difference in $[NSC]_{leaf}$ and also in $[NSC]_{stem}$ (except for the seedlings under CK1and D1), while $[NSC]_{root}$ values were only significantly different between CK1and D1. Also, except for the non significant interaction between species and treatment (CK1 and D1) on $[NSC]_{leaf}$, a significant interaction factor on $[NSC]$ was generally found between species and treatment (Table 1), implying that $[NSC]$ in three shrubs responded differently to treatments (CK1and D1; CK2 and D2; D1 and D2).

Compared with CK1, drought (D1) plants showed interspecific differences in $[NSC]_{root}$: a non-significant difference was observed in *S. xanthoxylon*, a significant decrease was noted in *B. diaphana*, a significant increase was detected in *S. moorcroftiana* (Table 1 and Fig. 2A). Similarly, D1 plants also showed interspecific differences

in $[NSC]_{stem}$: a significant decrease was observed in *S. xanthoxylon* and *S. moorcroftiana* and a significant increase was detected in *B. diaphana* (Table 1 and Fig. 2B). Whereas, D1 plants showed identical changes in $[NSC]_{leaf}$: a significant increase in $[NSC]_{leaf}$ was observed in all three species (Table 1 and Fig. 2C). Compared with CK2, D2 plants showed significant decrease in $[NSC]_{leaf}$ in all three species (Table 1 and Fig. 2C). For $[NSC]_{root}$ and $[NSC]_{stem}$, D2 seedlings generally had similar or lower levels compared to CK2 seedlings (Figs. 2A and 2B). In addition, compared with D1, D2 provoked significant decreases in $[NSC]_{leaf}$ in all three species (Table 1 and Fig. 2C), and D2 seedlings had similar or lower levels of $[NSC]_{root}$ and $[NSC]_{stem}$ (Figs. 2A and 2B).

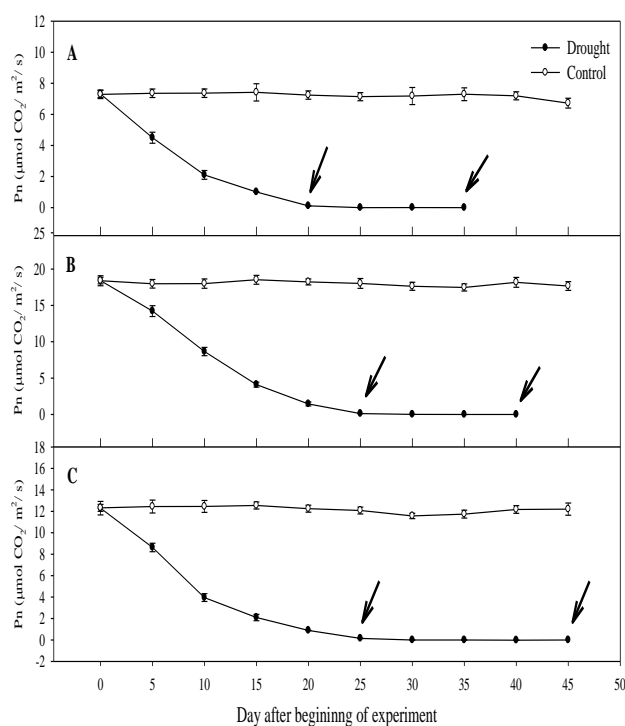


Fig. 1. Effects of treatment on the instantaneous net photosynthetic rate (Pn) in *Sarcozygium xanthoxylon* (A), *Berberis diaphana* (B) and *Sophora moorcroftiana* (C). The arrows indicate the harvest time (left: first harvest; right: second harvest).

Table 1. Summary of two-way ANOVA testing of the effect of drought in different shrub species on non-structural carbohydrate (NSC) concentration ($[NSC]$) in roots ($[NSC]_{root}$), stems ($[NSC]_{stem}$) and leaves ($[NSC]_{leaf}$). CK1 and CK2 seedlings were well watered during the experimental period. D1 seedlings refer to seedlings in which photosynthesis ceased due to drought. D2 seedlings are those that succumbed to drought.

Response variable	Effect	CK1 vs. D1			CK2 vs. D2			D1 vs. D2		
		df	F ratio	p value	df	F ratio	p value	df	F ratio	p value
$[NSC]_{root}$	Species	2	83.887	<0.001	2	151.089	<0.001	2	8.803	0.007
	Treatment	1	4.484	0.045	1	9.726	0.087	1	3.219	0.085
	Treatment \times species	2	19.736	<0.001	2	2.702	<0.001	2	192.954	<0.001
$[NSC]_{stem}$	Species	2	22.991	<0.001	2	24.149	<0.001	2	19.019	<0.001
	Treatment	1	3.685	0.067	1	9.225	<0.001	1	95.610	<0.001
	Treatment \times species	2	20.158	<0.001	2	4.170	0.028	2	4.336	0.025
$[NSC]_{leaf}$	Species	2	36.232	<0.001	2	6.183	0.007	2	5.982	0.008
	Treatment	1	152.330	<0.001	1	258.911	<0.001	1	4.102	0.029
	Treatment \times species	2	3.039	0.067	2	4.292	0.026	2	17.467	<0.001

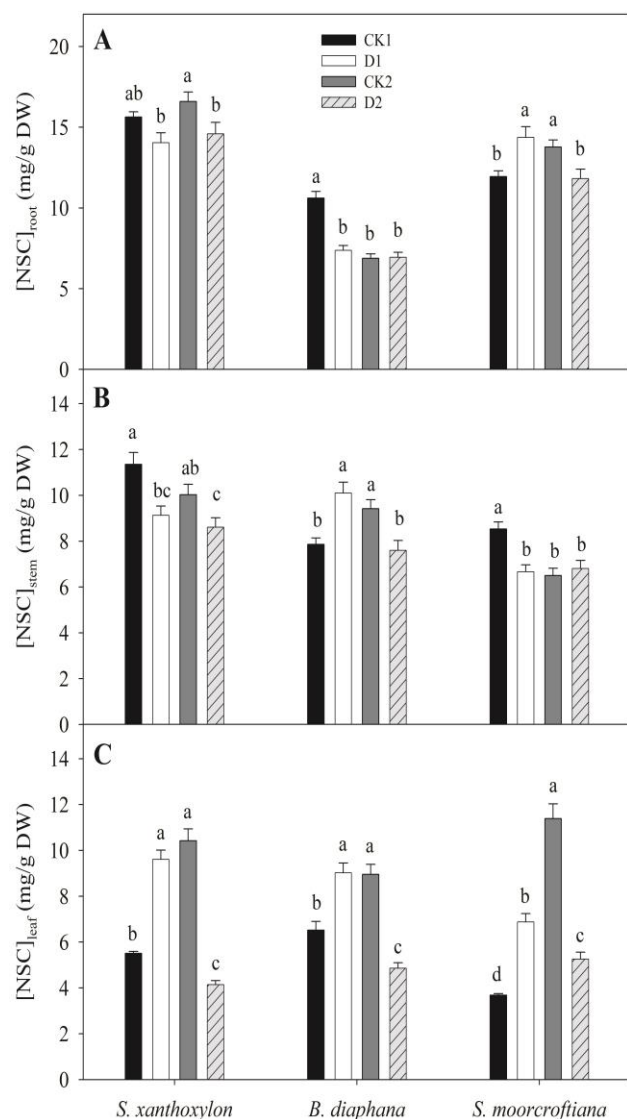


Fig. 2. Treatment (CK1, D1, CK2, and D2) effects on [NSC]_{root} (A), [NSC]_{stem} (B), and [NSC]_{leaf} (C) in three shrubs seedlings. Units correspond to mg/g dry weight (DW). Values are the average \pm standard error (SE, $n = 5$). For each shrub, the differences between treatments were examined using one-way ANOVA and Duncan's multiple range test, and different lowercase letters indicate significant differences, the same below.

In the D1 group, a significant decrease in NSC content was observed in *S. xanthoxylon* compared with CK1 (Fig. 3A), while for *B. diaphana* and *S. moorcroftiana*, no differences between D1 and CK1 in NSC content were detected (Figs. 3B and 3C). Compared with CK2, the D2 group showed significant decreases in NSC content (26.79–30.99%) in all shrub species (Figs. 3A, 3B, and 3C). In terms of the changes of NSC content between D2 and D1, our data observed that D2 seedlings of *S. xanthoxylon* and *S. moorcroftiana* had similar levels of NSC content compared with D1 seedlings (Figs. 3A and 3C), while the D2 group performed a significant loss in NSC content in *B. diaphana* compared with D1.

Stems generally comprised the largest portion of NSCs in all seedlings (Fig. 4), demonstrating that the stem is the main functional organ for NSC storage. We observed the effects of treatment on NSC allocation strategies based on observed changes in the portion of NSC content from roots,

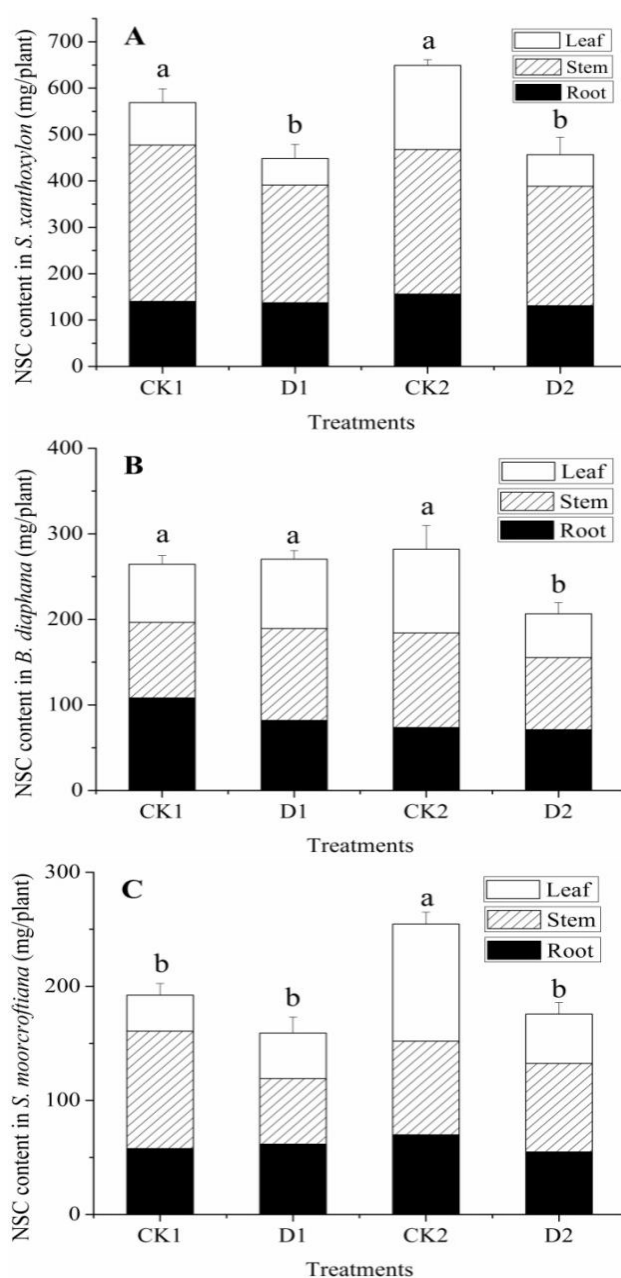


Fig. 3. Treatment (CK1, D1, CK2, and D2) effects on NSC content in three shrubs seedlings.

stems and leaves under the different treatments (CK1 and D1; CK2 and D2). Our data observed that stems of three shrubs all generally showed no obvious difference between treatments in the percentage of NSC content (Fig. 4). Compared with CK1 seedlings, an increase in the percentage of NSC content was detected in roots of D1 *S. xanthoxylon* and *S. moorcroftiana* seedlings, whereas a decrease the percentage of NSC content was observed in roots of D1 *B. diaphana* seedlings. Moreover, we observed that leaves of D1 *S. xanthoxylon* and *B. diaphana* seedlings had a lower level of percentage of NSC content, whereas leaves of D1 *S. moorcroftiana* seedlings had a higher level of percentage of NSC content. In the D2 seedlings of three shrubs, roots were all observed to comprise a higher portion of NSCs, whereas stems were all detected to account for a lower portion of NSC content (Fig 4), indicating that all seedlings at D2 tend to preferentially store more NSCs in the roots and limited NSCs in the leaves.

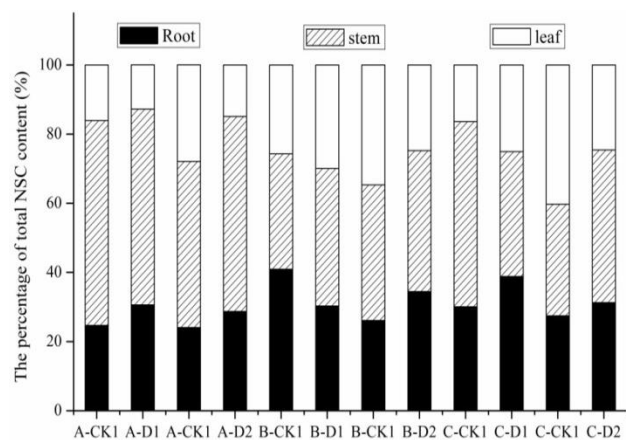


Fig. 4. NSC allocation in CK1 D1, CK2, and D2 seedlings of *S. xanthoxylon* (A), *B. diaphana* (B), and *S. moorcroftiana* (C). Root, stem, and leaf in the figure represent the portion of NSC content from roots, stems and leaves of shrubs, respectively.

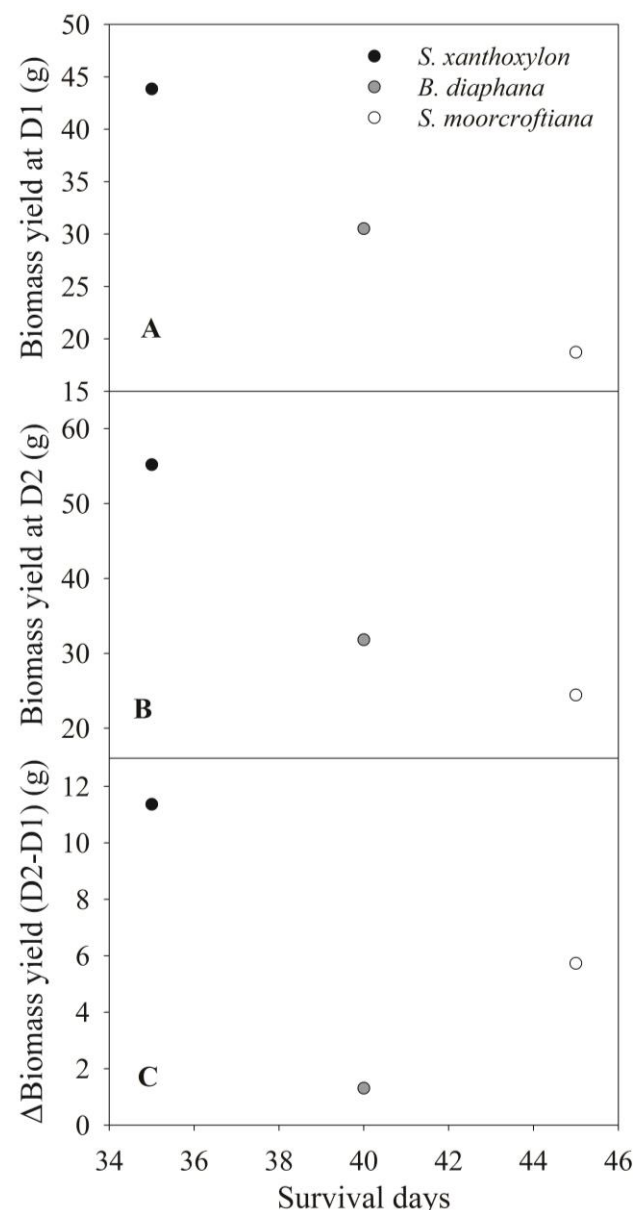


Fig. 5. The relationships between survival time and biomass yield at D1 (A) and at D2 (B), and Δ biomass yield (D2-D1).

As shown in Fig. 1, we noted that seedlings survived the drought for different lengths of time. We thus respectively classified *S. xanthoxylon*, *B. diaphana* and *S. moorcroftiana* as a fast-dying shrub, intermediate-dying shrub and slow-dying shrub according to their respective survival days. As indicated in Figs. 5A and 5B, we observed that biomass yield of D1 and D2 seedlings correlated closely with survival time, and the shrub (*S. xanthoxylon*) seedlings that survived the shortest had the biggest size of biomass yield, while slow-dying shrub (*S. moorcroftiana*) had the smallest size. Also, we found that D2 seedling of the fast-dying shrub (*S. xanthoxylon*) had the largest net increment in biomass yield compared with D1 seedlings (Fig. 5C), whereas, the D2 seedlings of intermediate-dying shrub (*B. diaphana*) and slow-dying shrub (*S. moorcroftiana*) was noted to perform a relatively less growth (Fig. 5C).

Discussion

NSCs are important for energy supply and comprise necessary temporary solute source for transportation metabolism and osmoregulation in plant growth and metabolism (Koch, 1996). [NSC] and content are physiological traits charactering ecologically adaptive strategies (Myers & Kitajima, 2007; Poorter & Kitajima, 2007). Furthermore, changes in plant NSCs can be measured to effectively analyze carbohydrate metabolism status (i.e. storage, supply, and consumption) in plants subjected to long-term drought. Our results demonstrate that [NSC] and content in seedlings at death due to long-term drought generally decrease, indicating that loss of NSC storage occurs in plants succumbing to drought and that the lost NSCs were mainly consumed to defend against drought stress. Photosynthesis will cease when a plant suffers serious drought (Akram *et al.*, 2007; Piper, 2011; Yang *et al.*, 2014; Shabbir *et al.*, 2015), which was observed in our study (Fig. 1). The main supply source of NSCs is cut off when photosynthesis ceases and the plant must consume stored NSCs to maintain metabolism and defence (McDowell, 2011).

Consistent with the need to quantify [NSC] and content in plants that are dying or have already died (Piper *et al.*, 2009; McDowell *et al.*, 2010; McDowell, 2011), our data emphasize the importance of observing NSC dynamics in shrubs that succumb to drought. Our results are consistent with those of a predictive simulation that an NSC surplus occurs during early drought, but NSC content begins to decline when photosynthesis is significantly reduced by long-term drought (McDowell, 2011). It was observed that D2 seedlings of three shrubs generally exhibited a loss compared with CK2 in [NSC] in different nutritive organs (Fig. 2) and NSC content (Fig. 3), whereas, D1 shrubs generally had similar NSC content levels compared with CK1 seedlings (except for *S. xanthoxylon*; Fig. 3) and exhibited an increase in [NSC]_{leaf} (Fig. 2C) and [NSC]_{root} (except for *S. xanthoxylon*; Fig. 2A). Also, compared to D1, a significant loss was detected in [NSC]_{leaf} in D2 *S. xanthoxylon* seedlings, in [NSC]_{leaf}, [NSC]_{stem}, and NSC content of D2 *B. diaphana* seedlings, and in [NSC]_{leaf} and [NSC]_{root} in D2 *S. moorcroftiana*. The possible reason was that NSC loss

leading to death ensued when photosynthesis ceased (McDowell, 2011). Due to NSC loss, carbon starvation occurred when carbon acquisition and storage did not meet the need for maintaining metabolism, increasing the risk of plant death (Sala *et al.*, 2010). Our data show that shrubs can die of carbon starvation due to long-term drought; however, we did not determine when carbon starvation occurs. We preliminarily propose that carbon starvation occurs after photosynthesis ceases, but the specific timing is unknown. Similarly, our data did not determine the threshold [NSC] and content in shrubs for carbon starvation and determining this is an important area of future research.

The NSC allocation strategies of plants can be comprehensively influenced by biological and environmental factors and are generally correlated closely with long-term survival and ecological adaptation strategies (Barbaroux *et al.*, 2003; Litton *et al.*, 2004; Rachmilevitch *et al.*, 2006). Specifically, plants will allocate NSC reserves to roots and shoots in varying amounts when subjected to changes in environmental conditions. Furthermore, regarding NSC allocation strategies, differences exist among tree species (Barbaroux *et al.*, 2003). Our results show that D2 seedlings of three shrub species all tend to preferentially supply stored NSCs to roots compared with CK2 seedlings (Fig. 4). Increased available NSCs in the roots may be used for root growth and defence against drought especially when subjected to NSC deficiency (Imaji & Seiwa, 2010), as plants will extend their roots to increase the range of water absorption and promote drought resistance (Imaji & Seiwa, 2010; Wishart *et al.*, 2014). However, our data do not answer why the examined shrub species preferentially supply NSC stores to different nutritive organs at D1: *S. xanthoxylon* (roots) *B. diaphana* (leaves), and *S. moorcroftiana* (roots and leaves) (Fig. 4), although this may be partly explained by the fact that different plants defend and adapt to environmental drought through various NSC allocation strategies when liberally supplied with NSCs.

In terms of the relationship between survival time and the size (biomass yield), our data observed that shrub seedlings that survived the shortest had the biggest size of biomass yield (Figs. 5A and 5B), and it may be attributed to that plant need allocate more NSCs for maintenance of tissue metabolism (i.e. autotrophic respiration) due to its big size (McDowell, 2011). Also, Doughty *et al.*, (2015) proposed that trees prioritized growth by reducing autotrophic respiration during drought, and thus weakened tissue maintenance and defence investment may increase post-drought tree mortality. Similarly, during drought-induced mortality, as the fast dying shrub in present study, *S. xanthoxylon* was also observed to exhibit the largest increment in biomass yield, while the intermediate-dying shrub (*B. diaphana*) and slow-dying shrub (*S. moorcroftiana*) exhibited a relatively less growth (Fig. 5C). The possible reason was that the fast dying shrub (*S. xanthoxylon*) prioritized growth by consuming more NSCs for tissue growth, resulting in reducing metabolism and defence, in this way, reducing investment in tissue maintenance and defence may cause the increase in post-drought shrub mortality. Further, we suggested that this finding can be partly explained for the

differences in drought tolerance between shrub species, because our data observed that *S. xanthoxylon* relatively prioritized growth rather than metabolism or defence during long-term drought, leading to its weaker drought tolerance in the absence of NSCs, however, *B. diaphana* and *S. moorcroftiana* relatively prioritized metabolism or defence by reducing growth when subjected to drought, resulting in increasing their drought tolerance. Hence, we cautiously deduce that the size (biomass yield) and growth data may provide information to determine survival time and drought tolerance of shrubs (Franklin *et al.*, 2012; Doughty *et al.*, 2015).

As reported by Piper (2011), drought induced different changes of [NSC] in different tree species of differential drought resistance. Our data also observed that D1 and D2 both provoked different variations of [NSC] in shrub species with differential drought resistance, also, the variations of [NSC] and content in seedlings between D2 and D1 differ among shrub species. Further, for each shrub observed in present study, their responses of NSCs to drought differ at two levels ([NSC] at tissue-level and content at individual-level). Thus, we recommend that NSC concentration and content dynamics should be conventional measurements. Moreover, we need to explore the threshold NSC at two levels, the tissue and the individual, in future studies on plant mortality. Defining plant death is challenging and determining when an individual plant dies is difficult (Anderegg *et al.*, 2012a), but all experiments on the mechanism of tree mortality must determine the final steps in the mortality process. For example, in conifer trees, which do not resprout, an absence of shoot respiration and needle color changes can be used as indicators of mortality, confirmed by staining leaf and twig samples (Sevanto *et al.*, 2014). Our study materials consisted of deciduous broad-leaved shrubs, so we used leaf colour changes and defoliation rather than lack of respiration (due to defoliation) as indicators of mortality. Furthermore, previous study showed that defoliation might affect the risk of carbon starvation in roots (Landhäusser & Lieffers, 2012), and it was widely accepted that conifer and broad-leaved trees generally performed disparate responses to drought stress. Hence, we suggest that the indicators chosen to confirm plant death should consider the various types (evergreen or deciduous; conifer or broad-leaved) of trees, and for trees that can resprout, we recommend determining root and stem death.

As described by various researchers, trees can die due to both hydraulic failure and carbon starvation (Hartmann *et al.*, 2013; Hereş *et al.*, 2014; Sevanto *et al.*, 2014). Anderegg *et al.*, (2012b) reports that no evidence exists to indicate that drought results in the depletion of carbohydrate reserves. Therefore, the drought-induced mortality mechanism in plants remains unresolved. Overall, we tend to agree with the view that carbohydrate reserves and allocation play an important role in determining plant survival time, in addition to hydraulic parameters (Sevanto *et al.*, 2014). From this perspective, the quantification of NSC dynamics in plants during long-term drought stress is a good starting point to test the carbon starvation hypothesis (McDowell, 2011), and an experimental test of carbon starvation in shrubs, in addition to trees, provides a necessary basis for better understanding plant mortality mechanisms.

Conclusions

Our results provide a means to test the carbon starvation hypothesis of the mechanism of drought-induced mortality in shrubs. As described by McDowell *et al.*, (2011), drought limits photosynthesis, resulting in a depletion of carbohydrate reserves and carbon starvation. Our data clearly show that drought leads to NSC loss in three shrub species at death, and carbon starvation seems to occur after photosynthesis ceases. Our data also show that the size of biomass yield and priority of NSC supply (growth or metabolism and defence) may determine survival time of post-drought shrubs, and the post-drought shrub which had the biggest size and prioritized growth was observed to survive the shortest. Our findings have some limitations because of our study materials, methods and experimental control conditions, rather than the use of natural drought conditions. We strongly understand that hydraulic failure should be factored into future studies on the mechanisms of plant mortality, and experiments should be designed to test carbon starvation and hydraulic failure hypotheses and determine the critical NSC to verify the mechanism of plant mortality.

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