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Article

Timing of Drought Affected the Growth, Physiology, and Mortality of Mongolian Pine Saplings

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Abstract: Background and Objectives: More frequent and severe droughts are occurring due to climate change in northern China. In addition to intensity and duration, the timing of droughts may be decisive for its impacts on tree growth, mortality, and the whole forest ecosystem. The aim of this study was to compare the effect of drought occurring in the early- and mid-growing season on the growth and physiology of Mongolian pine (*Pinus sylvestris* var. *mongolica* Litv.) saplings. Materials and Methods: Four-year-old container saplings that were about to sprout were exposed to three treatments: (i) regular irrigation throughout the growing season (CTRL), (ii) no irrigation in the early growing season (weeks 1–5) followed by regular irrigation (EGD), (iii) no irrigation in the mid growing season (weeks 5–10), and regular irrigation in the early and late growing season (MGD). We measured the root and shoot growth, sapling mortality, and the physiological changes in the roots and needles periodically. Results: Drought in the mid growing season was more harmful than in the early growing season in terms of chlorophyll fluorescence, electrolyte leakage of needles, needle length, stem diameter increment, and sapling mortality. The high mortality in the mid growing season might be attributed to the joint effect of drought and high temperature. Drought in the early growing season decreased root growth, and the starch and soluble sugars in roots as much as the drought in the mid growing season. Abscisic acid concentration increased in fine roots, but decreased in old needles after drought. Conclusions: Special attention should be paid on forest sites susceptible to drought during afforestation in the face of ongoing climate change.

Keywords: abscisic acid; chlorophyll fluorescence; drought timing; electrolyte leakage; root length; soluble sugar; starch

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1. Introduction

The global land areas exposed to drought have remarkably increased over the past decades [1]. Frequent and severe droughts have occurred in different parts of the world [2]. Severe droughts are expected to strongly impact forest ecosystems through affecting the growth, mortality, and regeneration of many tree species. In addition to intensity and duration, the timing of droughts has impacted plant productivity [3]. Huang et al. (2018) found that legacies from extreme drought events during the dry season lasted longer and impacted stronger on tree growth than those from extreme drought events during the wet season, suggesting that the timing of drought is a crucial factor in determining the impact on tree recovery [4]. Timing of drought may affect the mode, direction, and magnitude of the impact on terrestrial ecosystems [5,6].

Previous studies have mainly focused on the effects of summer drought on tree functioning. An irrigation experiment showed that summer drought limited Scot pine (*Pinus sylvestris* L.) forest establishment by reducing growth and increasing seedling mortality [7].

Zang et al. (2012) reported that radial growth of Scots pine trees of two different diameter groups were both limited by summer drought [8]. The severe late summer drought caused visual drought-damage symptoms of trees in Finnish boreal forests [9], and the ecosystem level water use efficiency showed a decrease during the severe soil drought [10].

In recent years, the effects of spring drought on the tree growth have been studied increasingly. Spring drought strongly reduced the radial growth of European larch (*Larix decidua* Mill.) and Norway spruce (*Picea abies* (L.) Karst) in Switzerland [11]. In temperate mesic forests of Eastern North America, drought-induced stem radial growth reductions in tree growth were the greatest when the droughts occurred at the beginning of the growing season [12]. In the northern part of China, the drought conditions in recent decades have been more severe than in other regions of China due to decreasing precipitation and increasing temperature [3]. Moreover, spring drought occurs frequently, causing stress to all plants in the early growth phase [3]. A study of Mongolian pine (*Pinus sylvestris* var. *mongolica* Litv.) forests in northeastern China also showed that the effect of temperature increases, caused by climate change, on tree growth depends on water availability during the early growing season [13]. However, comparative studies between the effects of drought occurred in the early- and mid- growing seasons on boreal and temperate tree species are lacking. It is probable that air temperature interacts with the drought stress, thus affecting the physiology and growth of tree saplings depending on the phase of the growing season.

Mongolian pine is a widely used tree species in afforestation in desertified areas of North China, Northeast China, and Northwest China. It has been cultivated for water and soil conservation and ecosystem recovery due to its quality of wood, fast growth, strong resistance to adverse environments, and adaptability. Four-year-old container saplings have been commonly used in afforestation. According to forest practices, the best plantation time is in the early spring. However, with the warming and drying of climate, water stress seems to be one of the main factors causing a low sapling survival rate, and low growth rate and dieback of this species in aging forests [14,15]. It is not well-known in which season the saplings may tolerate drought stress the best. That knowledge would be helpful for the forest practitioners to carry out the necessary irrigations.

In this study, we aimed to explore the impact of the timing of drought, particularly when occurring in the beginning (after bud burst) and middle of the growing season, on the above- and below-ground physiology and growth of Mongolian pine saplings. Different physiological and growth parameters were measured to gain a comprehensive view of sapling responses. Chlorophyll content and chlorophyll fluorescence were measured to assess the photochemistry of photosynthesis [16]. Non-structural carbohydrates' (mainly soluble sugar and starch) allocation in different organs that are vital for the metabolism and growth were monitored to evaluate the carbon balance under drought conditions [17,18]. Abscisic acid (ABA) is considered as a possible mediator of root-to-shoot communication, and it is translocated from the drought-sensing roots to the shoots via the transpiration stream, leading to the subsequent stomatal closure [19–21]. Therefore, ABA concentrations in roots and needles were measured. Electrolyte leakage of the needles were measured to evaluate the injury of the cellular membranes [22]. Root and needle length, stem diameter increment, sapling mortality were measured to assess the growth. Therefore, we assumed that these parameters would indicate sapling responses to drought during different phases of growing season. We hypothesized that the Mongolian pine seedlings are more susceptible to drought in the middle than in the early growing season, due to their different physiological status, phase of growth and the higher air temperature in the middle growing season.

2. Materials and Methods

2.1. Plant Cultivation and Drought Treatments

Four-year-old Mongolian pine saplings (height 46 ± 6 cm, basal stem diameter 8.56 ± 3 mm) (mean ± SE) were cultured in the containers with the mineral soil from the local nursery in Longtoushan Seedling Farm, Mulan State Forest Management Bureau

(41°99′ N, 117°70′ E, 884 m above sea level), Hebei Province, from seeds originating from local orchards. A total of 600 dormant container saplings were transported to Baoding, Hebei Agricultural University (38°50′ N, 115°26′ E) in March 2018. Immediately after arrival, the saplings with the soil clod (height 15 cm, diameter 10 cm) were replanted into plastic pots (diameter 18.5 cm at the top and 12.5 cm at the bottom, height 22 cm) with commercial organotrophic soil (N, P, K content >6%, organic matter content >45%, pH = 5.5–6.5). The saplings were raised for one month with irrigation at 1-week intervals before starting the drought treatments on April 26, when the saplings were about to start sprouting.

The saplings were distributed into four replicate blocks in a plastic shed. The roof was covered by plastic cloth to avoid the rain, and the four sides were open in order to enhance the air circulation, except for rainy days. Each block comprised three plots; the saplings of each drought treatment were put into one plot (50 saplings in one plot) and the plots were randomly distributed in each block. The drought treatments were applied within the first 10 weeks, when the buds burst and new needles started elongating (weeks 1–5), and shoot elongation was ending and needles was elongating quickly (weeks 6–10), followed by the post-treatment growth phase (weeks 11–18). The three treatments were: (i) regular irrigation throughout the growing season (CTRL); (ii) no irrigation in the early growing season (weeks 1–5) followed by regular irrigation (EGD); and (iii) no irrigation in the middle of the growing season (weeks 6–10) and regular irrigation in the beginning and end of the growing season (MGD). Control seedlings were irrigated at intervals to maintain adequate soil water content, and there was no irrigation in the EGD and MGD periods. After the end of the drought treatment at week 10, all the saplings were watered weekly.

The daily maximum and minimum air temperatures in the plastic shed were recorded during the experiment using a digital thermometer (HTC-1, Bing You, Shanghai, China). The maximum and minimum air temperatures in the shelter were 32 °C and 8 °C during the early growing season (weeks 1–5) and 38 °C and 15 °C during the middle of the growing season (weeks 6–10), respectively. The average air temperatures were 20 °C during the early growing season and 26 °C during the middle of the growing season, respectively (Figure 1a).

The volumetric soil water content in the randomly selected 20 pots per treatment was measured (TDR100, Spectrum Technologies, Inc., Plainfield, IL, USA) at 3 p.m. daily. The corresponding soil relative water content (SRWC) was calculated based on the volumetric soil water content, the field moisture capacity of soil (32.50% ± 1.2%) and soil bulk density (1.53 ± 0.1 g/cm^3) [23] as: SRWC = [(soil volumetric water content/soil bulk density)/field moisture capacity] × 100. The soil relative water content in CTRL pots remained higher than 60%, while it was from 36% to 21% between weeks 3 and 5 in EGD pots and it was from 36% to 20% between the weeks 8 and 10 in MGD pots (Figure 1b).

Twenty-four saplings (2 saplings × 4 replicate blocks × 3 treatment) were taken to the lab for the physiological measurements at week 3 (3 weeks EGD), week 5 (5 weeks EGD), week 8 (3 weeks MGD), week 10 (5 weeks MGD), week 13 (3 weeks after ending MGD) and week 16 (6 weeks after ending MGD).

2.2. Physiological and Growth Measurements

2.2.1. Chlorophyll Content and Chlorophyll Fluorescence of Needles

Previous-year and current-year needles (sample size 0.2 g fresh weight) was randomly sampled from the middle of the previous-year shoots and middle of the current-year shoots, respectively, in each of eight saplings per treatment (two saplings of each replicate block) for total chlorophyll content measurement by the method described in Zhang et al. (1986) [24]. The needles were cleaned and cut into pieces, followed by soaking in the mixed solution of ethanol and acetone with ratio of 1:1 for 24 h in a dark place. When the needle pieces turned white, extracting solution was measured spectrophotometrically (U-5100 Spectrophotometer, Hitachi High-tech Science, Tokyo, Japan) at 645 nm and 663 nm.

For the measurement of dark-acclimated chlorophyll fluorescence (F_v/F_m), five previous-year needles were picked randomly from the middle of each sapling. The needles were attached to a tape side by side and after dark-acclimation for 25 min at room temperature F_v/F_m was measured with a portable chlorophyll fluorescence meter (Handy-PEA, Hansatech Instruments, King's Lynn, UK).

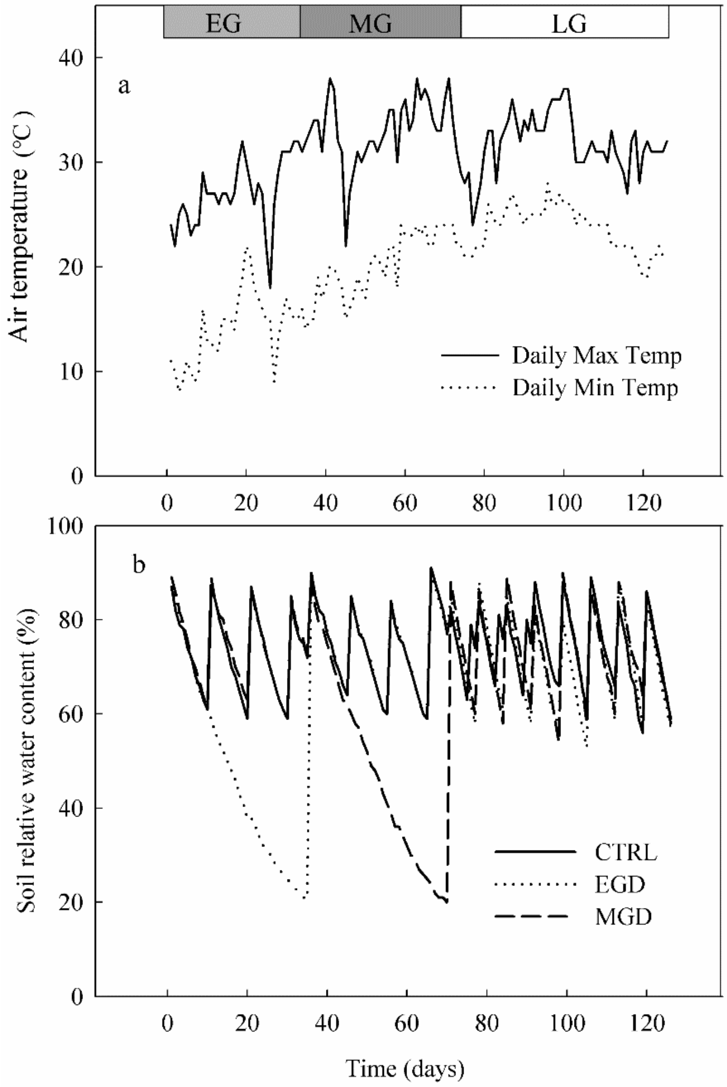

Figure 1. Air temperatures (**a**) and soil relative water content (**b**) during the experiment that started on April 26. CTRL indicates the control; EGD and MGD indicate the drought stress treatment in the early- (EG, days 0–35) and mid- (MG, days 36–70) growing season, respectively. LG refers to the late growing season (days 71–126). Time indicates days from the beginning of the experiment.

2.2.2. Relative Electrolyte Leakage (REL) of Needles

Four previous-year and current-year needles were randomly selected from the middle of the previous-year shoots and middle of the current-year shoots, respectively, in each of eight saplings per treatment (two seedlings of each replicate block) for relative electrolyte leakage (REL) measurement. In total, 32 previous-year needles and 32 current-year needles in each treatment at each sampling time were used. The samples, 10 mm in length, were cut from the middle of the needles and rinsed with deionized water, then put into four test tubes randomly with eight segments in each. Then, 12 mL of deionized water was added into each tube. The tubes were shaken for 22 h, then the first conductivity value (C_1) (DDS-307 conductivity meter with DJS-1C electrode, INESA Scientific Instrument Co. Ltd., Shanghai, China) was measured. The samples were then heat-killed in a water bath (92 °C for 30 min) and shaken again for 22 h before the second conductivity (C_2) was recorded. The REL was calculated as [25]:

$$\mathrm{REL} = \frac{C_1}{C_2} \times 100 \quad (1)$$

2.2.3. Soluble Sugar and Starch Content

Eight current-year needles, one 5 cm long piece of stem and about 2 g of fine roots (diameter < 2 mm) were randomly sampled from each of the saplings, that were used for the root morphological and physiological measurements, for determining soluble sugar and starch contents. The samples were dried at 40 °C to a constant weight and then ground into a powder. The soluble sugars were extracted using 80% aqueous ethanol. The concentration of total soluble sugars was determined spectrophotometrically at 630 nm after the reaction with anthrone; D-glucose was used as the standard. The starch was extracted from the residue using 35% perchloric acid and its content was determined spectrophotometrically at 625 nm with anthrone and using starch in 30% perchloric acid as a standard. The contents are given as percentage of dry mass of the needles [26].

2.2.4. Abscisic Acid (ABA) Determination

Five previous-year needles from middle of the previous-year shoots and about 0.2 g of fine roots were collected from each of the seedlings that were used for the physiological measurements at each sampling time and stored in liquid nitrogen for determining the concentration of abscisic acid (ABA) by enzyme-linked immunosorbent assay (ELISA) [27].

2.2.5. Sapling Growth

Before the onset of the drought treatment, two saplings in each treatment of each block (24 saplings in total) were randomly marked to measure the basal stem diameter and the length of the current-year needles later on. The stem diameter was measured with a 6″ electronic caliper (PD-151, Prokit's industries Co., Ltd, Taipei, Taiwan) at a marked position above the root collar. In addition, a current-year shoot was labelled and five needles were randomly selected for length measurement. The measurements of the same saplings were repeated weekly.

At the sampling times, in the beginning of the experiment (Week 0), after five weeks of EGD (Week 5) and MGD (Week 10) and resuming irrigation (Week 16), the roots of two saplings from each of the four replicate blocks, i.e., eight saplings per treatment for a total of 24 saplings used for physiological measurements were harvested. The whole root system was cut out at the root collar and separated from the soil, cleaned with tap water and deionized water, and then scanned to assess root morphology (Epson Expression 1640Xl scanner, Epson, Quebec, QC, Canada). The root length was analyzed using the WinRHIZO program (WinRhizo, Régent Instruments Inc., Sainte-Foy, QC, Canada).

2.2.6. Sapling Mortality

The number of dead saplings was counted in all treatments one week after each sampling. Mortality was calculated as the cumulative number of dead saplings divided by the total number of saplings in each treatment in the beginning of the experiment.

2.3. Statistical Analysis

The effects of the treatments on the physiological and growth parameters were analyzed by means of a mixed linear model (procedure MIXED in SPSS 20.0, SPSS Inc., Chicago, IL, USA). The model used was $y = \mu + \text{treatment} + \text{time} + \text{treatment} \times \text{time} + \varepsilon$, where μ is a constant. The 'treatment' (i.e., CTRL, EGD and MGD) and 'time' (i.e., sampling time) were regarded as fixed factors and ε as a random term. The significance of the difference between the treatments at different sampling times was tested by contrasts using Bonferroni-corrected significance levels. Normality and homogeneity of the variance of the residuals were checked. Root length data were log-transformed to fulfill the assumptions of the analyses. The mean value of the saplings in the replicate block was used in the statistical analyses of the variables. The number of replicate blocks was four.

3. Results

3.1. Chlorophyll Content and Chlorophyll Fluorescence of Needles

Chlorophyll content of previous-year needles in CTRL increased and reached the maximum at week 13 (Figure 2a). After three and five weeks from the start, it was significantly lower in EGD than CTRL ($p < 0.05$). After resuming the irrigation, it increased approximately to the same level as in CTRL, but then decreased afterwards except for the last sampling time. After three weeks of MGD (week 8), it was similar to that of CTRL, but after five weeks it was significantly lower in MGD than CTRL (week 10, $p < 0.01$), as well as after three weeks of resuming irrigation.

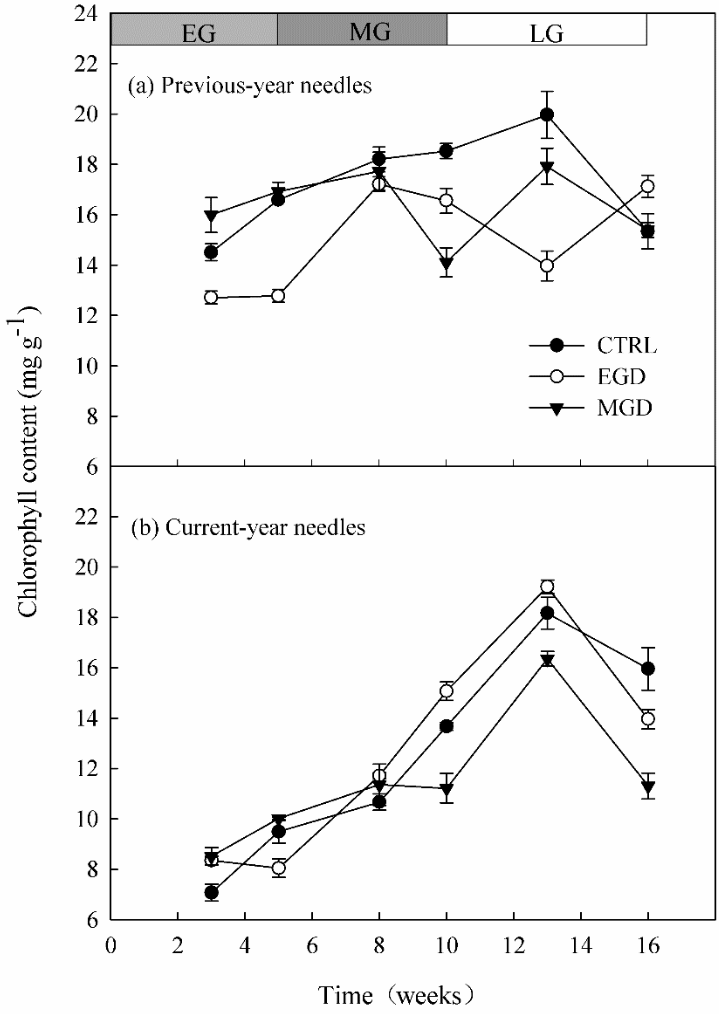

Figure 2. Chlorophyll content in the previous-year (**a**) and current-year (**b**) needles of Mongolian pine saplings by treatment. CTRL indicates the control. EGD and MGD indicate drought stress treatment in the early- (EG) and mid- (MG) growing seasons. EG, MG and LG refer to the early- (weeks 1–5), mid- (weeks 6–10) and late- (weeks 11−16) growing season, respectively. Time indicates weeks from the beginning of the experiment. Bars indicate standard error ($n = 4$).

Similar to the previous-year needles, chlorophyll content of current-year needles in CTRL increased during the experiment and reached the maximum at week 13 (Figure 2b). It was significantly lower in EGD than in CTRL after five weeks of EGD (week 5, $p < 0.05$). However, chlorophyll content of current-year needles was not reduced after three weeks of EGD like in the previous-year needles. After resuming the irrigation, chlorophyll content in EGD increased quickly and were even higher than in CTRL at two sampling times. MGD had similar effects on the chlorophyll content of the previous-year as current-year needles, except in the last sampling at the end of experiment, the chlorophyll content of the current-year needles was lower in MGD than in CTRL (Figure 2a,b).

In CTRL, F_v/F_m of the previous-year needles was fairly stable between 0.79–0.83 during the whole study period (Figure 3). F_v/F_m was lower in EGD than in CTRL after five weeks of drought and difference lasted until week 8, even though irrigation was resumed. F_v/F_m was significantly lower in MGD than in CTRL and EGD after five weeks of drought (week 10, $p < 0.05$). At the last two sampling times with similar irrigations in all treatments, there were no significant differences in F_v/F_m between the treatments.

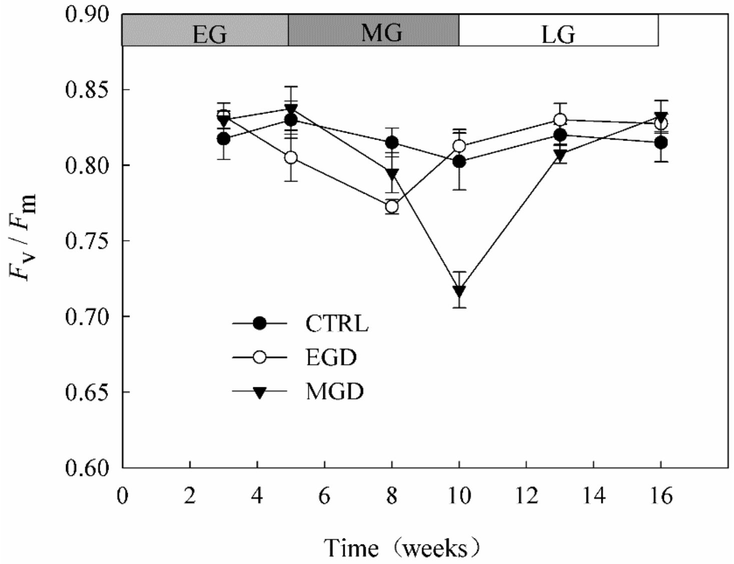

Figure 3. Dark-acclimated chlorophyll fluorescence (F_v/F_m) in the previous-year needles of Mongolian pine saplings by treatment. CTRL indicates the control. EGD and MGD indicate drought stress treatment in the early- (EG) and mid- (MG) growing seasons, respectively. EG, MG and LG refer to the early- (weeks 1–5), mid- (weeks 6–10) and late- (weeks 11–16) growing season, respectively. Time indicates weeks from the beginning of the experiment. Bars indicate standard error ($n = 4$).

3.2. Relative Electrolyte Leakage (REL) of Needles

In EGD, there was no immediate change in REL during the treatment in either previous- or current-year needles (Figure 4). However, in the previous-year needles the differences between EGD and CTRL were found at weeks 8 and 13 after resuming the irrigation ($p = 0.073$ and $p < 0.05$, respectively) (Figure 4a). In the current-year needles, the differences between EGD and CTRL were found after irrigation at weeks 8 and 10 ($p < 0.01$ and $p < 0.01$, respectively) (Figure 4b). In contrast, REL increased significantly right after three and five weeks of drought in MGD in both previous- and current-year needles ($p < 0.01$ for each), being 1.6 and 2.5 times higher in MGD than in CTRL at week 10 for these two kinds of needles, respectively (Figure 4a,b). In the latter part of the growing season with similar irrigations, there were no significant differences in REL between the treatments for the previous-year needles at week 16 and for the current-year needles at weeks 13 and 16.

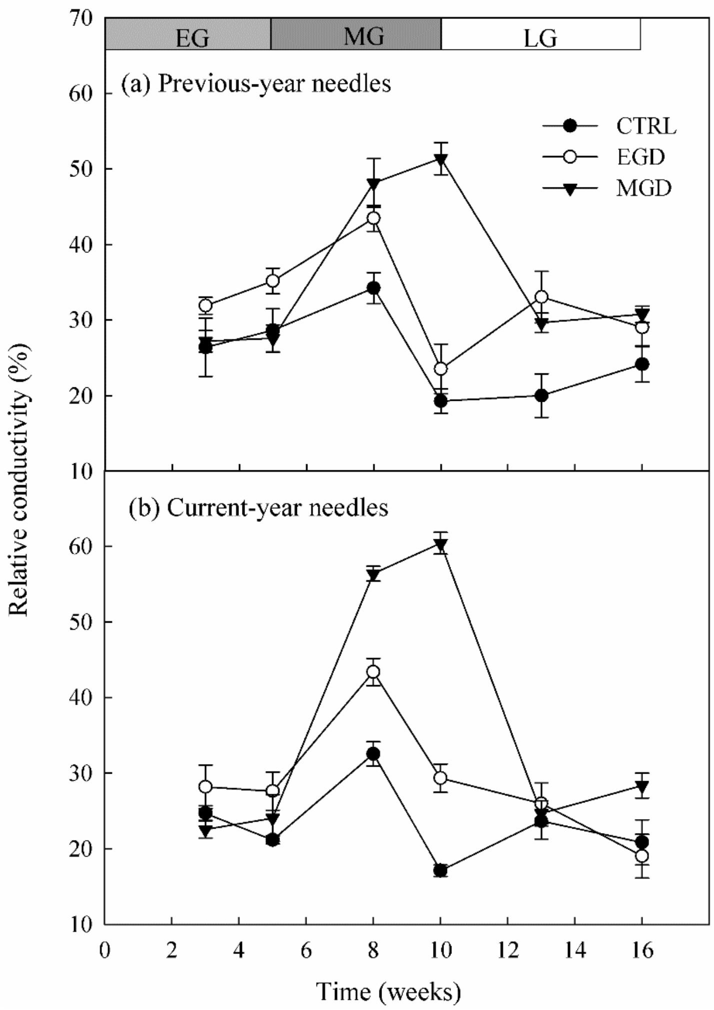

Figure 4. Electrolyte leakage in the previous-year (**a**) and current-year (**b**) needles of Mongolian pine saplings by treatment. CTRL indicates the control. EGD and MGD indicate drought stress treatment in the early- (EG) and mid- (MG) growing seasons, respectively. EG, MG and LG refer to the early- (weeks 1–5), mid- (weeks 6–10) and late- (weeks 11–16) growing season, respectively. Time indicates weeks from the beginning of the experiment. Bars indicate standard error ($n = 4$).

3.3. Non-Structural Carbohydrates (NSC) in Roots, Stems and Needles

The starch and soluble sugar content of fine roots did not differ between EGD and CTRL after three and five weeks of drought (Figure 5a,b). However, after resuming the irrigation, the starch and sugar contents were significantly lower in EGD than in CTRL at week 8 ($p < 0.01$ for both variables), week 10 ($p < 0.01$, $p = 0.086$, respectively) and week 13 ($p < 0.05$, $p < 0.001$, respectively). The starch content was significantly lower in MGD than in CTRL after three weeks of drought, and the difference lasted for the rest of sampling times ($p < 0.05$ for each) even though the irrigation had resumed (Figure 5a). The soluble sugar content was significantly lower in MGD than in CTRL after three weeks of drought ($p < 0.05$) and during the post-drought period at weeks 13 and 16 ($p < 0.001$ and $p < 0.01$, respectively) (Figure 5b). There were no significant differences in starch and soluble sugar contents between EGD and MGD, except for the soluble sugar content at week 16.

In stems, starch content was significantly lower in EGD than in CTRL at week 3 ($p < 0.01$) and week 8 ($p < 0.05$) (Figure 5c). It was significantly lower in MGD than in CTRL after five weeks of drought (week 10, $p < 0.05$) and after resuming the irrigation (week 13, $p < 0.05$). The soluble sugar content increased significantly in EGD after three and five weeks of drought ($p < 0.01$) (Figure 5d). However, it was significantly lower in EGD than in CTRL during the post-treatment period at weeks 10 ($p < 0.01$) and 16 ($p < 0.05$). The soluble sugar content was significantly lower in MGD than in CTRL at weeks 10 ($p < 0.001$) and 16 ($p < 0.01$).

In the current-year needles, starch content was lower in EGD than in CTRL and MGD after three ($p < 0.01$) and five weeks of drought and even after three weeks of resuming irrigation, but the statistical differences disappeared gradually (Figure 5e). Similarly, starch content in MGD decreased after three weeks of drought ($p < 0.001$) and the significant differences disappeared later. There were no significant differences in soluble sugar content between EGD and CTRL, despite the slight reduction in EGD (Figure 5f). The soluble sugar content was significantly lower in MGD than in CTRL after three and five weeks of drought (week 8, $p < 0.05$; week 10, $p < 0.01$), while it was slightly higher in MGD than in CTRL during the post-treatment period.

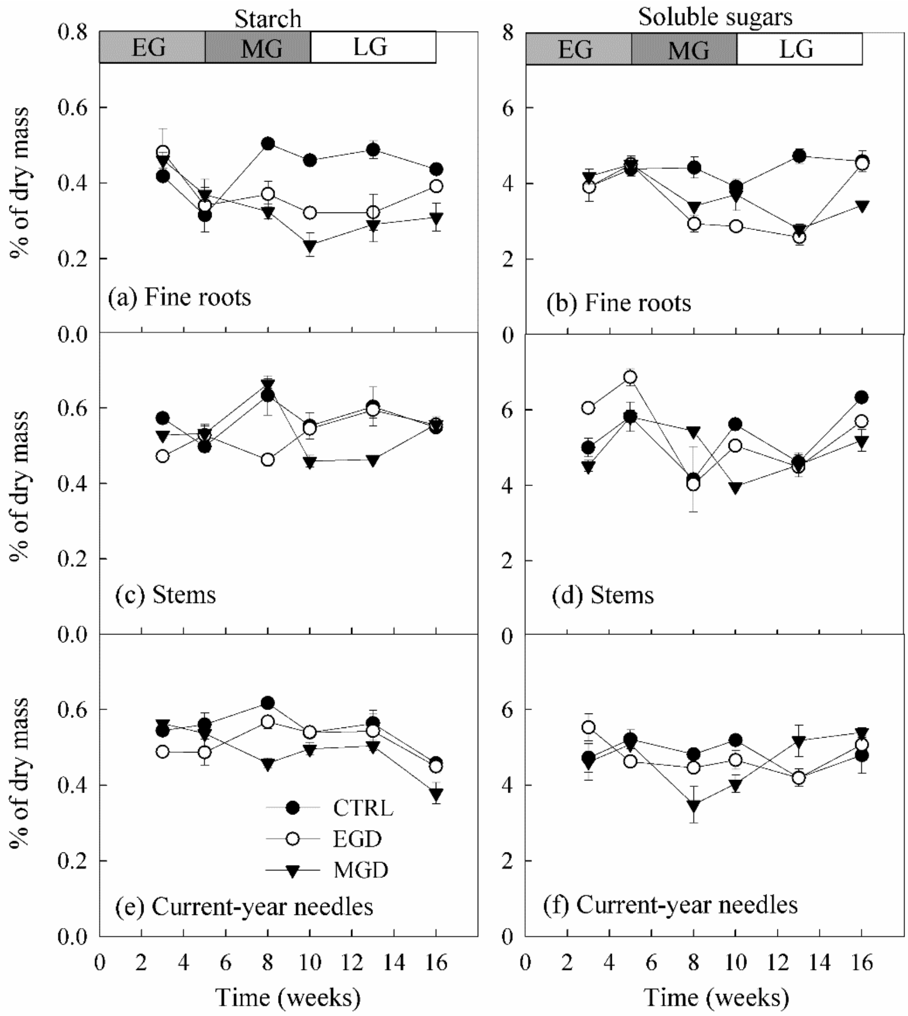

Figure 5. Starch content (left) and soluble sugar content (right) in fine roots (**a**,**d**), stems (**b**,**e**) and current-year needles (**c**,**f**) of Mongolian pine saplings by treatment. CTRL indicates the control. EGD and MGD indicate drought stress treatment in the early- (EG) and mid- (MG) growing seasons, respectively. EG, MG and LG refer to the early- (weeks 1–5), mid- (weeks 6–10) and late- (weeks 11–16) growing season, respectively. Time indicates weeks from the beginning of the experiment. Bars indicate standard error ($n = 4$).

3.4. ABA Concentration in Roots and Needles

In fine roots, the ABA concentration was significantly higher in EGD and MGD than in CTRL after three and five weeks of drought, and the differences lasted for five weeks after resuming irrigation ($p < 0.001$ for each) (Figure 6a). The ABA concentration was significantly higher in MGD than EGD after three weeks of MGD until the end of the experiment ($p < 0.001$ for each sampling time).

In contrast to the fine roots, the ABA concentration in the previous-year needles was significantly lower in EGD and MGD than in CTRL after three and five weeks of drought (Figure 6b). The differences lasted for few weeks after resuming irrigation ($p < 0.01$ for each), but disappeared at the end of the experiment.

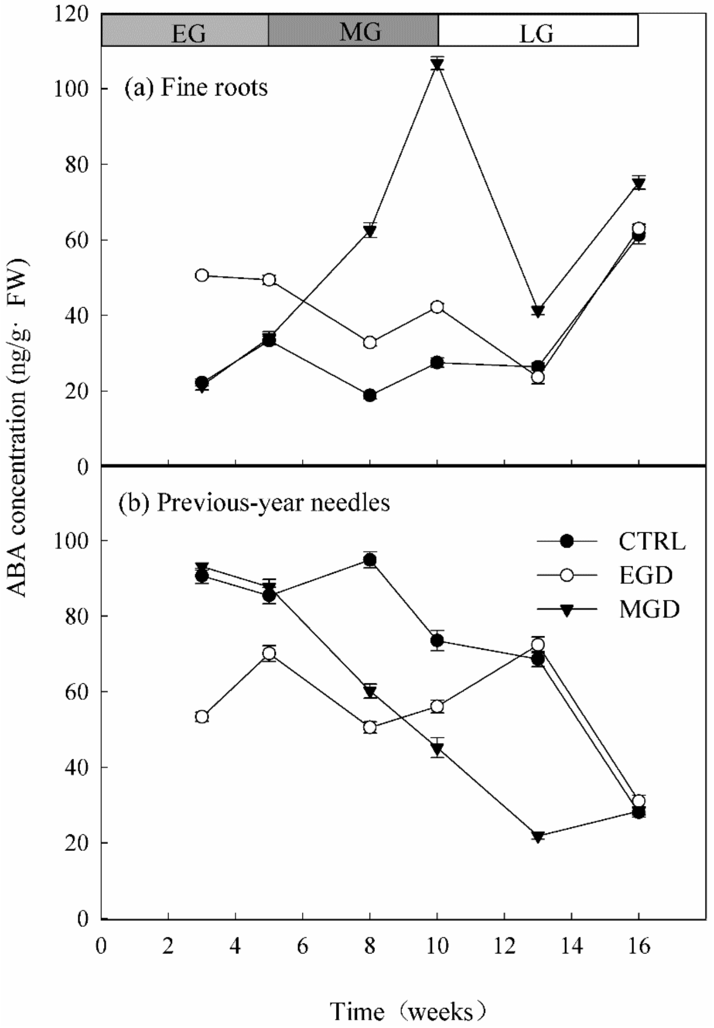

Figure 6. Abscisic acid (ABA) concentration in fine roots (**a**) and previous-year needles (**b**) of Mongolian pine saplings by treatment. CTRL indicates the control. EGD and MGD indicate drought stress treatment in the early- (EG) and mid- (MG) growing seasons, respectively. EG, MG and LG refer to the early- (weeks 1–5), mid- (weeks 6–10) and late- (weeks 11–16) growing season, respectively. Time indicates weeks from the beginning of the experiment. Bars indicate standard error ($n = 4$).

3.5. Length of Current-Year Needles

The length of the current-year needles increased in the CTRL treatment until week 15 (Figure 7a). After four weeks of EGD, the length of the new needles was shorter compared to CTRL and the significant differences lasted until week 10 ($p < 0.05$ for each). After three weeks of MGD, the growth rate of the needle length decreased, and afterwards they were always shorter than in CTRL ($p = 0.069$ at week 8, $p < 0.05$ for the later sampling times). At the end of the experiment, length of current-year needles in MGD were the shortest among the treatments.

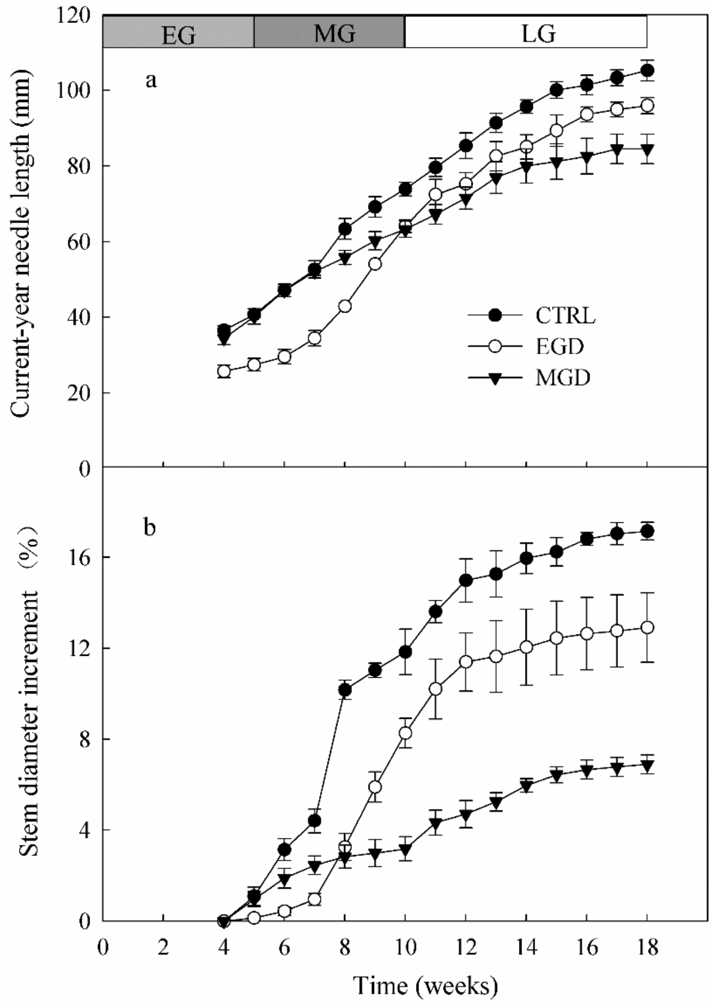

Figure 7. Mean current-year needle length (**a**) and basal stem diameter increment (**b**) of Mongolian pine saplings by treatment. CTRL indicates the control. EGD and MGD indicate drought stress treatment in the early- (EG) and mid- (MG) growing seasons, respectively. EG, MG and LG refer to the early- (weeks 1–5), mid- (weeks 6–10) and late- (weeks 11–18) growing season, respectively. Time indicates weeks from the beginning of the experiment. Bars indicate standard error ($n = 4$).

3.6. Basal Stem Diameter Increment

The basal stem diameter of the saplings increased quickly between weeks 4 and 11 in the CTRL treatment (Figure 7b). The stem diameter in the EGD saplings did not increase significantly between weeks 5 and 8 but increased thereafter. There were no statistically significant differences in stem diameter increment between MGD and CTRL at week 5 and 6. After two weeks of MGD (week 7) until the end of the experiment, stem diameter increment was significantly smaller in the MGD than CTRL saplings ($p < 0.05$ at week 7, $p < 0.001$ for the other times). Stem diameter increment was significantly smaller in MGD than in EGD ($p < 0.05$ for week 10, $p < 0.01$ for weeks 11–18).

3.7. Root Length

The total root length was just slightly lower in EGD than that in CTRL after five weeks of drought stress (week 5). However, it was significantly smaller in EGD than in CTRL five weeks after resuming irrigation (week 10) and afterwards ($p < 0.01$, $p < 0.05$, respectively). The total root length was significantly lower in MGD than in CTRL after five weeks of drought stress (week 10, $p < 0.001$) and after resuming irrigation. There were no significant differences in total root length between EGD and MGD in the end of the experiment (Figure 8).

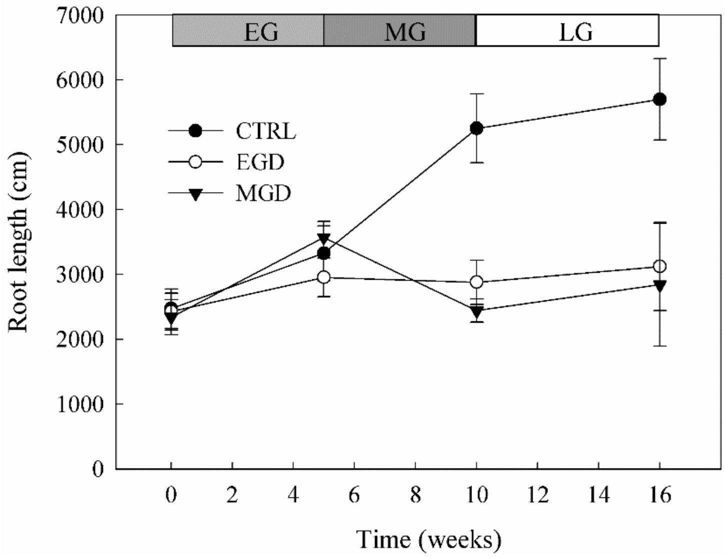

Figure 8. Root length of Mongolian pine saplings by treatment. CTRL indicates the control. EGD and MGD indicate drought stress treatment in the early- (EG) and mid- (MG) growing seasons, respectively. EG, MG and LG refer to the early- (weeks 1–5), mid- (weeks 6–10) and late- (weeks 11–18) growing season, respectively. Time indicates weeks from the beginning of the experiment. Bars indicate standard error (*n* = 4).

3.8. Sapling Mortality

The total sapling mortality was low in CTRL throughout the whole experiment, being 6.5% at the end of the experiment. In EGD, mortality increased after four weeks of drought and even after resuming irrigation (at weeks 6 and 9), being 22.6% at the end of the experiment. In MGD, the mortality increased after four weeks of drought (week 9) and continued to increase quickly after resuming irrigation (at weeks 11, 14 and 17), reaching a final mortality of 45.2% (Table 1).

Table 1. Sapling cumulative mortality of Mongolian pine saplings by treatment. CTRL indicates the control. EGD and MGD indicate drought stress treatment in the early- and mid- growing seasons, respectively.

Time (Weeks)	**Sapling Cumulative Mortality (%)**		
	CTRL	**EGD**	**MGD**
4	4	12.9	4.8
6	4	16.1	6.5
9	4.8	21.4	8.9
11	4.8	21.4	19.4
14	4.8	22.6	34.7
17	6.5	22.6	45.2

4. Discussion

We studied the physiological and growth response of Mongolian pine saplings to drought during the early (the buds burst and new needles started elongating) and middle of the growing season (shoot elongation ending and needles elongating quickly). The results showed differences in response to different drought timing. This is consistent with Forner et al. (2018) [28] that the timing of extreme drought events can affect trees more than drought intensity. Our results confirmed our hypothesis that the saplings are less resistant to drought in the middle than in the beginning of the growing season.

The drought in the early- and mid- growing season decreased stem diameter increment and needle length. This is in accordance with the previous study for European larch and Norway spruce concerning the effects of spring drought [11]. The MGD reduced the stem diameter even more than EGD, which was probably due to the timing of MGD in the rapid phase of radial growth in the middle of the growing season [12]. Conversely, the EGD saplings (drought until week 5) could compensate for the reduced growth when the irrigation started.

The total chlorophyll content and chlorophyll fluorescence of saplings decreased when the soil relative water content dropped down to 20% in both the early- and mid-growing season, indicating that the photosynthetic apparatus and photochemistry of photosynthesis were negatively affected. On the contrary, the chlorophyll fluorescence of European beech seedlings (*Fagus sylvatica* L.) was not affected by drought stress during the period of leaf development [29]. In previous-year needles, chlorophyll content was lower in EGD compared to CTRL when the soil relative water content dropped down to 36% without the reduction of F_v/F_m. This might be explained because the chlorophyll synthesized quickly in control saplings during the early growing season, and the drought inhibited the synthesis. The elevated REL of needles of the MGD saplings indicates severe cell membrane injuries, which might have contributed to the reduction of chlorophyll fluorescence and increased mortality, as compared to EGD and CTRL saplings.

The concentration of ABA in fine roots increased with drought during both growing phases. This is in accordance with Ding et al. (2016) results that showed that root ABA accumulation was induced by drought stress in rice seedlings. Elevated ABA concentration in roots increased the aquaporin expression and activity with the drought [30], which is associated with enhanced root hydraulic conductivity [31] and regulating the root hair growth [32]. However, ABA concentration in previous-year needles was reduced by drought in this study. This is inconsistent with the study for Scots pine and Sitka spruce (*Picea sitchensis* (Bong.) Carr.), where the ABA concentration of xylem sap extruded from current-year shoots increased 11 times as the drought progressed [33]. ABA synthesized in roots is translocated to leaves, leading to stomatal closure and plant growth reduction to adapt to drought stress [34]. The lower ABA concentration in the previous-year needles than current-year shoot after drought might be related to the age class of the measured needles. We assume that ABA was transported via transpiration stream more to the current-year shoots and needles than to the old needles, therefore stomata of current-year needles were probably closed, and CO_2 could not enter into the mesophyll cell, which would lead to a decrease in starch content, as it proved to be.

Starch content of needles, stems and roots decreased already during EGD and MGD, and/or just after resuming irrigation (e.g., in EGD of roots). In addition, soluble sugar content of needles was not affected by EGD, but increased in stems and decreased with a delay in roots. In MGD, soluble sugar content decreased in needles, stems and roots, but increased in needles after resuming irrigation. Therefore, production of carbohydrates by photosynthesis was not affected by drought only, but its timing during the growing season was also important too. In addition, drought seemed to change the allocation of starch among the organs to support respiration and structural and reproductive growth [35]. Transportation of energy-rich compounds to roots decreased, thus leading to carbon starvation and shortage of energy for root function [36,37]. This was corresponding to the reduced root length during the post-treatment period. Root growth was also reduced in two-year-old Scots pine saplings after drought stress [38].

Drought occurring both in the early- and mid- growing season decreased the survival rate of the saplings. Tree mortality associated with drought has been observed frequently both in the boreal forest and globally [39,40]. As shown in this study, the timing of drought also affected the mortality. The mortality of the saplings remarkably increased when drought occurred in the mid growing season, when air temperature was temporarily high and reached 38 °C at maximum. With the climate change, the extreme high temperature is expected to increase in the distribution area of Mongolian pine in northern China [41]. In this study, the mortality of the control saplings did not increase, even though they experienced the same temperature as the saplings in the drought treatments. This indicates that the high temperature alone did not increase the sapling mortality. The joint effect of high temperature and drought probably led to the sapling mortality. Many studies demonstrated that mortality can occur faster in hotter drought in a review by Allen et al. (2015) [42]. This is because that hotter drought increased risks of both hydraulic failure (irreversible xylem dysfunction) and carbon starvation [43]. Carbon starvation might have occurred since the starch and sugar content in fine roots, current-year needles, and stems were reduced by drought in the mid growing season. In this study, the high sapling mortality in MGD treatment might be also related to hydraulic failure, which was enhanced by rising atmospheric moisture demand [44], as atmospheric moisture demand increases largely with hotter temperature when accompanying drought [45].

5. Conclusions

The impacts of drought in the middle of the growing season on the Mongolian pine saplings was more harmful in terms of the needle length, stem diameter, electrolyte leakage of needles and sapling mortality. In spite of the less effect of drought in the early growing season on the saplings, it reduced root growth and allocation of non-structural carbohydrates to roots as much as the drought in the middle of the growing season. Special attention should be paid on forest sites susceptible to drought during afforestation in face of ongoing climate change.

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