



Prolonged drought from winter to spring affected the phenology, growth, and physiology of differently pretreated *Pinus sylvestris* var. *mongolica* seedlings

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Abstract

Key message Continuing drought from winter to spring delayed the spring bud phenology of seedlings, and seedlings experiencing colder winter previously were less influenced by the severe drought.

Abstract Water availability at the beginning of the growth phase, and even before it, is decisive in the phenology and annual cycle of forest trees, consequently affecting carbon sequestration and forest ecosystem balance. This is a novel experimental study on the effects of continuous drought throughout winter and early spring on tree performance. Two groups of *Pinus sylvestris* var. *mongolica* seedlings were overwintered in a Chinese solar greenhouse (BIG seedlings) and a plastic tunnel (SMALL seedlings). The seedlings were subjected to continuing droughts from winter to an extra 0 (control), 15 (D15), 30 (D30) and 45 days without irrigation (D45) after soil thawing next spring. Bud phenology, tree growth and physiology were examined. Bud phenological development, tree aboveground growth and root biomass growth were delayed in treatments D30 and D45 in both seedling types. SMALL seedlings had earlier bud phenology and were less influenced by drought than BIG seedlings. The drought-induced changes in spring phenology were associated with higher ABA and lower GA3 concentrations of needles. The phenology and growth differences between the seedling types might relate to soluble sugar concentrations of roots, needle chlorophyll content, needle chlorophyll fluorescence and acclimated morphological changes, such as root-shoot ratio. We suggest that in forest management, a big seedling size does not guarantee growth success, but attention should be paid to the proper conditions in overwintering and storing of the seedlings prior planting. Prolonged drought throughout winter and early spring should be avoided especially before planting the seedlings in an area prone to drought.

Keywords Mongolian Scots pine · Aridity · Bud phenology · Nonstructural carbohydrates · Endogenous hormones · Seedling size

Introduction

Drought intensity and frequency and land area affected by drought continue to increase with global warming (Dai et al. 2013; Trenberth et al. 2014; Samaniego et al. 2018).

Drought has become a serious problem restricting tree growth, increasing tree mortality, and declining forest productivity, thus affecting the carbon sequestration of forest ecosystems (Allen et al. 2010). The timing of drought may affect tree growth as importantly as drought intensity and duration do. Moreover, the timing of drought is a crucial factor in response to tree recovery after drought (Huang et al. 2018). Many studies have focused on the effects of summer drought on tree functioning. However, in recent years, winter and spring droughts have occurred frequently (Huang et al. 2019), and their implications for trees deserve further studies.

Water available from snowfall in winter is critical for tree growth during the subsequent growing season (Wu et al. 2019). Winter snow had a compensation effect on subsequent growing-season larch growth, especially in dry years

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in snow-rich regions in Northeast China (Li et al. 2021). On the contrary, drought in winter resulted in a dramatic decline in stem moisture of three conifer species, a 50% reduction in spring starch accumulation, and a concomitant decline in tree growth in California, USA (Earles et al. 2018). Winter drought also changed foliar metabolic substances (thiol and amino acid concentrations) similarly to summer drought did in date palm (*Phoenix dactylifera*) (Du et al. 2021). Spring drought stresses plants in their early growth phase and it has been reported to reduce stem diameter growth (D'Orangeville et al. 2018; Bose et al. 2021) and total annual aboveground tree growth (Swidrak et al. 2013). However, the impacts of continued drought throughout winter to spring and different durations of winter and spring droughts on forest trees have not been addressed well.

In late winter and early spring, tree sap begins to flow and tree growth starts with rising air and soil temperatures (Juice et al. 2016; Repo et al. 2021). During this time, irrigation is usually applied to meet the initial growth of trees in urban landscape, whereas natural forests depend on precipitation. If drought continues from winter to spring, spring phenology of trees might change. Spring phenology, such as bud swelling and bud burst, is driven by preceding winter chilling, the increasing length of the photoperiod and by rising temperature (Basler and Körner 2014). Freezing damage can be avoided and a long growing season ensured by a timely bud development. Several previous studies on pre-season drought have shown contrasting findings on the spring phenology of trees. Pre-season droughts delayed the start of the growing season in forests in the North China Plain (Ji et al. 2021). Rain reduction delayed the needle emergence of *Pinus edulis* while treatment effect was small on *Juniperus monosperma* in New Mexico, USA (Adams et al. 2015). Conversely, spring drought initiated an earlier spring growth in *Erica multiflora* shrub in Catalonia, Spain (Bernal et al. 2011). A study on the sensitivity of plants to pre-season droughts in Northeast China Transect found the earlier start of the growing season in forest ecosystems too (Yuan et al. 2020). The change of spring phenology of shoots and roots by pre-season drought might affect tree growth negatively during the growing season (Zeng et al. 2021).

The phenological changes are associated with physiological responses including phytohormones, phytochromes and carbohydrates (Chao et al. 2007). The cause of death of individual Scots pines at their southern distribution edge in eastern Spain was suggested to connect with winter drought and the hydraulic failure (Voltas et al. 2013). Carbon starvation is another main mechanism to explain tree death (McDowell 2011; Choat et al. 2018). Nutrients can directly impact both carbon and water uptake and use, but their effects on tree mortality have not been considered as the main mechanisms in regard to drought (Gessler et al. 2017). Chlorophyll content and chlorophyll fluorescence have been used

as parameters to indicate photosynthesis (Baker et al. 2008). Non-structural carbohydrates (mainly starch and soluble sugars) in roots reflect available carbon reserve (Wiley et al. 2013). The change of endogenous hormones under drought stress have been associated with the regulation of the stomatal conductance and transpiration (Jacobsen et al. 2009; Fang et al. 2015; Zhou et al. 2022).

Mongolian Scots pine (*Pinus sylvestris* var. *mongolica*) is an ecologically and economically important tree species in northern China where it has effects on the soil and water conservation and ecosystem recovery. Two to four-years-old container seedlings have been commonly used in afforestation. Seedling height and root collar diameter are important morphological traits for judging the seedling quality in a plantation. Initial seedling size was an important variable in predicting survival, diameter, and dominance (Pinchot et al. 2018). Seedling survival and growth in afforestation were strongly related to height (Jiang et al. 2022). However, a low sapling survival rate in young forests, low growth rate and frequent dieback in adult forests have occurred, which can relate to water stress (Li et al. 2020; Liu et al. 2018). The previous growing conditions of the seedlings might also affect the resistance to environmental stress. The different overwinter storage methods might be one of the reasons affecting the performance of seedlings under drought stress. In the nursery practice, it is common to leave the container seedlings in the field and fill the gap between the containers by soil, or move the containers to a solar greenhouse to avoid freeze damage to root systems, while bare-rooted plants are usually left in soil (beds). The container seedlings are also stored in a freezer to overwinter in Northern Europe and North America (Pantová et al. 2021).

Previously we found that drought in the early growing season (after bud burst) reduced the growth and starch and soluble sugar concentrations in roots (Qian et al. 2021). However, the effects of continuous drought prior to the start of tree growth on the phenology and physiological changes of tree seedlings are not yet studied. In this study, we put half of the seedlings to overwinter in a solar greenhouse where the seedlings started to grow early in next spring and therefore developed larger in size. The other half of the seedlings overwintered in a plastic tunnel (commonly known as a cold shed), started growth later in spring, and thus grew smaller. The different pretreatments led to statistically significant differences in seedling heights and the two groups are therefore hereinafter referred to as BIG and SMALL seedlings. We aimed to find out how continuous winter and spring drought affect the phenology and growth of Mongolian Scots pine seedlings, to study whether the responses of the two kinds of pretreated seedlings differ, and to consider possible underlying physiological mechanisms in the drought responses. We generally hypothesized that the spring phenology, growth and physiology of all Mongolian Scots pine seedlings are

affected by the drought treatments. A further hypothesis is that the two pretreated seedlings perform differently during and after drought, and in more detail, SMALL seedlings would have better resistance to winter and spring drought since they experienced tougher conditions previously and thus might be better acclimated than BIG seedlings.

Materials and methods

Experimental set-up

In the beginning of September 2019, one-year-old bare-root seedlings of Mongolian Scots pine were purchased from a nursery in Weichang County, Hebei Province, China (42°01'N, 117°37'E). The roots of the seedlings were dipped in mud and shipped to Hebei Agricultural University in Baoding, Hebei Province, China (38° 84'N, 115°48'E). Upon arrival, the seedlings were planted in round plastic pots (20 cm in diameter, 15 cm in height) in fertilized growing media (peat:vermiculite:perlite 3:2:1 in volume, pH neutral, bulk density $0.38 \pm 0.03 \text{ g/cm}^3$), and two months of growth in a plastic tunnel was applied for conditioning of the seedlings to the experimental site. The plastic tunnel (14 m in length, 4 m in width) had a steel frame covered by a plastic sheet. The plastic sheet was opened or closed from the ground to one-meter height according to the weather.

In November 2019, half of the seedlings were moved to an adjacent Chinese solar greenhouse (34 m in length, 7 m in width) for overwintering while the other half of the seedlings remained in the plastic tunnel. Seedlings can overwinter in the solar greenhouse without the risk of winter freezing injury (Luo et al. 2023), however, the average air temperature in the plastic tunnel could be below-zero during winter. The seedlings in the solar greenhouse started to grow in early February 2020, whereas the growth of seedlings in the plastic tunnel started between mid and late March. The seedlings were irrigated regularly after their growth started. The seedlings were moved from the solar greenhouse to the plastic tunnel from April 25th–27th, and all the seedlings were watered with tap water once a week and weeded in summer until the drought treatments began in winter of 2020 to 2021. Before the start of drought treatments, the seedlings previously overwintered in the solar greenhouse were on average 40.7 ± 0.5 (mean \pm SE) cm tall and the seedlings previously overwintered in the plastic tunnel were 29.3 ± 0.3 cm tall. The two seedling groups differed significantly in height (*t*-test, $P < 0.05$), therefore, the taller and shorter seedlings are called BIG seedlings and SMALL seedlings, respectively.

Both 192 BIG seedlings and 192 SMALL seedlings were randomly divided into four treatments, and each treatment consisted of four replicate blocks with 12 seedlings in each

replicate block. The soil volumetric water content of all the seedlings was maintained at 16–20% until the soil was irrigated with amply water on November 20, 2020. After that, no watering was carried out throughout the winter. Drought treatments were initiated on February 17, 2021, when the daily average air temperature had stabilized above 0°C for one week, and the soil was fully thawed (soil temperature $> 0^\circ\text{C}$ at 10 cm depth). The four treatments were (i): control, the soil was watered normally after thawing (CK); (ii): continuing drought for 15 days (D15); (iii): continuing drought for 30 days (D30); (iv): continuing drought for 45 days (D45). Watering was resumed after each drought treatment ended. The growing conditions of seedlings were continuously monitored in the plastic tunnel.

Air temperature and humidity were monitored and recorded every ten minutes with a temperature and humidity recorder T20R (Miaoguan Technology Co., Ltd., Shenzhen, China) that was placed in the middle of the plastic tunnel. Soil temperature was monitored and recorded every hour with three soil thermometers T10R (Miaoguan Technology Co., Ltd., Shenzhen, China) that were distributed at edges and in the middle of the plastic tunnel. The temperature probe was buried at a depth of 3 cm under the soil in the middle of the pot and the data was exported by THtool-V200.

The seedlings in each replicate block of each treatment were randomly selected every other day to measure the soil moisture by means of a soil moisture meter (HH2, Delta-T, UK). A 10-cm-long humidity probe (WT200) was completely inserted into the soil, was measured thrice in different positions in each pot, and the soil volumetric water content was obtained by calculating the means.

Spring bud phenology, shoot and root growth

After the start of different drought treatments, the bud phenological development of Mongolian Scots pine seedlings were observed twice a week. The phenology of buds was divided into five phenophases based on a discrete class system according to the classification for Scots pine in Ewa Jach and Ceulemans (1999): 1 = bud closed (bud dormant and unchanged in size), 2 = bud swelling slightly, 3 = swollen bud, 4 = bud elongation, 5 = bud burst and needle emergence (Fig. 1). Forty seedlings were always selected at random from each treatment to observe the phenology and the number of buds in each phenophase were recorded. Bud development degree (%) in each observation time was calculated. The bud phenological numerical value (1, 2, 3, 4, 5) in each seedling was used in the bud phenology statistical analysis.

One seedling per replicate block in each treatment ($4 \times 4 = 16$ seedlings for each seedling type, 32 seedlings in total) was designated for plant height and basal stem diameter measurements at 2-week intervals. The plant



Fig. 1 Bud phenological development: 1 = bud closed (bud dormant and unchanged in size), 2 = bud swelling slightly, 3 = swollen bud, 4 = bud elongation, 5 = bud burst and needle emergence

height was measured using a tape measure, while the basal stem diameters were measured twice perpendicularly 2 cm above the soil on the main stem using vernier calipers. Proportional height or diameter increment was calculated as the increment at each measurement times divided the original height or diameter.

On day 60, one seedling from each of the four replicate blocks in each treatment ($4 \times 4 = 16$ seedlings for each seedling type, i.e. 32 seedlings in total) was randomly selected for biomass measurement. The shoots and roots were separated from each other. The entire root system was carefully extracted from the pot and separated from the soil and possible detached roots were collected from the surrounding soil. All the roots and shoots were gently rinsed with tap water and deionized water, and then dried in an oven at 40 °C to constant weight, respectively, as root biomass and shoot biomass. The root-shoot ratio was calculated accordingly.

Physiological measurements of needles and roots

Since the start of the drought treatments, the physiological parameters of aboveground and underground tree parts were measured every 15 days for a total of six times. The six sampling times took place on days 0, 15, 30, 45, 60, and 75, respectively. At each sampling time, one seedling was randomly taken from each of the four replicate blocks in four treatments for both BIG seedlings and SMALL seedlings, that is, 16 seedlings per seedling type, and 32 seedlings in total.

The fresh needles from the middle of the side branches of the previous-year shoots weighing 0.1g were rinsed with deionized water and cut into smaller pieces and immersed in a mixed solution of ethanol and acetone (volume ratio, 1:1) for 24 h in dark. The extracted solution was measured spectrophotometrically at 645 nm and 663 nm using a U-5100 Spectrophotometer (Hitachi High-tech

Science, Tokyo, Japan). The total chlorophyll content was quantified.

Five intact needles were randomly selected from the middle of the side branches of the previous-year shoots in each seedling per treatment as a sample. The five needles in each sample were horizontally aligned and securely affixed to a transparent tape strip to ensure a seamless connection between them. The samples were subsequently subjected to a dark-acclimation period of 20 min at room temperature prior to determining dark-adapted chlorophyll fluorescence parameters using a portable fluorescence meter (Handy-PEA, Hansatech, UK) under continuous exposure to saturated excitation light of $3000 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ for 10 s (F_v/F_m).

The rinsed roots were collected from each seedling and packed in tinfoil, inserted briefly in liquid N and stored in a freezer at -80°C for further analysis. The frozen roots were thawed at room temperature and dried to constant weight. Subsequently they were milled, and 0.1 g of dry powder was weighed to determine the content of soluble sugar and starch by the method in Hansen and Møller (1975). The soluble sugars were extracted using 80% ethanol. The concentration of total soluble sugars was determined spectrophotometrically at 620 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.

To explore the change of hormone and nutrients of needles with the time of drought, we selected the BIG seedlings in CK and D45 treatments for the measurements. At each sampling time, fresh needles of ca. 0.5 g were picked from the middle of lateral branches of the previous-year shoots of BIG seedlings in CK and D45 treatments in each of the four replicate seedlings for the hormone determination. The rinsed needles were packed in tinfoil, inserted briefly in liquid N and stored at -80°C for further determining the content of abscisic acid (ABA), gibberellin (GA3) and indoleacetic (IAA) by enzyme-linked immunosorbent assay (ELISA) (Yang et al. 2001). Similar to the hormone determination, fresh needles of 0.5 g were picked from the middle of lateral branches of the previous-year shoots of BIG seedlings in CK and D45 treatments in each of the four replicate seedlings and stored at -80°C before they were dried and milled to a powder for the macroelement content determination. Prior to measurement, the samples underwent boiling with sulfuric acid and hydrogen peroxide. Total nitrogen content was determined using a flow injection analyzer, phosphorus content was analyzed through vanadium-molybdenum yellow absorbance spectrophotometry, and potassium content was measured by flame photometry (Ministry of Agriculture of China 2011).

Statistical analysis

The effects of the drought treatments on the variables were analyzed by means of linear mixed models (procedure MIXED in SPSS 27.0, SPSS Inc., Chicago, IL, USA) (as by Wang et al. 2013). The model used for bud phenology, growth, chlorophyll content, F_v/F_m of needles, and the soluble sugar and starch of roots was $y = \mu + \text{treatment} + \text{time} + \text{size} + \text{treatment} \times \text{time} + \text{size} \times \text{treatment} + \text{size} \times \text{time} + \text{treatment} \times \text{time} \times \text{size} + \varepsilon$, where μ is a constant. The ‘treatment’ (i.e., CK, D15, D30, D45), ‘time’ (i.e., sampling times) and ‘size’ (BIG and SMALL seedlings) were regarded as fixed factors, and ε as a random term. The significance of the difference between the treatments at different sampling times in each seedling type was tested by pairwise comparisons using Bonferroni-corrected significance levels. The model for root biomass and root-shoot ratio was $y = \mu + \text{treatment} + \text{size} + \text{treatment} \times \text{size} + \varepsilon$, where treat was CK, D15, D30, D45, size was seedling types, others are the same as in the above models. The differences in root biomass and root-shoot ratio between the treatments in different seedling types were tested by a Bonferroni-corrected pairwise test. The model for hormone and nutrients was $y = \mu + \text{treatment} + \text{time} + \text{treatment} \times \text{time} + \varepsilon$, where treatment was CK and D45, time was sampling times, others are the same as in the above model. The significance of the difference between the two treatments at different sampling times was tested by Bonferroni-corrected pairwise test. The normality and homogeneity of the variances of the residuals were checked by means of scatter plots and Q–Q plots. The selection of the covariance structure was based on Akaike’s information criteria. The replicative numbers for bud phenology ($n=40$), and for other variables ($n=4$) in each treatment were used in the statistical analyses.

Results

Air and soil conditions

During the drought treatments, the daily average air and soil temperature was always above 0°C and below 30°C . Before the onset of the experiment (day 0), the daily average temperature was above 0°C for 7 consecutive days. The average air and soil temperature was around 6°C during days 0–15, $12\text{--}13^\circ\text{C}$ during days 15–45, and $17\text{--}18^\circ\text{C}$ during days 45–75 (Fig. 2a, b). The daily average air humidity was 44–59% during the experiment (Fig. 2c). The soil volumetric water content at the onset of the continuing drought treatment (day 0) was ca. 4.6%. It decreased to 4.4% after 15 days continuing drought (D15), to 0.5% after 30 days continuing drought (D30) and to 0.2% after 45 days of continuing drought (D45). The soil volumetric water content of CK

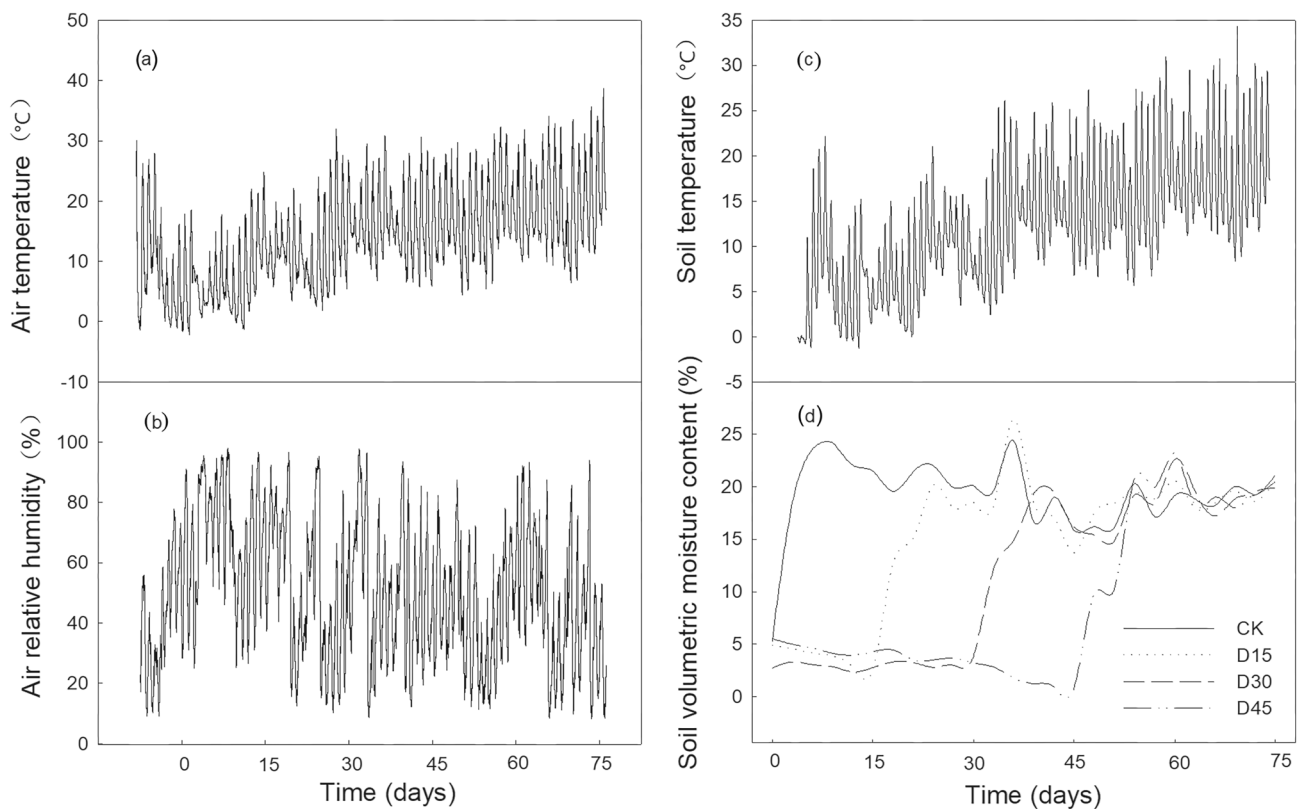


Fig. 2 The daily average air (a) and soil temperature (b), air relative humidity (c) and soil volumetric water content in the pots (d) during the experiment in the plastic shed

seedlings was maintained at ca. 20% with regular irrigation after day 0. After the end of each drought treatment period, soil volumetric water content was ca. 20% in the drought treatments as in the control (Fig. 2d).

Bud phenological development

The bud phenological development in SMALL seedlings was a little earlier than in BIG seedlings at the same observation timepoint ($P_{T \times \text{Size}} = 0.08$, $P_{T \times \text{Tr} \times \text{Size}} = 0.058$, Table 1; Fig. 3). In CK seedlings, the differences can be seen on days 39, 42, 45 when the buds entered to the next phenophase in SMALL seedlings, but not in BIG seedlings (Fig. 3a, e). In D15 seedlings, there were significant differences in bud swelling proportion between BIG and SMALL seedlings on day 31 ($P = 0.042$). In D45 seedlings, buds swelled on day 39 in SMALL seedlings, but it was seen on day 49 in BIG seedlings. All the buds swelled in SMALL seedlings, but still some buds were closed in BIG seedlings on day 55, showing significant differences between the two seedling types in D30 ($P = 0.015$) and D45 ($P = 0.004$) (Fig. 3d, h).

There were significant differences between the drought treatments in bud phenological development ($P_{\text{Tr}} < 0.001$, Table 1). D15 treatment had no remarkable differences with

CK, but at each observation timepoint, the proportion of the buds in higher phenological state was a bit larger than in CK (Fig. 3). However, treatments D30 and D45 delayed the bud phenology significantly for both BIG and SMALL seedlings in each time ($P < 0.001$ for each). For both the BIG and SMALL seedlings, the majority (over 85%) of the buds of the seedlings in CK and D15 treatment was in slight bud swelling state on day 31, whereas the slight bud swelling state in majority was seen later on days 39 and 49 in D30 and D45 treatments, respectively (Fig. 3).

Increment of basal stem diameter and plant height

There were significant differences between the drought treatments, and the interaction of seedling type and sampling time was nearly significant in basal stem diameter increment ($P_{\text{Tr}} < 0.001$, $P_{T \times \text{Size}} = 0.084$, Table 1). The basal stem diameter of both BIG and SMALL seedlings started increasing continuously in the CK treatment on day 30. In both BIG and SMALL seedlings, proportional basal stem diameter increment was smaller in D30 and D45 treatment on day 30 than in other treatments ($P = 0.028$, $P = 0.037$ for SMALL), and was significantly smaller in D45 treatment on day 45 than in other treatments ($P < 0.001$ for each in BIG and $P < 0.01$

Table 1 Summary of statistical tests (degrees of freedom, *F*-value, *P*-value) of the effects of drought treatment, sampling time, seedling types and their interactions on the measured parameters

Response	df1; df2; <i>F</i> ; <i>P</i>						
	Time	Treatment	Seedling type	Time × Treat- ment	Time × Seed- ling type	Treat- ment × Seed- ling type	Time × Treat- ment × Seedling type
Bud phenology	6; 312.0; 1888.5; < 0.001	3; 312.0; 359.5; < 0.001	1; 312.0; 2.0; 0.158	18; 312.0; 163.2; < 0.001	6; 312.0; 1.9; 0.080	3; 312.0; 0.2; 0.916	18; 312.0; 1.6; 0.058
Proportional height incre- ment	4; 24.0; 136.7; < 0.001	3; 24.0; 3.5; 0.032	1; 24.0; 5.2; 0.032	12; 24.0; 6.5; < 0.001	4; 24.0; 3.1; 0.036	3; 24.0; 0.2; 0.889	12; 24.0; 2.6; 0.022
Proportional diameter increment	4; 24.0; 209.6; < 0.001	3; 24.0; 21.5; < 0.001	1; 24.0; 0.2; 0.674	12; 24.0; 14.1; < 0.001	4; 24.0; 2.3; 0.084	3; 24.0; 2.2; 0.118	12; 24.0; 1.6; 0.169
Chlorophyll content (nee- dles)	4; 32.3; 106.5; < 0.001	3; 33.1; 8.6; < 0.001	1; 33.2; 16.9; < 0.001	12; 32.9; 3.4; 0.002	4; 32.3; 2.0; 0.126	3; 33.1; 4.0; 0.015	12; 32.9; 4.2; < 0.001
<i>F_v/F_m</i> (needles)	4; 31.5; 22.5; < 0.001	3; 32.2; 2.4; 0.083	1; 32.2; 1.1; 0.310	12; 31.9; 3.1; 0.006	4; 31.5; 0.5; 0.738	3; 32.2; 0.5; 0.718	12; 31.9; 0.8; 0.678
Starch content (roots)	4; 78.3; 12.6; < 0.001	3; 29.1; 2.0; 0.135	1; 29.1; 1.5; 0.236	12; 78.3; 5.7; < 0.001	4; 78.3; 2.8; 0.030	3; 29.1; 0.7; 0.532	12; 78.3; 2.8; 0.003
Soluble sugar content (roots)	4; 73.4; 45.9; < 0.001	3; 36.6; 5.5; 0.003	1; 36.6; 2.5; 0.123	12; 77.2; 7.1; < 0.001	4; 73.4; 2.9; 0.030	3; 36.6; 3.3; 0.030	12; 77.2; 2.9; 0.002
Root-shoot ratio		3; 24.0; 0.4; 0.743	1; 24.0; 2.3; 0.141			3; 24.0; 2.7; 0.065	
Root biomass		3; 20.0; 3.0; 0.055	1; 20.0; 6.5; 0.019			3; 20.0; 1.0; 0.426	
ABA (needles in BIG seed- lings)	2; 5.2; 216.5; < 0.001	1; 4.0; 341.0; < 0.001		2; 5.2; 166.2; < 0.001			
IAA (needles in BIG seed- lings)	2; 4.0; 0.9; 0.470	1; 4.0; 12.0; 0.026		2; 4.0; 12.9; 0.018			
GA3 (needles in BIG seed- lings)	2; 9.1; 24.9; < 0.001	1; 6.5; 48.9; < 0.001		2; 9.1; 5.1; 0.033			
N (needles in BIG seed- lings)	2; 4.0; 2.7; 0.179	1; 4.0; 0.0; 0.899		2; 4.0; 0.1; 0.937			
P (needles in BIG seed- lings)	2; 4.0; 0.1; 0.870	1; 4.0; 3.3; 0.145		2; 4.0; 1.5; 0.325			
K (needles in BIG seed- lings)	2; 7.1; 0.4; 0.691	1; 3.9; 0.5; 0.518		2; 7.1; 5.1; 0.041			

P values ≤ 0.05 are in bold

for each in SMALL), but the growth in D30 was recovered almost to the same level with CK after resuming irrigation (days 45, 60, 75). However, the diameter increment was still lower in D45 than in other treatments on day 60 (*P* < 0.01 for each in BIG, *P* < 0.05 for each in SMALL). In SMALL seedlings, the increment of the basal stem diameter was significantly higher in D15 than in CK on days 30 (*P* = 0.005), 45 (*P* = 0.02), and 60 (*P* = 0.006) (Fig. 4 a, b).

There were significant differences between the drought treatments and between the seedling types in height increment (*P_{Tr}* = 0.032, *P_{Size}* = 0.032, Table 1). The plant height of BIG and SMALL seedlings started to increase continuously in CK treatment during days 30 and 45. In BIG seedlings, the plant height increment was smaller in D45 compared to the CK on days 60 (*P* = 0.003) and 75

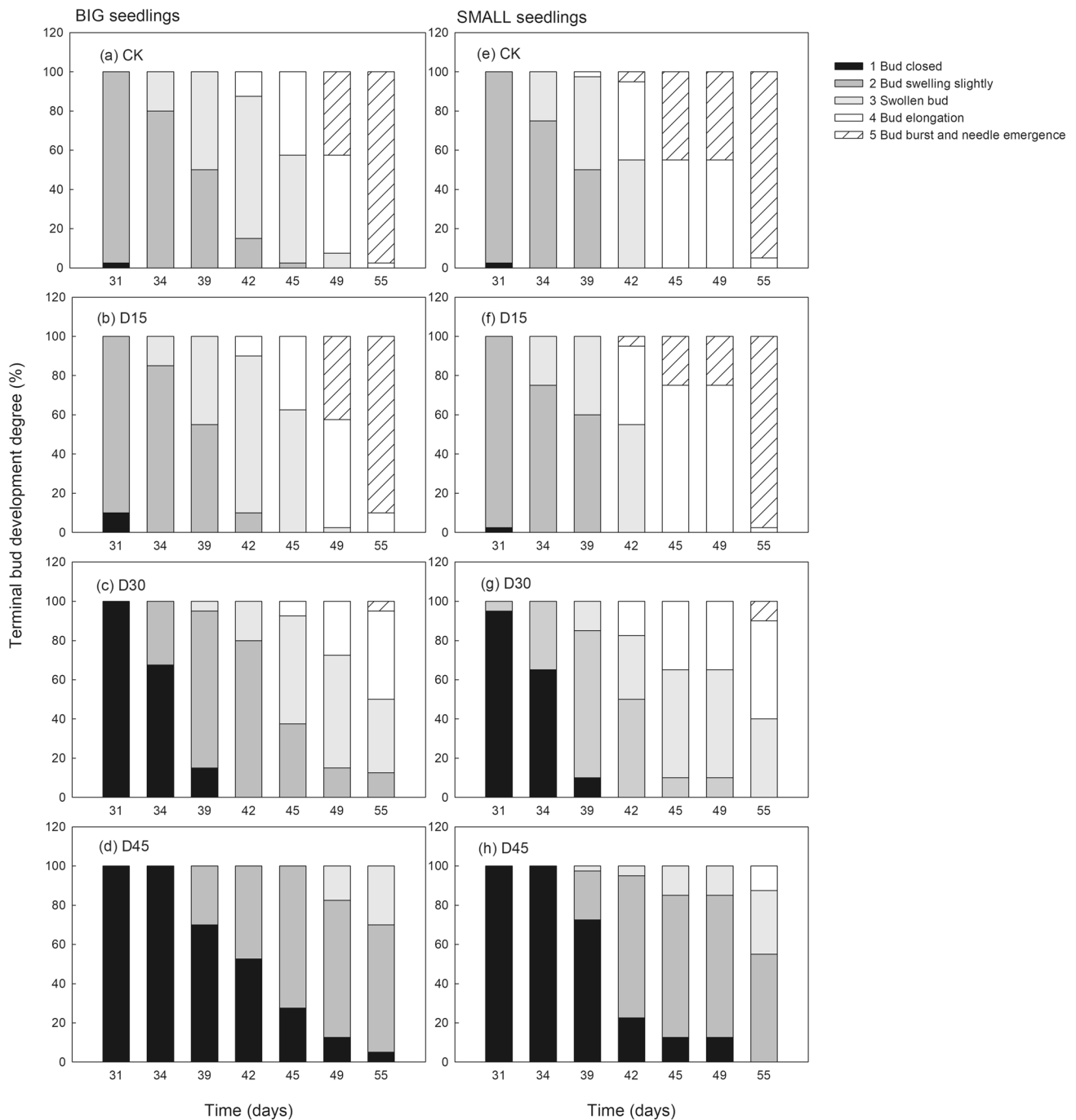


Fig. 3 Terminal bud development degree in BIG (left panel) and SMALL (right panel) seedlings under different drought treatments. **a–d** were the terminal bud development degree in CK, D15, D30,

D45, respectively, in BIG seedling group. **e–h** were the terminal bud development degree in CK, D15, D30, D45, respectively, in SMALL seedling group

($P=0.002$). In SMALL seedlings, the plant height increment was significantly lower in D45 than in CK and D15 on day 60 ($P=0.009$, $P=0.011$, respectively) and it recovered to a similar level to CK later (Fig. 4 c, d).

Root biomass and root-shoot ratio

There were nearly significant differences between the treatments and significant differences between the two seedling types in root biomass ($P_{Tr}=0.055$, $P_{Size}=0.019$ respectively,

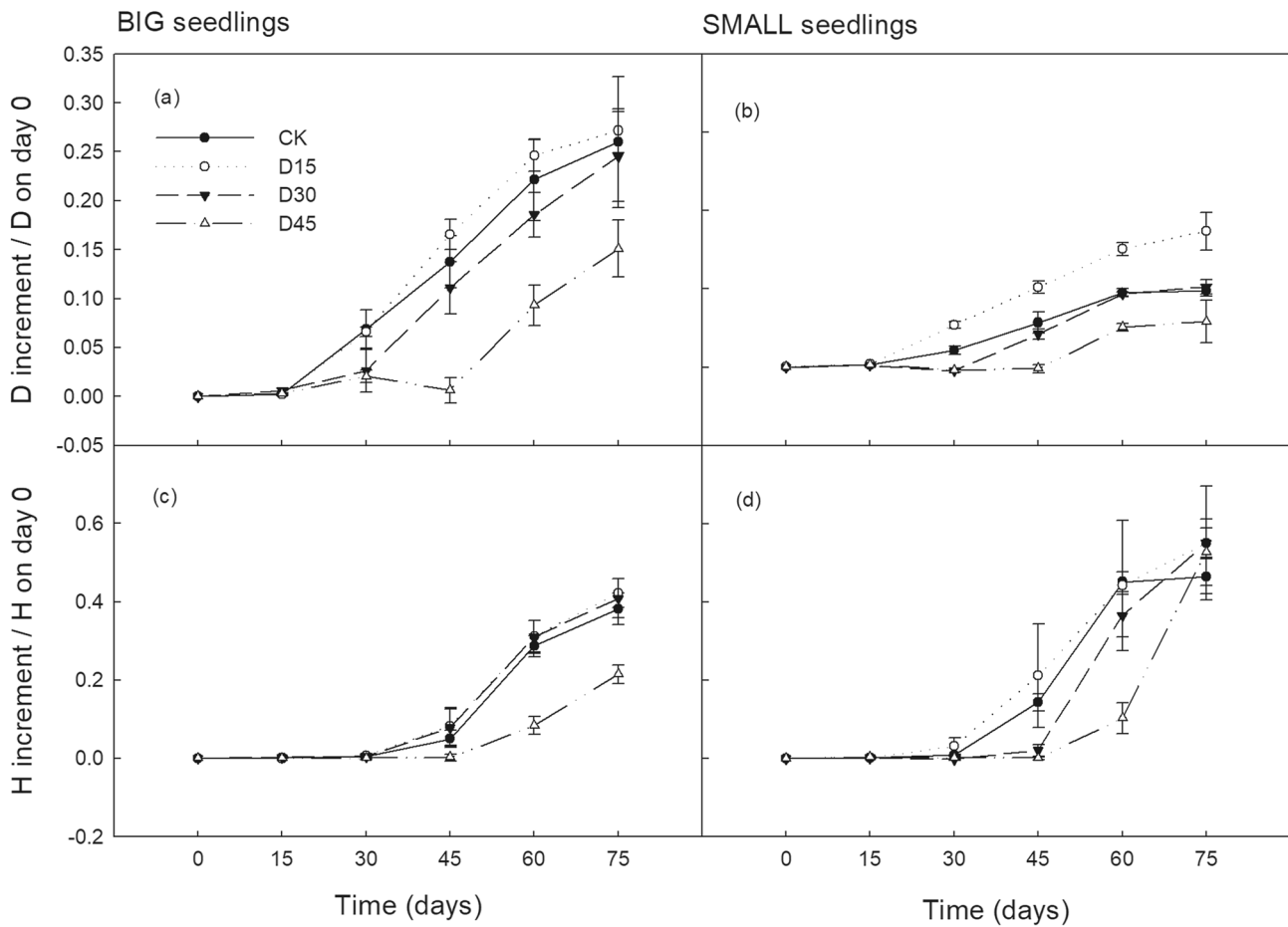


Fig. 4 Increment of basal stem diameter (a, b) and plant height (c, d) of Mongolian Scots pine seedling types under different drought treatments. Mean \pm SE values are shown, $n = 4$

Table 1). When considering both seedling types together, the root biomass in D45 was nearly significantly lower than that in CK ($P = 0.060$). However, there were no significant differences between treatments in root biomass for either BIG or SMALL seedlings separately (Fig. 5a).

The interaction of drought treatments and seedling type was nearly significant ($P_{Tr \times Size} = 0.065$, Table 1). In BIG seedlings, there were no significant differences between the treatment in the root-shoot ratio, but in SMALL seedlings severe drought treatment slightly, but insignificantly, increased the root-shoot ratio the largest difference being between CK and D45 ($P = 0.069$). In D45, the BIG seedlings had a significantly smaller root-shoot ratio than SMALL seedlings ($P = 0.008$, Fig. 5b).

Total chlorophyll content and F_v/F_m of needles

There were significant differences between the sampling times, treatments, seedling types and in their interactions in chlorophyll content ($P < 0.001$ for each, Table 1). In BIG

seedlings, the chlorophyll content was higher in D15, D30 and D45 than in control during days 0–45, and the difference between D45 and CK was significant on day 45 ($P = 0.013$) (Fig. 6a). The difference between D45 and CK lasted until they reached the same level at the last sampling time. On the contrary, in SMALL seedlings, the total chlorophyll content was lower in CK than D30 on day 15 ($P = 0.022$), lower in D15 ($P = 0.027$) and D30 ($P = 0.028$) than in CK on day 45, and lower in D45 than in CK and D30 on day 60 (Fig. 6b).

There were significant differences between the sampling times and in the interactions between the sampling times and treatments in F_v/F_m of needles ($P_T < 0.001$, $P_{T \times Tr} = 0.006$, Table 1). F_v/F_m of the needles was higher in SMALL seedlings than in BIG seedlings at the onset of the drought treatment (day 0) (Fig. 6c, d). It increased between days 0 and 30 and reached the maximum value in both pretreated seedling types, therein F_v/F_m was a bit higher in SMALL than in BIG seedlings. In both BIG and SMALL seedlings, there were no significant differences between drought treatments in F_v/F_m (Fig. 6 c, d).

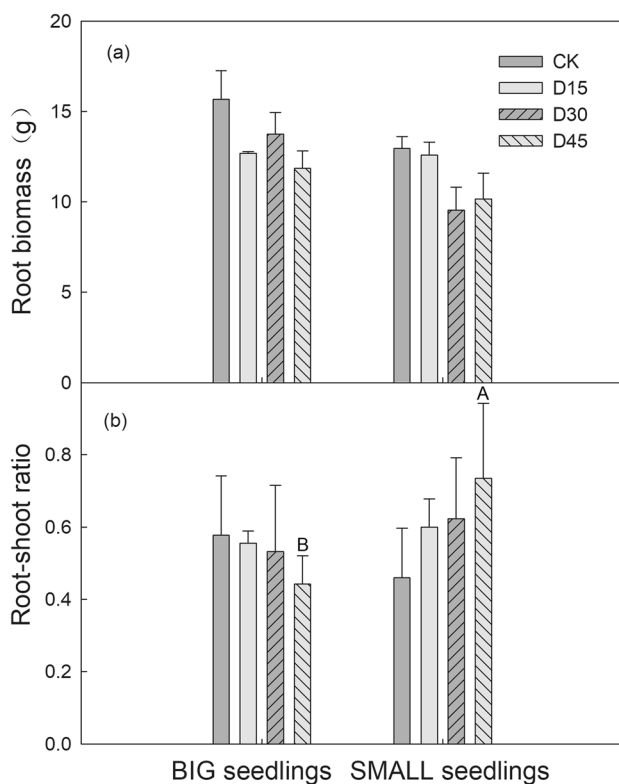


Fig. 5 Root biomass (a) and root-shoot ratio (b) of Mongolian Scots pine seedling types by drought treatments. Mean \pm SE values are shown, $n=4$. The different capital letters indicate significant differences between the seedling types in each sampling time ($P < 0.05$)

Soluble sugar and starch concentration of roots

There were significant differences between the sampling times, treatments and in the interactions in soluble sugar concentration ($P_T < 0.001$, $P_{Tr} = 0.003$, $P_{T \times Tr \times Size} = 0.002$, Table 1). The soluble sugar concentration of roots was clearly higher in SMALL seedlings than in BIG seedlings on day 0. In CK, the soluble sugar concentration of roots decreased sharply during days 0–30, then maintained stable in SMALL seedlings; whilst in BIG seedlings it decreased during days 15–45 and then slightly rebounded. In BIG seedlings, the soluble sugar concentration of roots was higher in D15, D30 and D45 on day 15 ($P < 0.001$ for each), in D30 ($P = 0.095$) and D45 on day 30, and in D45 on day 45 ($P = 0.005$) than in CK. Similarly, in SMALL seedlings, it was slightly higher in D15 ($P = 0.091$), insignificantly higher in D30 and D45 on day 15, in D30 ($P = 0.002$) and D45 on day 30 ($P < 0.001$), and in D45 ($P < 0.001$) on day 45 than in CK (Fig. 7a, b).

There were significant differences in starch concentration between the sampling times, and interactions were also significant ($P_T < 0.001$, $P_{T \times Tr \times Size} = 0.003$, Table 1). The starch concentration of roots did not differ between SMALL and

BIG seedlings on day 0. The starch concentration of roots increased significantly during days 0–15 and then decreased gradually until day 60 in BIG seedlings, whereas it increased significantly from days 15 to 30, then decreased continuously until the last sampling time in SMALL seedlings. In BIG seedlings, the starch concentration of roots was lower in D15 ($P = 0.009$), D30 and D45 on day 15, in D30 ($P = 0.009$) and D45 on day 30, in D45 on day 45 than in CK. In SMALL seedlings, it was significantly lower in D45 than in CK on days 30 and 45 ($P < 0.001$, $P = 0.002$, respectively). The starch concentration of roots in drought treated seedlings recovered to the same level as control on days 60 and 75 in BIG seedlings, while in SMALL seedlings, it was lower in D15 but higher in D30 and D45 than in CK, showing significant differences between D15 and D30 ($P = 0.026$, $P = 0.014$, respectively) (Fig. 7c, d).

Endogenous hormones and N, P and K concentration of needles in BIG seedlings

There were significant differences between the treatments, samplings times and the interactions between them in ABA ($P < 0.001$ for each), IAA ($P_{Tr} = 0.026$, $P_{T \times Tr} = 0.018$) and GA3 ($P_{T \times Tr} = 0.033$, $P < 0.001$ for others) (Table 1). Foliar ABA, IAA and GA3 concentrations started to differ between drought-treated and CK seedlings on day 15. ABA concentration of D45 needles increased rapidly on day 30, reached the maximum on day 45, and was significantly larger than in CK needles ($P < 0.001$ for days 30 and 45). On the contrary, IAA concentration of needles in D45 was lower than in CK on day 15 ($P < 0.05$) and slightly lower on day 30 ($P = 0.056$). Moreover, the GA3 concentration of needles in D45 was lower than in CK on days 30 ($P < 0.001$) and 45 ($P = 0.022$) (Fig. 8a, b, c).

There were no significant differences between the treatments, sampling times and the interaction between them in N and P content. However, the interaction between sampling times and treatments was significant in K ($P = 0.041$, Table 1). The N and P concentrations of needles in the D45 treatment were similar to CK during days 0–45. The K concentration of needles was slightly higher in D45 treatment than in CK ($P = 0.088$) after 15 days of drought (day 15), whereas it was lower in D45 treatment than in CK after 30 days (day 30) ($P < 0.05$) and 45 days of drought (day 45) (Fig. 8d, e, f).

Discussion

This study shows that continuous drought from winter to spring delays the bud phenological development and decreases the growth of shoots and roots during the following growing phase in Mongolian Scots pine seedlings. The

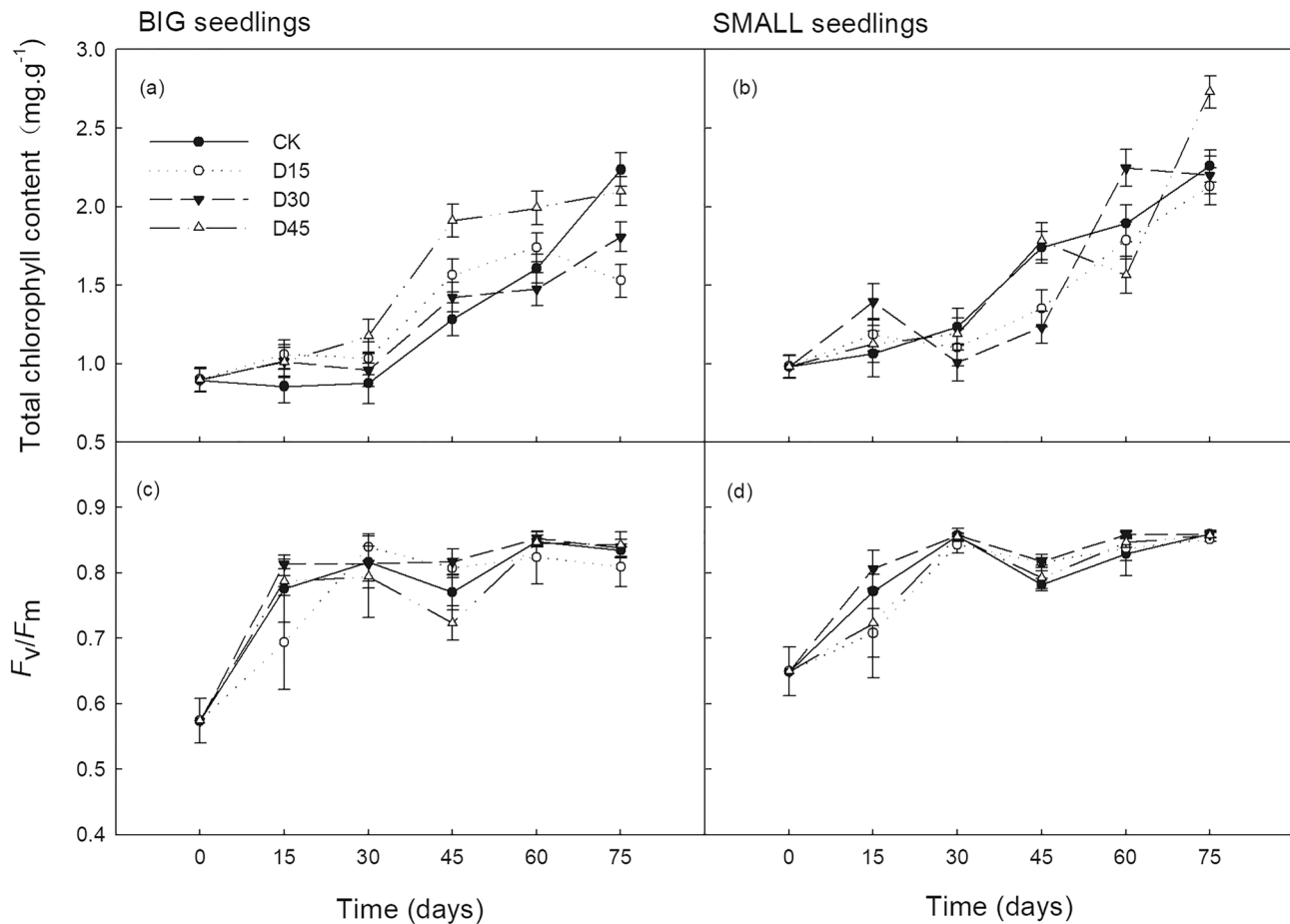


Fig. 6 Total chlorophyll content (**a, b**) and F_v/F_m (**c, d**) of needles in Mongolian Scots pine seedling types under different drought treatments. Mean \pm SE values are shown, $n=4$

results supported our hypothesis that spring phenology and growth of Mongolian Scots pine seedlings are affected by the drought treatments and the two groups of seedlings are affected differently. This was especially prominent for the continued drought for 30 days and 45 days.

With the rising temperature in spring, most of the buds slightly swelled on day 31 in the control seedlings. It has been proposed that the onset of bud swelling indicates that the transition from endodormancy to ecodormancy has occurred (Saure 1985; Pallardy 2008). Based on the calculation of the number of slightly swelling buds, D30 and D45 delayed the bud phenology for 8 days and 18 days, respectively. This finding was in accordance with the results of an experiment in which drought delayed the emergence of needles on primary axis branches of piñon pine (*Pinus edulis*) on average by 19 days and combined rain reduction and air warming by 57 days (Adams et al. 2015). The bud phenological development in D15 was not delayed, which may be due to the low environmental temperature during days 0–15, and the seedlings were probably still in the late

endodormancy phase. Therefore, the drought during this period did not affect the bud phenology. The endodormancy of buds is generally released after sufficiently long exposure to cool, non-freezing temperatures to fulfil the chilling requirement (Perry 1971; Sarvas 1974). During days 15–31, the seedlings in CK and D15 might finish the endodormancy and gradually enter into the ecodormancy as the slight buds swelling were seen on day 31.

The delay of bud phenology in D30 and D45 might be associated with the increase of ABA concentration and the decrease of GA3 concentration of needles that was visible on days 30 and 45. ABA and GA are the two most important hormones that regulate bud endodormancy induction, maintenance, and release (Wang et al. 2015; Yang et al. 2021). A high level of endogenous ABA is the primary factor in maintaining bud endodormancy (Zheng et al. 2018), while GA is responsible for endodormancy release (Zhuang et al. 2013). The IAA concentration of needles increased between days 0 and 15 and then decreased, which was in line with the studies on two tea (*Camellia sinensis*) cultivars, where the

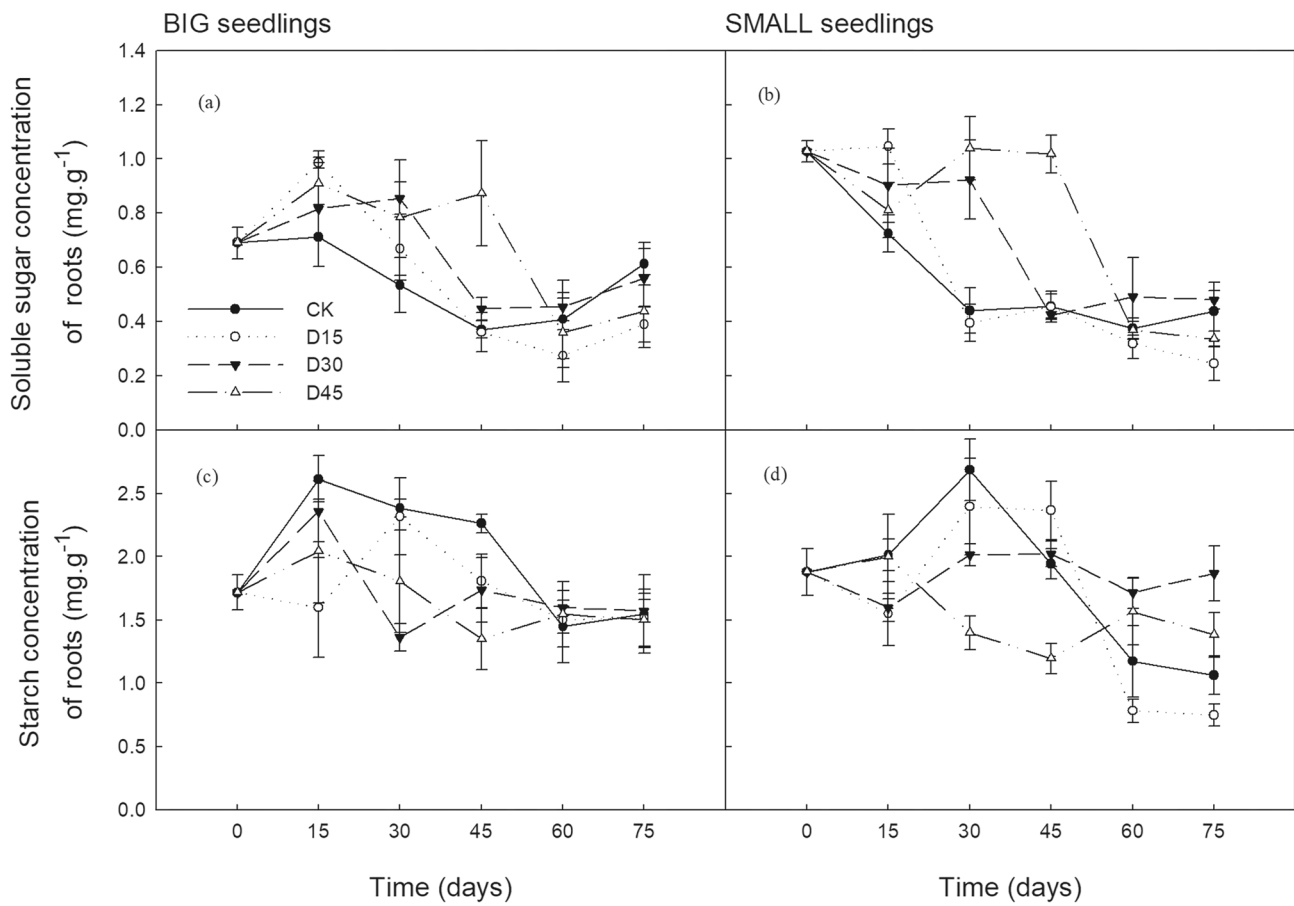


Fig. 7 Soluble sugar (a, b) and starch concentration (c, d) of roots in Mongolian Scots pine seedling types under different drought treatments. Mean \pm SE values are shown, $n = 4$

IAA content in terminal and lateral buds increased gradually during endodormancy release (Hao et al. 2019), and with the studies on the adventitious buds of leafy spurge (*Euphorbia esula*), where the IAA content was higher in endodormancy than in ecodormancy (Chao et al. 2017). Based on our results and the above literature, we suppose that day 15 was the timepoint of endodormancy release and ecodormancy initiation.

In addition to the phytohormones, the phenological changes are associated with phytochromes and carbohydrates (Chao et al. 2007). The SMALL seedlings had earlier bud phenology than the BIG seedlings. When the chilling requirement for endodormancy release is fulfilled, metabolic activity increases, hydrolytic enzymes are activated and carbohydrate reserves gradually become mobilized (Basler and Körner 2014). In CK, the soluble sugar concentration of roots in SMALL seedlings decreased sharply during days 0–30, whereas it decreased during days 15–45 in BIG seedlings. This indicated the connection between the sugar and the endodormancy release, as sugars have been thought to play a role in signaling pathways required for endo- and

ecodormancy (Chao et al. 2007). Before the most buds swelling on day 31, chlorophyll content and the maximum photosynthesis efficiency of needles in SMALL seedlings were a bit higher than in BIG seedlings, indicating an earlier start of metabolic activity. The chlorophyll content and F_v/F_m ratio increased during day 0 to 15, likely due to warmer temperatures and stronger sunlight reactivating dormant chloroplasts in the needles. This revived chlorophyll production, turning the needles from pale yellow-green to bright green, while also restoring Photosystem II activity (Xu et al. 2020; Wang et al. 2023). Plant hormones such as gibberellins (GA) and IAA may further enhance chlorophyll synthesis by activating related genes (Liu et al. 2020). The chlorophyll content of needles in BIG seedlings increased under drought treatment, which might be explained by the unchanged N content and lower water content of needles. The unchanged N content showed the strong drought resistance of Mongolian Scots pine seedlings. Foliar potassium content was increased first but decreased later with prolonged drought. Unlike nitrogen and phosphorus which exist as organic compounds in plant cells, potassium exists as ions. Its increase was involved in

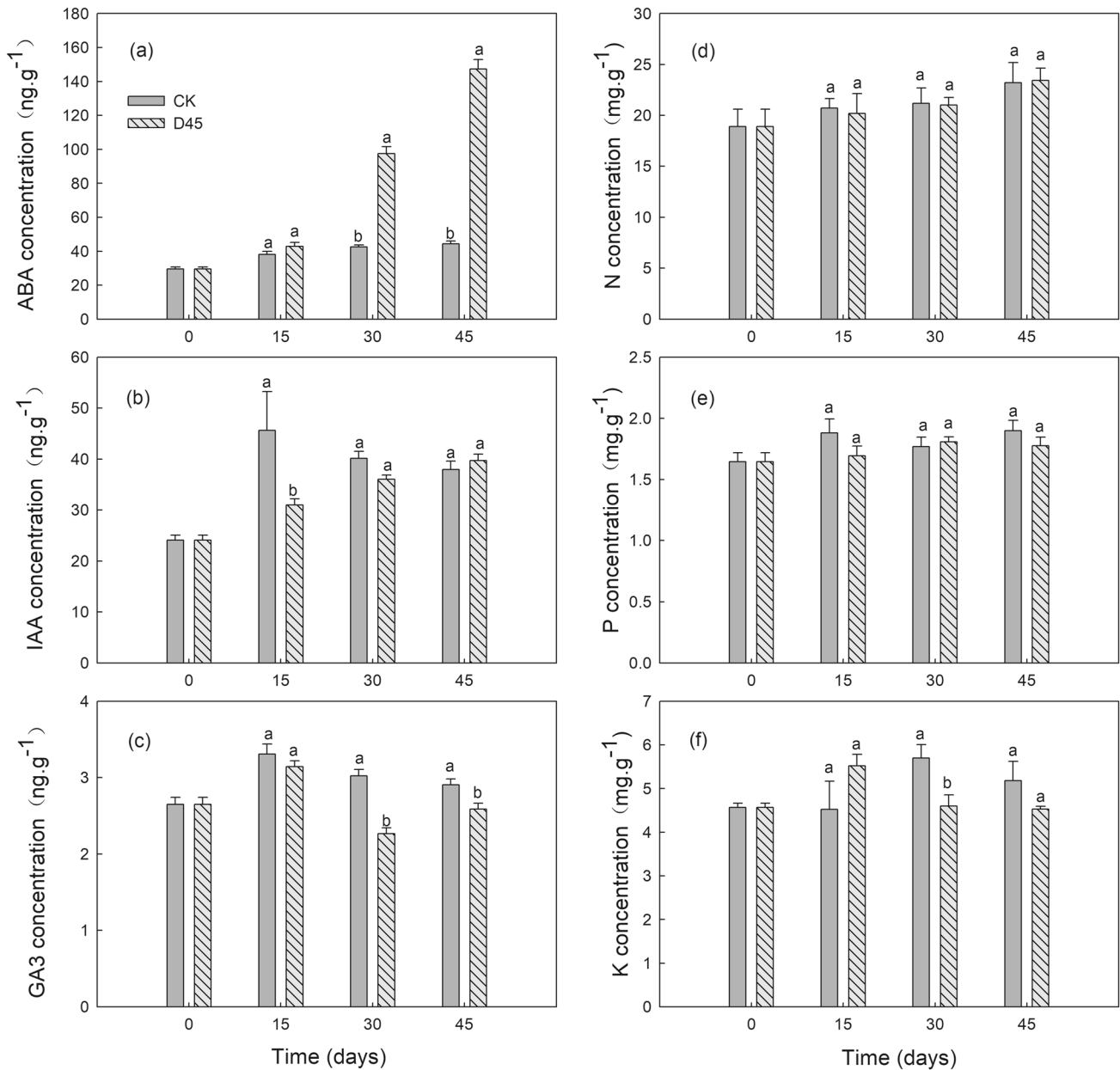


Fig. 8 Change of ABA, IAA and GA3 (a, b, c) and N, P, and K (d, e, f) concentrations of needles in BIG seedlings during the drought treatment. Mean \pm SE values are shown, $n=4$. The different letters indicate significant differences between the treatments in each sampling time ($P < 0.05$)

cell osmotic adjustment, and its decrease can be attributed to decrease in the nutrient uptake capacity with prolonged drought (Johnson et al. 2022).

In our study, the onset of basal stem diameter growth occurred during days 15–30, which was followed by the onset of plant height growth (during days 30–45), which was in accordance with a study in which the radial growth onset was considerably preceded by the onset of shoot height growth in *Pinus sylvestris* (Swidrak et al. 2013). The most prolonged drought (D45) impeded the stem diameter and plant height growth significantly in both BIG

and SMALL seedlings, with better resumption in SMALL seedlings during the post-treatment period. Moreover, the slight drought (D15) raised the diameter increment in SMALL seedlings. This might suggest that SMALL seedlings experienced harsh conditions before and were acclimated to the adverse future environment. Water transport in trees is also dependent on the plant size (Mencuccini 2015). The SMALL seedlings may have smaller vessels that may be less susceptible to drought-induced xylem embolism and help to regulate the water flow under drought (Brunner et al. 2015). In our study, the seedlings

that experienced a colder winter grew smaller than seedlings that experienced a warmer winter, however, we could not conclude that smaller seedlings in any case have better resistance to drought stress. In the future, to separate the response of seedling size and the pre-acclimated winter conditions to the drought stress, it is better to control the other condition when one is in question.

Even though D45 slightly decreased the root biomass in both BIG and SMALL seedlings during the post-treatment period, it seemed that the SMALL seedlings increased the root-shoot ratio in the face of the severe drought. This is in accordance with numerous studies in which fine root biomass was reduced by drought (Eldhuset et al. 2013; Zang et al. 2014). Responses of tree roots to drought include root biomass adjustments, anatomical alterations, and physiological acclimations that enable them to avoid and tolerate the stress (Brunner et al. 2015). Water loss can be limited by stomatal closure and over the longer term by restricting shoot growth, leading to an increased root-shoot ratio.

The soluble sugar concentrations of roots in BIG and SMALL seedlings were increased by the prolonged drought, especially in D30 and D45, which can be interpreted as a response to soil drought, as accumulation of sugar lowers the osmotic potential in the root tissue to enhance the water uptake (Brunner et al. 2015). The starch content in roots was decreased in D30 and D45, which was consistent with the reduction of nonstructural carbohydrates in Norway spruce tree roots under drought conditions (Hartmann et al. 2013). Drought treatment may reduce the accumulation of photosynthetic products, thus significantly reducing the starch content of roots (Mitchell et al. 2013), and the starch can be converted into soluble sugars in drought stress for osmotic adjustment of root cells (Dickman et al. 2014).

In conclusion, a prolonged drought for 30 and 45 days after soil thawing in early spring delayed the bud phenological development and decreased the aboveground growth and root biomass in Mongolian Scots pine seedlings. The SMALL seedlings that experienced a colder winter and spring in the previous year had earlier bud phenology and were less influenced by the severe drought than the BIG seedlings. The differences between the two seedling types with different overwintering conditions were attributed to physiological and morphological changes. Therefore, we suggest that in forest management, a big seedling size is not a prerequisite of growth success, but proper conditions in overwintering and storing the seedlings prior planting are more important. Prolonged drought throughout winter and early spring should be avoided especially before planting the seedlings in a drought-prone area.

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Author contributions The study was designed by A.W. and J.K. The experiment was carried out by H.Q. The data were analyzed by H.Q., F.Z., A.W., Y.L., and Y.G. The manuscript was written by F.Z., A.W. and J.K., with some revisions from B.D. All authors approved the final manuscript.

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Data availability Data will be made available on request.

Declarations

Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

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