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RESEARCH

Morphological and physiological response of conifer seedlings to drought conditioning

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Abstract

Increased frequency, severity, and duration of droughts and increased wildfire severity are impacting many conifer forests globally. Reforestation in these changing disturbance regimes requires tree seedlings capable of establishing in hotter and drier climates. We evaluated the morphological and physiological effects of drought conditioning on second-year ponderosa pine (*Pinus ponderosa)*, western white pine (*Pinus monticola*), and western larch (*Larix occidentalis)* seedlings. Treatments included a well-watered control (75% of container capacity) and a water-limited (40% of container capacity) drought treatment. Ponderosa pine exhibited no significant treatment effects to above or below-ground biomass while treatment caused a significant reduction of height and root-collar diameter for both western white pine and western larch coupled with a significant reduction in root mass for western larch. The drought treatment resulted in significant reductions of gas exchange rates across all species, although mid-day water potentials and $\delta^{13}C$ indicate lack of cumulative stress. Drought conditioned ponderosa pine seedlings resulted in decreased total root nonstructural carbohydrates (NSCs) and droughted western white pine displayed increased total needle NSCs driven by increased needle soluble sugars. Drought conditioning responses in second-year seedlings in large pots proved to be species dependent allowing for further investigation of treatment intensity and duration for drought-adapted stocktypes.

Keywords Seedling · Conifer · Drought · Nursery · Reforestation · Nonstructural carbohydrates

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Introduction

The adaptive capacity of forests depends on successful seedling regeneration following disturbance. Shifts in disturbance regimes (Moritz et al. 2012) coupled with directional changes in climate (Moss et al. 2024; Dai 2013) are driving regeneration failure in many forest ecosystems (Davis et al. 2019; Kemp et al. 2019; Martínez-Vilalta and Lloret 2016; Will et al. 2013; Simeone et al. 2019). Global reforestation initiatives that involve artificial regeneration, or planting nursery-cultured seedlings, have been proposed to restore degraded ecosystems, promote biodiversity, meet ecosystem service needs, and to mitigate the effects of climate change (IUCN 2011; Newmark et al. 2017; Bastin et al. 2019; Griscom et al. 2017). However, reforestation efforts face many challenges (Dobrowski et al. 2024; Fargione et al. 2021) including increased reforestable area due to fire (Dumroese et al. 2019) and area impacted by drought (Naumann et al. 2018; Dai 2013; Sheffield and Wood 2008), leading to the idea that climate change will outpace the ability of forest ecosystems to adapt naturally (Aitken et al. 2008; Keane et al. 2018). Seasonally dry forests represent a particularly vulnerable landscape where soil moisture deficits severely limit seedling establishment (Petrie et al. 2016) and outplanting success, especially after fire (Hanson et al. 2018; Tepley et al. 2018; Harvey et al. 2016). To encourage successful reforestation and survival of planted seedlings, there is a need to develop nursey grown seedlings capable of establishing in arid, post-disturbance landscapes. Drought conditioning, the practice of restricting irrigation during seedling cultivation in the nursery (also termed drought hardening and drought preconditioning), is a technique hypothesized to enhance survival and performance of planted seedlings (Puértolas et al. 2024).

Conifer seedlings exhibit a continuum of morphological and physiological mechanisms to prevent irreversible turgor loss and subsequent mortality due to drought. If atmospheric water demands exceed the soil moisture supply, plants may experience embolism, hydraulic failure, and irreversible turgor loss (Tyree and Sperry 1989). Due to inevitable water-loss through stomata during photosynthesis, plants rely on species-specific traits to cope with moisture-stress on both short-term (daily) and long-term (drought) time scales. Certain hydraulic traits can prevent large xylem tensions that lead to embolism, such as decreased stomatal conductance, decreased sapwood area-to-leaf area ratios (Martínez-Vilalta et al. 2009), or other traits that enhance water absorption via deep roots (Padilla and Pugnaire 2007) and solute accumulation using carbohydrate reserves (Chapin et al. 1990; O'Brien et al. 2014; Sala et al. 2012).

Some plants tolerate low water potentials by accumulating solutes allowing for osmotic adjustment (Martínez-Vilalta and Garcia-Forner 2017). Soluble sugars in the form of sucrose or other low molecular weight oligosaccharides facilitate osmotic adjustment and influence a plant's carbon balance (Morgan 1984; Dietze et al. 2014). Together with starches, these compounds can be stored as nonstructural carbohydrates (NSCs) and retrieved to support not only osmoregulation, but also metabolism, biomass accumulation, defense, and recovery from biotic and abiotic stressors (Dietze et al. 2014; Hartmann and Trumbore 2016). How seedlings regulate these carbon stores during periods of stress could confer drought resistance traits and mechanisms for growth and survival.

Selecting and cultivating well-adapted plant material is crucial for ensuring seedling establishment, requiring the evaluation of drought resistance metrics related to morphology, physiology and performance. Seedling morphological traits are proxies for physiological

functioning and play a key role in plant architecture and water balance, influencing a plants survival under drought (McDowell et al. 2008). Easy to measure traits often correlated with seedling drought resistance, and ultimately survival, include height and root-collar diameter (RCD) (Pinto 2011). While tall plants with extensive photosynthetic tissue would be best suited for sites with high competition for light, short stock with extensive root systems could be better suited to sites with high competition for limited soil moisture (Grossnickle 2012; Pinto et al. 2012). For this reason, root-to-shoot ratios can be a useful indicator of drought resistance when culturing seedlings that balance transpirational area (shoot) with water absorbing area (root) (Haase 2008). Physiological measurements that quantify plant function related to water, mineral, and carbohydrate transport offer insight into mechanisms of stress resistance. For example, plant water potential offers an integrative yet instantaneous physiological measure of plant water status and can also serve as an indirect measurement of a plant's water absorbing capabilities (Hsiao 1973), while carbon isotope ratios ($\delta^{13}C$) can infer cumulative responses of stomata and water use efficiency on longer time scales (Farquhar et al. 1982). The combination of morphology and physiology are thought to correlate with performance (e.g., growth and survival) of seedlings in the field, and influence how nursery managers cultivate seedlings (Grossnickle 2018).

While different species have different strategies to cope with drought, plants can exhibit high levels of plasticity in response to their environment, allowing for increased drought resistance (Dudley 1996; Alpert and Simms 2002). Plasticity of this nature may be something that could be exploited through nursery culture. The Target Plant Concept is a framework (Landis et al. 2010; Davis and Pinto 2021) that emphasizes cultivating seedlings under desired conditional constraints for specific outplanting conditions. One prediction from the TPC is the hypothesis that exposing seedlings to limited soil moisture regimes in the nursery could influence growth and water relations, enhancing survival and growth in outplanted seedlings.

Cultivating stock types for stressful, dry sites by influencing plant water relations, and carbon acquisition, use, and storage could ultimately promote planted seedling survival and performance (Puértolas et al. 2024). Irrigation regimes that limit water during growth phases, known as drought conditioning, are a nursery culturing technique applied to a variety of native species. The application of this technique to conifer seedlings has shown that some species can morphologically and/or physiologically adjust (Villar-Salvador et al. 1999; Royo et al. 2001; Seiler and Johnson 1988; Grossnickle 2012; Pinto et al. 2023), potentially allowing for survival under unfavorable conditions (Kozlowski et al. 1991; Cregg 1994; van den Driessche 1991). Previous studies in which irrigation was manipulated during first-year growth indicated improvement in drought tolerance with changes to pre-dawn water potentials and osmotic potentials in the nursery (Kandiko et al. 2011; Hennessey and Dougherty 1984; Deligoz and Gur 2015). While most research has been conducted on first-year seedlings, little has been conducted on second-year stock when seedlings have transitioned from relying on primary xylem to more embolism-resistant secondary xylem (Miller and Johnson 2017). Exploring the effects of drought conditioning at this stage in growth could allow for insight into cultivating larger conifer seedling stock types best suited for hot, dry, landscapes where soil surface temperatures frequently cross lethality thresholds for smaller, younger seedlings (Rank et al. 2022; Holden et al. 2024; Conlisk et al. 2018).

This study examined how drought conditioning via a restricted irrigation regime during the second year of growth in containerized ponderosa pine (*Pinus ponderosa* Lawson &

Lawson var. *ponderosa* C. Lawson), western larch (*Larix occidentalis* Nutt.), and western white pine (*Pinus monticola* Douglas ex D. Don) influenced above- and below-ground biomass, gas exchange rates, carbon isotope composition, and nonstructural carbohydrate concentrations. Although ponderosa pine is more vulnerable to cavitation than *Pseudotsuga menziesii*, it tends to avoid drought stress through tighter stomatal regulation and a higher sapwood-to-leaf area ratio (Piñol and Sala 2000). Western white pine may employ similar drought avoidance strategies, though direct physiological evidence is limited. In contrast, the deciduous western larch is considered more drought tolerant due to its low vulnerability to cavitation, despite exhibiting limited stomatal control in mature trees (Swidrak et al. 2013; Piñol and Sala 2000). The three selected species represent a gradient of drought vulnerability and unique drought tolerance strategies, while all being important species for reforestation in the Inland Northwest and northern Rocky Mountain regions. The purpose of this research is to advance the physiological underpinnings of drought conditioning in conifer seedlings.

We hypothesized that (1) drought-conditioned seedlings would exhibit morphological and physiological changes associated with drought resistance (e.g., decreased height, increased root: shoot, osmotic adjustment); and (2) the response would be species-dependent, reflecting the different drought adaptations of the study species. This research is motivated by the need for improved nursery stock grown to withstand stressful hot and dry sites associated with climatic change and improved understanding of the ecophysiological responses of tree seedlings to drought.

Methods

First year seedling cultivation

All seeds were sourced from the U.S. Forest Service Nursery in Coeur d'Alene, Idaho. We used Supplemental Mass Pollinated (SMP) and Non-Supplemental Mass Pollinated (NSMP) sources for western larch and ponderosa pine, respectively. Western white pine was an F1 white pine blister rust resistant source with a general elevation seed zone (Forest Service seed record 7461-16). Two low-mid elevation western larch seed sources were used: western larch 1 (7663) and western larch 2 (7659-14-SMP). Ponderosa pine was a mid-elevation seed zone (7741-18-NSMP).

Western white pine, ponderosa pine, and western larch 2 were grown from seed at the University of Montana Memorial Greenhouse in Missoula, Montana. Prior to sowing, seeds were stratified in cold-moist conditions. Western larch 2 and ponderosa pine were stratified for 28 days, whereas western white pine was stratified for 90 days. Seeds were sown in March of 2021 in styrofoam block containers containing 77 cells, each cell being 164 ml in volume (Stryoblock 77/170, Beaver Plastics, Acheson, Alberta, Canada). Containers were filled with media comprised of 85% sphagnum peat moss and 15% vermiculite. When blocks dried down to 70–80% of their container capacity (CC) ("manager technique" described by Dumroese et al. 2015), blocks were rehydrated to saturation.

During the 2021 growing season, seedlings were fertilized with multi-purpose liquid fertilizer (Miracle-Gro 24-8-16, The Scotts Company, USA) while irrigating. In September 2021, hardening of seedlings was initiated using fertilizer (Flora Micro 5-0-1, General

Hydroponics, Santa Rosa, CA, USA, and Beastie Bloomz 0-50-30, FoxFarm Soil & Fertilizer, Fairhaven, CA, USA) concentrated with nitrate and phosphorus to increase basal diameter, slow foliar growth, and concentrate root development at the end of the growing season (Landis et al. 2010; Oliet et al. 2013).

One-year old western larch 1 seedlings were purchased from the USDA Forest Service, Coeur D'Alene Nursery due to low germination of western larch 2. Western larch 1 seedlings were grown in an operational regime to meet target specifications in styrofoam block containers containing 112 cells (Styroblock V112105EXT, Stuewe & Sons, Inc, Tangent, Oregon, USA) at the Forest Service Nursery in Coeur D'Alene, Idaho. In preparation for cold storage, all seedlings were exposed to 400 h below 4 °C. Once hours were accumulated, seedlings (total of 1,344 seedlings) were packaged into plastic bags and placed into wax boxes. Seedlings were stored in a freezer (−4 to −2° C) from December 2021 to May 2022.

Nursery drought conditioning experiment

In May of 2022, seedlings were slowly thawed for two weeks at 3° C before being transplanted and grown in a greenhouse at the University of Montana Environmental Control for Organismal Research (ECOR) facility. Greenhouse environmental conditions were maintained around 22.5° C/16.5° C with a relative humidity of 61%/21% (day/night). Each seedling was transplanted into 1600 ml containers and placed in racks holding 16 containers (TP49 containers, MT49TI trays, Stuewe & Sons, Inc, Tangent, OR, USA). The planting media was comprised of 50% sphagnum peat moss and 50% vermiculite. Grit was added atop the media to discourage weed growth and limit evaporation. Each rack was given a unique identifier to facilitate statistical analysis. Ponderosa pine, western white pine, and western larch 1 were each transplanted into 24 racks while western larch 2 was transplanted into 12 racks due to prior low germination rates.

All seedlings were well-watered (80–90% CC) using the "scientific technique" from Dumroese et al. (2015) for the first 2–4 weeks after transplanting to facilitate seedling establishment. Racks were randomly assigned to one of two irrigation levels: a well-watered control (75% CC) or a water-limited drought conditioning treatment (40% CC). Both treatments for ponderosa pine, western white pine and western larch 1 had a population size of 192 seedlings while western larch 2 had 96 seedlings per treatment. After establishment, racks were successively dried down to target CC (75% or 40%) at increments of 10% per week. Racks were weighed daily to determine irrigation needs in accordance with irrigation treatments. Once a target level was reached for half or more of all racks within a treatment group, all racks were irrigated to 100% CC. During September of 2022, the target CC for the drought conditioning treatment (hereafter, drought treatment) was raised to 60% for both western larch 1 and western larch 2 due to premature needle reddening. Irrigation treatments concluded on December 10, 2022, when all blocks were irrigated to saturation in preparation for cold storage.

Fertilizer was applied during irrigation in a manner that resulted in each seed source receiving similar amounts of total nitrogen (mg N). All fertilizer was applied as an aqueous solution via a fertilizer injector (R3, Smith Pumps, Newbury Park, CA, USA). Phosphoric acid was added to increase acidity and to adjust the pH to 5.5. General fertilization target application rates (ppm) were identified throughout the growing season (establishment, rapid

growth, hardening) for each species and nutrient balances were set relative to nitrogen (N) (Ingestad and Ågren 1995). Larch, being an N-sensitive species, had a target N of 5–10 ppm, while ponderosa pine and western white pine had a target of 100 ppm and 150 ppm, respectively. Fertilizer application amounts were tallied throughout the growing season to track N additions. Nitrogen additions were modified to keep N totals similar for each treatment species combination. Needle tissue was sent to Washington State University's Stable Isotope Lab for nitrogen content analysis to assess fertilizer amounts post-treatment (Figs. S1–S3).

Morphological and physiological measurements

All morphological measurements were collected at the end of the drought treatment. Height and root-collar diameter (RCD) were measured on all seedlings while a subset of seven randomly selected seedlings for each species × treatment combination was selected for destructive samples, once at target container capacity (40%, 75% or 100%). Destructive sampling included measurements of total seedling dry mass (g), total root dry mass (g), and total shoot dry mass (g). Physiological measurements were taken prior to destructive sampling and included pre-dawn and mid-day water potentials, gas exchange rates, soluble sugar, and starch concentrations (roots, shoots, and needles), and tissue for carbon isotope analyses ($\delta^{13}C$). Water potential measurements and gas exchange rates were taken in peak summer (August/September) while tissue analysis for NSCs, $\delta^{13}C$, and nitrogen content were collected in December allowing for both instantaneous and cumulative effects of our drought treatment. To maintain population numbers during the growing season, no water potential measurements were collected for western larch 2. Needles for both pine species and branches for larch were collected for $\delta^{13}C$ and nitrogen content analysis.

Morphological characteristics

Total seedling height (measured from root-collar to terminal bud) and RCD (measured 1 cm above media surface) were measured on each of the seven sub-sampled seedlings. Seedlings were removed from their containers, roots were washed free from media, and placed in paper bags and oven-dried at 68° C for 48 h. Root dry mass was measured separately from needle and shoot biomass. Root:shoot ratios were calculated by dividing the root mass by the sum of the stem and needle masses for each seedling. After seedlings were destructively sampled, container weights were updated to reflect correct saturated CC and target CC.

Plant water status

Pre-dawn and mid-day water potentials were measured to quantify plant moisture stress, allowing for an instantaneous assessment of our drought treatments impact on plant water status. Measurements were taken once the rack had reached its target weight (75% or 40% CC) in August/September of 2022. Prior to destructively sampling selected seedlings, each seedling's water status was measured using a pressure chamber (Scholander PMS instrument, Corvallis, OR, USA). Mature secondary needles were used for both pine species whereas a lower branch was chosen for western larch.

Gas exchange

Gas exchange measurements were used to assess treatment effects on net photosynthesis, transpiration, and stomatal conductance. In August 2022, measurements were made on a mid-canopy branch of the seven randomly selected seedlings per experimental treatment for each seed source. Data was collected at both saturation (100% CC) and target dry down (75% or 40% CC) for each species. Measurements occurred between 1100 and 1700 h using a portable photosynthesis system (LI-6800, Li-Cor Biosciences, Lincoln, NE, USA) equipped with an LED light source (6400-02B, LI-COR, Inc., Lincoln, NE, USA). Instrument settings were as follows: chamber = 25 °C, reference CO_2 = 415 ppm, µmol mol^{-1}, flow rate = 500 µmol s^{-1}, and light source = 1,500 µmol m^{-2} s^{-1} photosynthetically active radiation (light-saturating photon flux density). Leaf area (cm^2) was obtained by taking photos of photosynthetic tissue used for measurements and were analyzed using computer imaging software (Image J Java 1.8.0_345, W.S. Rasband, Bethesda, MD, USA).

Nonstructural carbohydrates

Root, stem, and needle tissue were collected for NSC analysis to assess cumulative treatment effects on carbon storage after the experiment. Fresh needle, stem, and root tissue samples were frozen in liquid nitrogen to stop metabolic activity. Tissue samples were then oven-dried at 70° C for 72 h. Samples were first coarsely ground with a Wiley mill (20-gram mesh, Thomas Scientific, Swedesboro, NJ, USA) for woody tissue and mortar and pestle for needles, followed by grinding to a fine powder with a bead mill (TissueLyser III, Qiagen, Germantown, MD, USA). Soluble sugars (glucose, fructose, and sucrose) and starch were quantified enzymatically following Sapes et al. (2021). Approximately 12–14 mg of dried plant material was incubated in 1.6 ml ultra-pure water at 100° C for 60 min to extract soluble sugars. An aliquot of supernatant from the centrifuged extract was treated with phosphoglucose isomerase (P5381, Sigma-Aldrich, St. Louis, MO, USA) and invertase (I4504, Sigma-Aldrich) to convert fructose and sucrose into glucose, respectively. Total NSC concentration was obtained from a separate aliquot incubated in amyloglucosidase (10115, Sigma-Aldrich) at 50° C for 16 h followed by phosphoglucose isomerase to convert polysaccharides (starch and sucrose) and fructose to glucose. Glucose concentration in reacted aliquots was measured as absorbance at 340 nm following hexokinase-glucose 6-phosphate dehydrogenase enzymatic digestion (G3293, Sigma-Aldrich) on a 96-well plate reader (EL800, BioTek/Agilent, Santa Clara, CA, USA). Resulting glucose concentrations were converted to glucose-equivalent concentrations of soluble sugars, starch, and total NSC content in the tissue sample and expressed as a percentage of dry mass ($mg \cdot mg^{-1}$, %). Starch was calculated as the total NSC percentage minus soluble sugars.

Carbon isotope ratio ($\delta^{13}C$)

$\delta^{13}C$ provides a measure of cumulative stress due to stomatal closure over time. For this reason, samples of needle tissue from pines or branch tissue from larch, grown during the 2022 growing season, were gathered at the end of the drought treatment, oven-dried, and finely ground for analysis at the Stable Isotope Core Laboratory of Washington State University. The isotopic analysis procedure involves the conversion of samples into CO_2 using an ele-

mental analyzer (ECS 4010, Costech Analytical, Valencia, CA), followed by gas separation utilizing a 3 m gas chromatography (GC) column and subsequent analysis with a continuous flow isotope ratio mass spectrometer (Delta PlusXP, Thermofinnigan, Bremen) (Brenna et al. 1997; Qi et al. 2003). To ensure accuracy, isotopic reference materials are inserted intermittently for calibration purposes. The contribution of ^{17}O is adjusted using the Santrock correction method through the IRMS software (Santrock et al. 1985). Carbon isotope results were originally reported in permil relative to VPDB (Vienna PeeDee belemnite) by assigning a value of +1.95 per mill to NBS 19 $CaCO_3$ (Coplen 1994). Presently, the NIST calibration of VPDB relies on NBS 19 and L-SVEC as anchor points (Coplen et al. 2006).

Experimental design and analyses

This experiment was designed and analyzed as four experiments in which each seed source was randomly assigned one of two irrigation treatments. Seedlings were randomly assigned an irrigation treatment, and the resulting racks of a seed source × treatment combination were given a random location in the greenhouse. Statistical analyses and models were conducted using R statistical software (R Core Team 2023) using the 'lme4' package. Linear mixed effect models and linear models were used to understand the influence of treatment on each seed source. For both height and RCD, racks were the experimental units and thus rack-level random effects were included when possible. For destructively sampled variables, seedlings were randomly sub-sampled across racks resulting in sample sizes that were too small to incorporate random rack effects ($n=7$). Using the fitted linear models, ANOVAs were conducted ($\alpha=0.05$). All model outputs can be found in Supplementary material (S1-S6).

Results

Seedling morphology

Drought conditioning affected seedling morphology (height, RCD, root:shoot ratio, root mass and shoot mass), although the degree of treatment effect varied by species (Fig. 1). Drought conditioning significantly reduced seedling height and RCD for western white pine ($P=0.001$, <0.001) western larch 1 ($P=0.031$) and western larch 2 ($P=0.022$). While drought conditioned western white pine and both seed sources of western larch resulted in shorter and smaller diameter seedlings, drought treated ponderosa pine seedlings maintained similar average height and RCD compared to controls (Fig. 1a and b). Root:shoot ratios did not differ between irrigation treatments for any seed source (S1). Root mass was significantly reduced for western larch 1 seedlings ($P=0.001$). Shoot mass was significantly reduced for both western larch 1 ($P=0.025$) and western larch 2 ($P=0.017$), while no significant effect was observed for either ponderosa pine or western white pine (Fig. 1, Table S1).

Plant water status

Mid-day water potential of ponderosa pine, western white pine and western larch 1 did not differ between droughted and control groups (Fig. 2; Table S2). Pre-dawn water potential

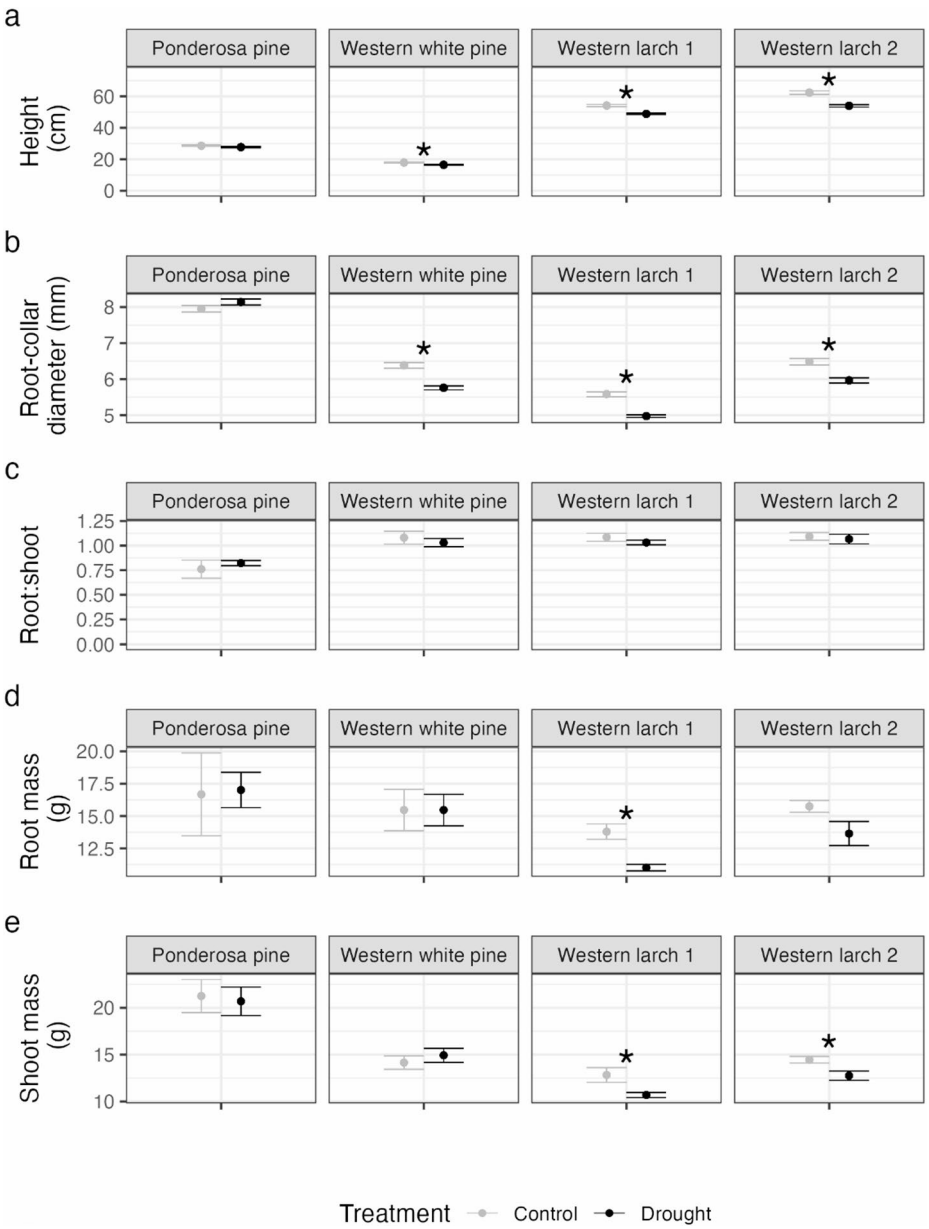

Fig. 1 **a** Height (cm), **b** RCD (mm), **c** root: shoot, **d** root mass (g), and **e** shoot mass (g) for all seed sources at the end of the drought treatment. Height and RCD reflect collections on all live seedlings while destructive samples of root: shoot, root mass, and shoot mass reflect a sample size of $n = 7$. Circles represent means and error bars represent +/- one standard error. Asterisks indicate a significant treatment effect ($P < 0.05$)

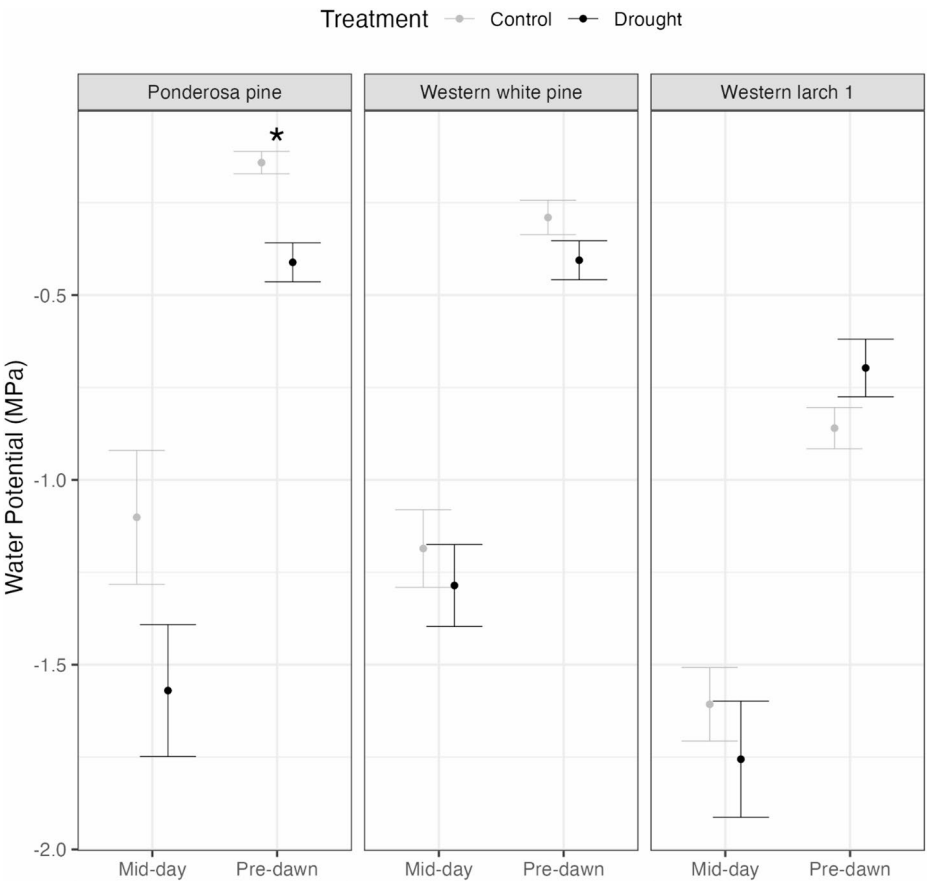

Fig. 2 Mid-day and pre-dawn water potentials (MPa) collected in August/September of the 2022 growing season for both treatments of ponderosa pine, western white pine, and western larch 1 ($n=7$). Circles represent means and error bars represent +/- one standard error. Asterisks indicate a significant treatment effect ($P<0.05$)

was significantly reduced for droughted ponderosa pine compared to well-watered controls (Fig. 2; $P=0.090$). Neither western white pine nor western larch 1 had statistical differences between treatment groups for pre-dawn water potentials.

Gas exchange

Gas exchange rates responded strongly to restricted irrigation, with significant treatment effects apparent for all four seed sources at target CC (Fig. 3). At target CC (i.e., the minimum container water content before irrigation to saturation), seedlings in the drought treatment had significantly lower photosynthetic rates than the control group for ponderosa pine ($P=0.002$), western white pine ($P=0.032$), western larch 1 ($P<0.001$) and western larch 2 ($P=0.022$) seed sources. However, when control and drought treatment groups were saturated (i.e., freshly irrigated to 100% CC), these differences in mean photosynthetic rate became negligible for ponderosa pine, western larch 1, and western larch 2. Photosynthesis

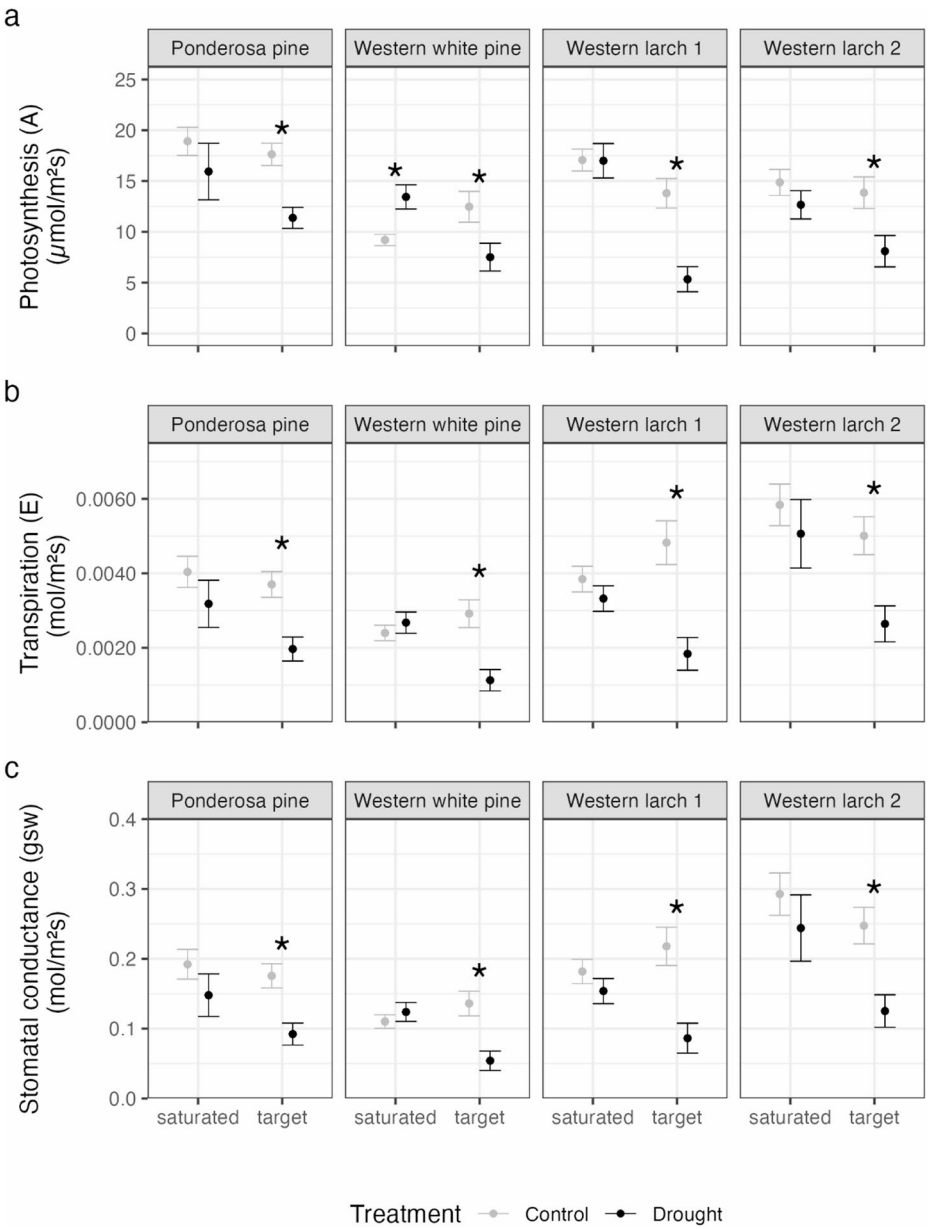

Fig. 3 **a** Photosynthesis (A) (μmol/m^{2}s), **b** transpiration (E) (mol/m^{2}s), and **c** stomatal conductance (gsw) (mol/m^{2}s) of all four seed sources measured at saturated container capacity (CC) or at target CC. Measurements were conducted on the same seedlings at target and saturation with a sample size of $n = 7$ within each seed source × treatment combination. Asterisks indicate a significant treatment effect ($P < 0.05$)

was significantly higher ($P=0.007$) for droughted western white pine compared to the control group (Fig. 3a).

Similar trends were seen between rates of transpiration and stomatal conductance (Fig. 3b and c). When watered to saturation, there was no statistical evidence of a treatment effect. However, when seedlings were dried down to target container capacities, a statistically significant treatment effect was observed between control and drought seedlings for all seed sources (Table S3). At target container capacities, rates of transpiration were reduced significantly for ponderosa pine ($P=0.004$), western white pine ($P=0.002$), western larch 1 ($P=0.002$) and western larch 2 ($P=0.006$). Similarly, stomatal conductance at target CC was reduced significantly for droughted ponderosa pine ($P=0.005$), western white pine ($P=0.003$), western larch 1 ($P=0.003$) and western larch 2 ($P=0.004$) compared to corresponding control groups for the respective seed sources.

Nonstructural carbohydrates

We found no evidence for differences between treatment groups in June, prior to the start of the experiment, for any seed source × tissue combination (Figs. 4 and 5). This result confirms uniform initial conditions in terms of NSC concentrations at the start of the experiment. Total NSC content in needle tissue for western white pine significantly differed by treatment

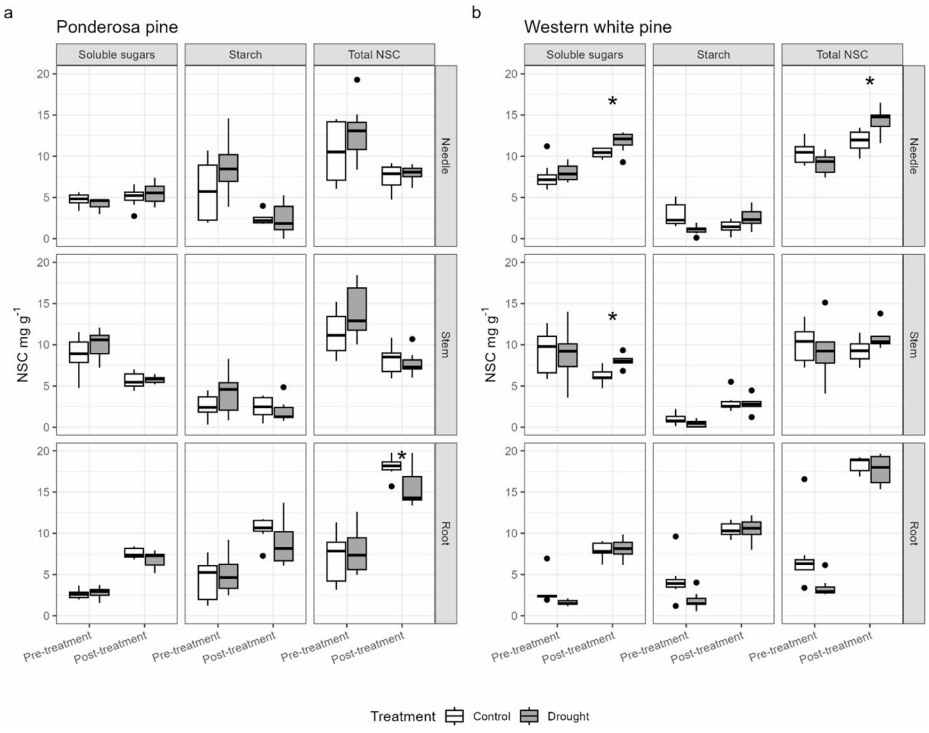

Fig. 4 Soluble sugars, starch, and total NSCs ($mg \cdot g^{-1}$) content both pre-treatment (June) and post-treatment (December) in three tissue types for each treatment (control or drought) for a ponderosa pine and b western white pine. Data represents a sample size of n = 7 for each tissue type. Asterisks indicate a significant treatment effect ($P < 0.05$)

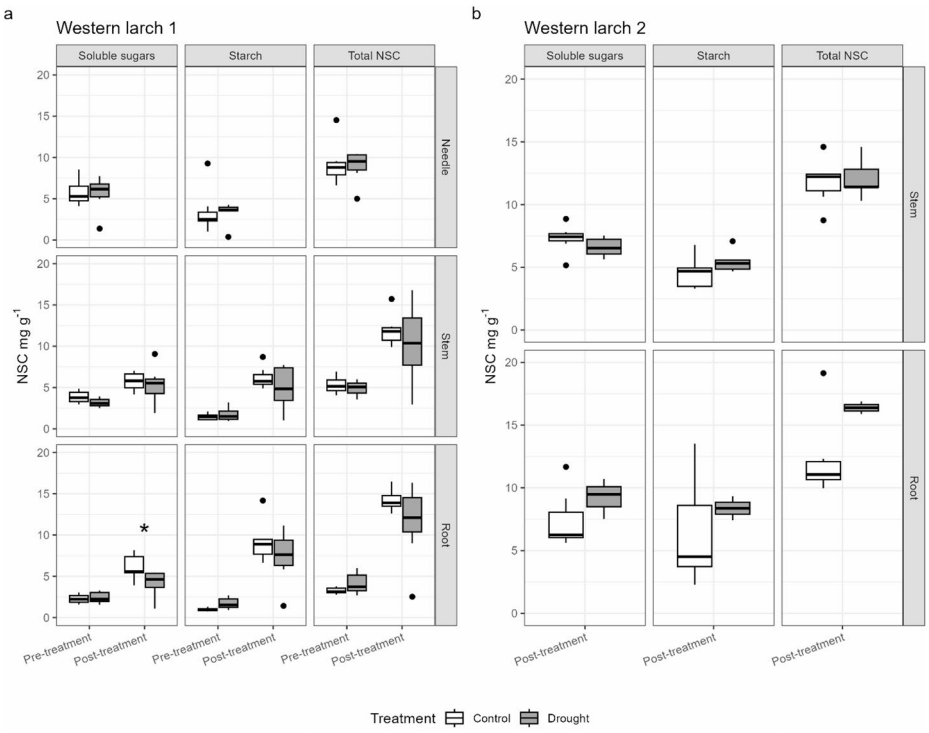

Fig. 5 Soluble sugars, starch, and total NSCs ($mg \cdot g^{-1}$) content both pre-treatment (June) and post-treatment (December) in three tissue types for each treatment (control or drought) for a western larch 1 and b western larch 2. Data represents a sample size of n = 7 for each tissue type. Due to the deciduous nature of western larch, no needles were processed in December; western larch 2 was only destructively sampled in December due to the smaller initial population size. Asterisks indicate a significant treatment effect ($P < 0.05$).

($P = 0.015$) in the post-treatment measurement. The higher mean total needle NSC concentration in droughted western white pine seedlings was driven by a significant increase in needle soluble sugars ($P = 0.043$) post-treatment; no difference among treatment groups was detected for western white pine needle starch. No significant treatment effect was observed for needle starches in ponderosa pine ($P = 0.984$). Needle tissue was only analyzed for pines due to the lack of needles present in December for the deciduous western larch.

Stem soluble sugar content post-treatment for western white pine proved to be statistically different among treatments ($P = 0.003$), with stem soluble sugar content elevated in the post-treatment droughted group relative to controls. No difference between treatment groups was detected in post-treatment western white pine stem starch content ($P = 0.720$), along with no difference in total stem NSCs ($P = 0.072$). No significant treatment differences were found for post-treatment stem soluble sugar, starch, or total stem NSC content for ponderosa pine, western larch 1, or western larch 2 (Table S4).

Total root NSC content differed significantly between treatments for ponderosa pine (Fig. 4a, $P = 0.032$) with droughted seedlings having lower root total NSC concentration compared to post-treatment controls. Root soluble sugar content proved significantly different between treatments for western larch 1 (Fig. 5a, $P = 0.032$) with droughted seedlings

having lower root soluble sugar concentration compared to post-treatment controls. No significant treatment effect was observed in any root tissues for western white pine or western larch 2 (Fig. 5).

Notably, both treatment groups of ponderosa pine, western white pine, and western larch 1 exhibited a marked increase in root soluble sugar, root starch and total root NSC from pre- to post-treatment (i.e., from June to December) (Figs. 4a, b and 5a). The magnitude of seasonal change in NSC concentrations was greater in root tissue than in stem or needle (evergreen species only) tissue for all seed sources (Figs. 4 and 5). The seasonal accumulation of NSCs in root tissues was consistent across the three species studied.

Seasonal trends of stem tissue NSC concentrations were species specific. Ponderosa pine total stem NSC concentration decreased from pre- to post-treatment, potentially driven by a decrease in stem soluble sugar concentration (Fig. 4a). In contrast, total stem NSC concentration in western larch 1 increased from pre- to post-treatment, corresponding to increases of both stem soluble sugar and stem starch concentration (Fig. 5a). Strong seasonal trends in stem NSC concentrations were not apparent for western white pine.

Seasonal trends in needle tissue NSC concentration differed between the two pine species. Ponderosa pine total needle NSC concentration trended modestly lower from pre- to post-treatment, tracking a similar decrease in ponderosa pine needle starch concentration (Fig. 4a). In contrast, western white pine total needle NSC concentration trended modestly higher from pre- to post-treatment, tracking a similar increase in western white pine needle soluble sugar concentration (Fig. 4b). Needle tissue NSC concentration was not analyzed for the deciduous western larch seed source.

Carbon isotope ratios

There was no significant treatment effect on carbon isotope ratios for any seed source (Fig. 6a, b; Table S5).

Discussion

Drought conditioning seedlings in nurseries via restricted irrigation regimes have been hypothesized to induce morphological and physiological adaptations advantageous for drought resistance (Puértolas et al. 2024). Our experiment—conducted on containerized second-year stock of ponderosa pine, western white pine and two seed sources of western larch—induced significant morphological and instantaneous physiological effects, although responses varied by species. Ponderosa pine exhibited no significant changes to above- or below-ground biomass, whereas western white pine and both seed sources of larch showed notable morphological responses to the drought conditioning treatment. Given the containerized nature of our study, it should be noted that root development may have been limited. Physiologically, our data indicates that our drought conditioning treatment sufficiently stressed ponderosa pine as seen in the significantly lower pre-dawn water potentials. Interestingly, neither western white pine nor western larch 1 exhibited this response. All drought conditioned species and seed sources experienced a significant reduction in gas exchange rates at target CC, but once rehydrated, gas exchange rates rebounded to levels similar to controls. Although the drought treatment reduced gas exchange rates for all seed sources

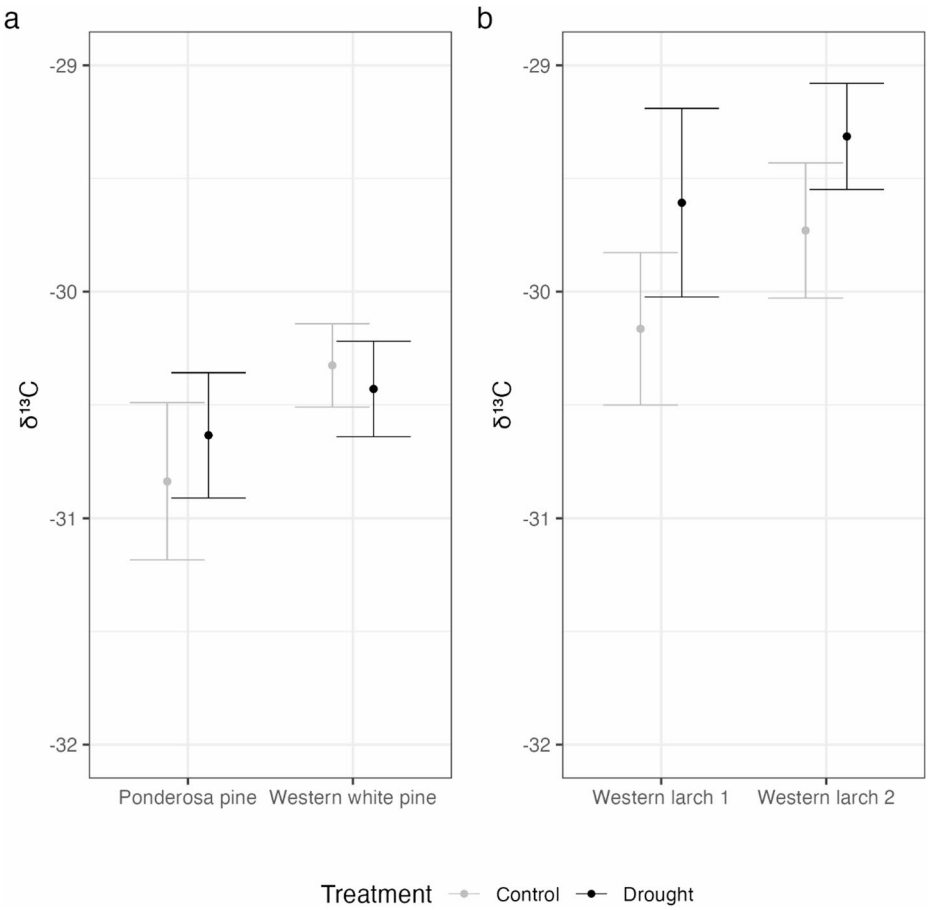

Fig. 6 **a** Ponderosa pine and western white pine needle tissue $\delta^{13}C$ and **b** western larch branch tissue $\delta^{13}C$ collected post-treatment for both control (grey) and drought (black) with a sample size of $n=7$. Circles represent means while error bars represent +/- one standard error. (Color figure online)

and induced plant moisture stress in ponderosa pine, the short-term stress imposed by the treatments during maximum dry-down did not induce significant changes in carbon isotope ratios, indicating a lack of cumulative or significant stress throughout the growing season. Our findings demonstrate that drought conditioning can induce morphological and instantaneous physiological responses and reveals differential sensitivity to restricted irrigation regimes across the study species.

Response of Pinus seedlings to drought conditioning

Our drought treatment resulted in significant morphological changes to western white pine while droughted ponderosa pine seedlings remained morphologically unaffected. We had hypothesized that seedlings would exhibit morphological plasticity beneficial for adapting to moisture-limitation by decreasing above ground biomass and increasing below ground biomass, thereby increasing root:shoot ratios (Toca et al. 2022). Increased root:shoot ratios

could be advantageous for moisture-limited sites where the plant has less photosynthetically active tissue vulnerable to water loss, and increased water absorption capabilities with increased root mass (Hasse 2008). While our study found almost no difference to ponderosa pine morphology, droughted western white pine experienced a significant reduction to RCD. This was similar to findings of Pinto et al. (2023) who found no effect of their reduced irrigation treatment on root mass or shoot mass of ponderosa pine seedlings but did find reductions in height and RCD. Other drought conditioning studies conducted on *Pinus* species also found reductions height and RCD, but like Pinto et al. (2023), the seedlings used were all conditioned within their first year (Royo et al. 2001; Deligoz and Gur 2015; Pinto et al. 2023). While ponderosa pine remained unaffected, the decrease in height and RCD in western white pine coupled with maintenance of root masses equivalent to controls, could prove beneficial once outplanted.

The response of western white pine is likely driven by the comparatively small stature and slow growth rates combined with the relatively large container size. Container type will influence moisture availability and the potential for root growth (Pinto 2011). Droughted western white pine experienced significant reductions in height and RCD relative to controls. Droughted western white pine maintained similar root mass with reduced RCD. While it was hypothesized that western white pine would be sensitive to the drought treatment, the treatment was not severe enough to induce strong responses. The lack of separation seen in mid-day water potentials indicate seedlings were under similar amounts of moisture stress in both treatments. The inherent morphology of this species with a significantly smaller shoot mass compared to ponderosa pine, accompanied by similar root masses, may have been advantageous. Though this species was morphologically sensitive, potentially the combination of having less photosynthetic tissue susceptible to water loss paired with a deep pot allowed western white pine sufficient access to water, effectively avoiding hydraulic stress under the drought treatment.

As soil moisture decreases, photosynthesis, transpiration, and stomatal conductance decrease simultaneously (Farquhar et al. 1989). The recovery of gas exchange rates for the droughted ponderosa pine seedlings once rehydrated to saturation coupled with minimal changes to morphology indicate that ponderosa pine was able to opportunistically use water when available while accumulating biomass (Marshall and Zhang 1994; Zhang et al. 1996, 1997; Pinto et al. 2023). Although gas exchange rates at target CC indicate an instantaneous response to the drought treatment, the lack of significant carbon isotope ratio differences among treatments, especially for the pines, indicate a lack of cumulative stress over the growing season.

While there may be opportunity to further limit irrigation as indicated above, both *Pinus* species presented some effects to NSC concentrations. Droughted ponderosa pine significantly reduced root total NSC driven by a decrease in starch and soluble sugar concentrations. A reduction in root NSCs has been reported in 1-year ponderosa pine left un-watered for 76 days (Woodruff et al. 2024), > 1-year-old *Pinus halepensis* with 20 day drying cycles (Royo et al. 2001), and with studies on more mature *Pinus halepensis* under atmospheric drought (Klein et al. 2014). Additionally, this was mirrored in *Picea abies* with decreased root NSC content coupled with homeostatic concentrations in stems and needles (Hartmann et al. 2013). This decrease could reflect decreased carbon assimilation via photosynthesis and reliance on these stores due to decreased stomatal conductance, or mobilization to maintain osmotic balance. Whether the decrease in total root NSC was allocated to growth,

respiration, or osmotic adjustment, this reduction may be disadvantageous as seedlings will require resources to invest in photosynthesis (Burdett 1990) largely allowing for growth (Grossnickle 2012), especially root development (Grossnickle and Ivetić 2022).

In contrast, droughted western white pine seedlings displayed no significant change to root NSC's but experienced an increase in soluble sugar concentrations in both needles and stems, along with an increase in total needle NSC. This is consistent with a study that found increased needle soluble sugars in *Pinus yunnanensis* seedlings under light and moderate drought (Xiao et al. 2024), in addition to studies on mature trees in the Mediterranean which found drought-stressed trees had lower NSCs in roots but not in shoots (Klein et al. 2014). In general, studies have found that drought can impair the transportation of NSC's from leaves to roots (Sala et al. 2010; Sevanto 2014; Savage et al. 2016) due to physiological restraints on phloem transport (Hartmann et al. 2013; Sevanto 2018). Our morphological data supports this as the seedlings experienced reduced growth coupled with high rates of photosynthesis once watered. While our drought treatment may reflect the plants' impaired ability to transport NSCs from sources (leaves) to sinks (roots) (McDowell 2011), there is a possibility that these sugars in needles and shoots also reduced osmotic potential allowing for turgor maintenance (Sala et al. 2012; Dietze et al. 2014) or could indicate an increase in cold tolerance. Studies have found that NSC dynamics are linked to phenological timing (Blumstein et al. 2024; Luo et al. 2024) along with temperature (D'Andrea et al. 2021) leading to the idea that trees can convert starches to soluble sugars for cold hardiness and/or stress tolerance (Kozlowski 1992).

Given the complex, dynamic nature of NSC's that are still under investigation, theorizing the specific mechanisms leading to our results remains difficult. Ultimately, if these enriched values of needle and stem NSCs remained available to the plant for use once planted, this could provide a survival advantage as seen in increased survival of NSC enriched tropical plants (O'Brien et al. 2014). Although drought-adaption of this sort is possible, the long-term biological availability of these enriched values is debated as research indicates a short residence time of NSCs in needles (Asao et al. 2024) along with potential NSC depletion during over-winter storage (Ritchie 1982).

Response of Larix seedlings to drought conditioning

Western larch exhibited the most significant morphological response to the drought treatment, with significant decreases in height and RCD as well as decreases in root and shoot mass. Lower root mass might not be beneficial for dry field sites, although the mirrored shoot mass reduction allowed for similar root: shoot ratios balancing root mass and transpirational area. Both larch seed sources experienced similar treatment effects on morphology, gas exchange rates, and carbon isotope ratios. Higgins et al. (1987) found that above −1.5 MPa, no effect to gas exchange was observed in *Larix decidua* and inferred that *Larix* may exhibit high rates of photosynthesis over the growing season. As a deciduous species, needle shedding would be expected to decrease carbon demand and improve carbon balance (Reich et al. 2009) which was seen in the premature needle reddening of western larch 1 in September 2022, which we interpret as a response to the drought treatment. However, our mid-day water potentials conducted in August did not indicate severe stress caused by our drought treatment.

In this drought conditioning experiment, our droughted western larch seedlings were exposed to short-term yet severe stress with a target CC of 40% of their saturated CC from June until September, then raised to 60% from September to December of 2022. Other drought conditioning studies conducted on 6.5-week-old western larch seed sources used similar irrigation methods but three different regimes: Low (50–65% CC), Moderate (70–75% CC) and High (75–100% CC) (Moler and Nelson 2021; Vale et al. 2021). The results from these studies indicated a significant increase in new root tissue mass as a ratio of new foliar tissue in the Low and Moderate treatments. Vale et al. (2021) also found a significant increase in the proportion of root mass contained in deep lateral roots compared to upper lateral roots. Although these experiments included different seed sources at different ages, their results are consistent with the idea that optimal irrigation regimes for drought conditioning may be ≥ 50% CC for larch seedlings.

Given the deciduous nature of larch, they may respond to the drought treatment via osmoregulation of solutes (Badalotti et al. 2000), however there are few studies reporting NSC dynamics in western larch. Western larch 1 exhibited increased soluble sugar concentrations in roots while no response was displayed for western larch 2. Instead, an increase in total root NSCs largely driven by starch reflects the allocation to storage carbohydrates (Fig. 5). This strong seasonal accumulation of NSCs may have outweighed any drought treatment effect. A study using mature *Larix principis-rupprechtii* indicated that temperature was the primary environmental factor influencing NSC concentrations (Yang et al. 2023). Another study using one-year *Larix kaempferi* and *Larix olgensis* seedlings indicated that elevated nitrogen fertilization reduced drought tolerance and suggested growing seedlings under water- and nitrogen-limited regimes (Kitao et al. 2022).

Conclusions and implications for future research

Developing seedlings with novel nursery culturing techniques provides an opportunity for nursery managers, land managers, and researchers to improve reforestation success by anticipating planting sites previously disturbed by wildfire and increasingly limited by soil moisture. We add to the understanding of how restricted irrigation regimes influence seedling morphology and physiology of conifer seedlings with differing functional traits with our investigation of responses to restricted irrigation by second year ponderosa pine, western white pine and western larch seedlings. Based on our results and findings from a recent review (Toca et al. 2022) and meta-analysis (Puértolas et al. 2024), drought conditioning treatments will have species-specific outcomes, and their intensity, duration and magnitude must be tailored accordingly.

We recommend future studies calibrate restricted irrigation regimes to the species studied. Puértolas et al. (2024) found that more intense drought conditioning applications tended to improve outplanting survival. Based on our results, we suggest investigating more restrictive irrigation regimes for ponderosa pine and western white pine (i.e., reduce target CC to below 40%). In contrast, more sensitive and/or deciduous species such as western larch may require less restrictive irrigation regimes (i.e., increase target CC to above 40%) to achieve drought conditioning outcomes without incurring acute stress and premature needle reddening and loss. Another avenue to explore is manipulation of the duration of drought by implementing a sustained restricted irrigation regime, rather than the dry down and re-

wetting cycles used in our experiment (Valliere et al. 2019). At maximum dry down, all three of our study species exhibited reduced gas exchange and photosynthesis. Yet, when rewetted these parameters rebounded to levels equivalent to those in well-watered controls, and we found little evidence for long-term drought-induced physiological acclimation in the carbon isotope, NSC, or pre-dawn water potential measurements. Thus, we suggest that a restricted irrigation regime that maintains chronic, constant moisture stress rather than cycles of repeated dry down and rewetting may induce a stronger acclimation response. Toca et al. (2022) found that manipulation of water and nutrient regimes have the greatest potential to manipulate root development, so assessing additive and interactive effects of water and nutrient manipulations may be useful in future studies. Finally, experimental assessment of the relative performance of one-year-old and two-year-old stock would provide insight into the tradeoffs associated with using older and larger containerized stocktypes for drought conditioning.

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Data availability Data is provided within the manuscript or supplementary information files.

Declarations

Conflict of interest The authors declare no competing interests.

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