New Forests (2026) 57:52
https://doi.org/10.1007/s11056-026-10198-x

Timing of water limitation moderates drought hardiness traits of western larch (*Larix occidentalis*) seedlings

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Received: 28 January 2026 / Accepted: 2 July 2026 / Published online: 21 July 2026
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Abstract
Water limitation during tree seedling cultivation can promote traits that improve drought hardiness. Nurseries apply water limitation mostly during a brief developmental window of seedling growth, which may restrict the range of useful traits achievable through water limitation if phenotypic responses differ across ontogeny. We characterized the diversity of western larch (*Larix occidentalis* (Nutt.)) seedling traits produced by four factorial combinations of low- and high-watering regimes spanning the first and second years of growth. Seedlings from all watering treatments were exposed to ten days of water withholding during a drought pulse trial at the end of year two preceding destructive measurements. Seedling stem water potential (Ψ) measurements recorded before, during, and after the drought pulse trial suggested that water-limited cultivation in the second year and consecutively across both years resulted in the greatest mid-day Ψ (least moisture stress) during the drought pulse and the greatest integrated water use efficiency. Seedlings cultivated with limited water in the second year and consecutively across both years expressed similar traits and performance during the drought pulse, suggesting that previous exposure to drought did not moderate seedling responses to subsequent water limitation. Water-limited cultivation in year one resulted in a greater number of root branch nodes and mass of egressed roots given ample water in year two, but not when water was limited in year two. Evidence suggests that seedlings express age-dependent phenotypic responses to water limitation that promote belowground growth best when applied during the first year except in dry transplanting environments.

Keywords Functional trait variation · Drought pulse · Target plant concept · Root egress · Drought hardening · Plant stress memory · Forest regeneration

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Introduction

Hot and dry conditions pose a challenge to forest regeneration across the globe (Allen et al. 2015; Clark et al. 2016; Hammond et al. 2022; Gebrechorkos et al. 2025). Seedlings often express stunting or early mortality shortly after planting (i.e. transplant shock; Close et al. 2005) due primarily to insufficient access to and uptake of soil water (Rietveld 1989, Burdett 1990). However, when exposed to controlled water limitation early in plant cultivation, seedlings can express a range of phenotypic traits that facilitate water access and retention, such as an increased ratio of root to shoot tissue mass (minimizing water demand relative to supply); increased root system surface area and depth (improving plant access to water stored in deeper soil horizons); and increased carbon assimilation per unit water lost (i.e. plant water use efficiency; Arnott 1975; Ding et al. 2012; Green et al. 2005; Grossnickle 2005; Guarnaschelli et al. 2006; Livingston and Black 1987a, b; Moler and Nelson 2021; Pritzkow et al. 2021; Sloan et al. 2020; Valliere et al. 2019; Vilagrosa et al. 2003).

Water limitation is used in temperate zone containerized seedling nurseries toward the end of the first season of seedling growth. While late season water limitation is compatible with the goal of achieving size specifications for marketable seedlings, reforestation under historically hotter and drier conditions has created a need for seedling cultivation methods that promote drought hardiness sufficient to achieve acceptable levels of seedling survival and growth in increasingly challenging environments. In contrast with the nursery practice of applying water limitation during a single brief period of seedling cultivation, the natural environments where many tree species evolved likely entailed drought exposure at various times during early seedling life stages. While it is known that plant age can influence plant responses to resource limitation (Gedroc et al. 1996), it remains unknown whether nursery practitioners could control a wider range of seedling traits by using a more flexible growing schedule for water-limited cultivation. Cultivation methods that allow a greater level of control over seedling trait expression are desirable and may be possible, but the extent to which the phenotypic effects of water limitation vary with the timing of water limitation exposure during early seedling growth is not well known. Furthermore, although some species respond to water limitation treatments with increased future root growth (Rook 1973; Moler and Nelson 2021), there is a need to better understand the conditions under which water limitation treatments in fact mitigate drought stress (Puértolas et al. 2024). For example, 1+0 *Pinus radiata* seedlings cultivated with limited water produced significantly more roots and longer roots than seedlings cultivated with ample water under both well-watered and water-restricted post-transplanting conditions, although water-restricted post-transplanting conditions constrained root number and length of both treatment groups relative to well-watered post-transplanting conditions (Rook 1973). And further illustrating the impact of water limitation on root growth, Day and MacGillivray (1975) found that roots of 2+0 white spruce seedlings (*Picea glauca* (Moench) Voss.) planted in sandy loam soils maintained at several soil water potential values grew poorly in soils at −0.06 MPa relative to treatments with higher water potential values, and they found that values of −0.15 MPa resulted in negligible root growth.

Drought hardening achieved by water-limited cultivation is an outcome of phenotypic plasticity (i.e. environmentally determined phenotypic expression; Pfennig et al. 2010) in response to nonlethal drought stress. For controlled water limitation to promote seedling establishment, seedlings must be phenotypically plastic for drought hardiness traits, and

traits achieved through phenotypic plasticity must remain expressed or be re-expressed after planting, but not impaired by water limitation in outplanting environments. Thus, it is perhaps unsurprising that a recent meta-analysis by Puértolas et al. (2024), including data from 22 studies of the effect of water limitation on seedling survival, found that outcomes were strongly context-dependent, with some lifeforms and outplanting conditions broadly more compatible with the technique than others. Previous studies demonstrate that outcomes of water limitation vary strongly with species (Ritchie and Dunlap 1980) and the timing of water limitation (Valliere et al. 2019). In a study of herbaceous, shrub, and grass species native to California, Valliere et al. (2019) employed a reciprocal drought conditioning experiment consisting of an initial drought period of 20-days followed by a second drought period of 60-days. The reciprocal treatments were separated by 30 days and showed divergent but species-dependent phenotypic effects across treatments. A study of *Pinus tabuliformis* showed that the effects of drought conditioning intensity and duration influenced seedling morphology and physiology in the nursery and following planting into the field, and a longer duration of drought conditioning in the nursery resulted in seedlings with greater root mass relative to shoot mass (Luo et al. 2021). In contrast, *Pinus roxburghii* seedlings subjected to moderate drought in a potted study showed an initial increase in the ratio of root: shoot dry mass relative to well-watered seedlings, but declining root: shoot dry mass with increasing drought intensity (Tewari and Mittal 2017).

Western larch (*Larix occidentalis* Nutt.) is an ecologically and commercially important species with known ecotypes across its limited geographic distribution spanning the USA and Canada in Western North America, where warmer and drier conditions are expected to limit the extent of habitat suitable for the species (Rehfeldt and Jaquish 2010). Artificial regeneration of western larch stands would benefit from seedling stocktypes able to withstand increasingly hot and dry field conditions. Previous work has shown that controlled water limitation applied to western larch seedlings during the first growth year can increase the length, number, and mass of roots measured early in the second growth year following germination (Moler and Nelson 2021; Vale et al. 2021). But the importance of temporal and sequential patterns of water limitation for seedling drought hardiness remains underexplored. For example, while drought exposure early in cultivation enhances subsequent root growth potential in *L. occidentalis* (Moler and Nelson 2021), it is unknown whether further trait improvement or the manipulation of different seedling traits can be achieved through novel timing or sequences of controlled water limitation. Answers to these questions would elucidate plant stress-acclimation processes and could lead to improved practices for cultivating seedlings with enhanced performance under drought.

We explored the diversity of seedling phenotypes achievable through factorial combinations of water limitation in a potted experiment spanning two years of growth by subjecting western larch seedlings to reciprocal water limitation treatments in the first and second growth years, followed by exposure to severe water withholding during a drought pulse trial at the end of year two. Multi-season reciprocal water limitation treatments included the following factorial combinations of high and low levels of watering during the first and second years of seedling cultivation:

i. Ample water in both growth years (high-high)
ii. Ample water in the first year and water-limited in the second year (high-low)
iii. Water-limited in the first year followed by ample water in the second year (low-high)

iv. Water-limited in both years (low-low)

We addressed two hypotheses to investigate the effects of seedling water limitation history on seedling morphology and water relations physiology during and after a drought pulse trial. We expected that the *timing* of seedling exposure to water limitation (i.e. water limitation during the first versus second year) and *recurrence* of water limitation (i.e. cultivation with limited water during both years versus only one year) would produce distinct seedling phenotypes because plant responses and ability to respond to stress and resource limitation can vary with plant age (Gedroc et al. 1996; Weinig and Delph 2001; Rankenberg et al. 2021) and plant responses to various types of stress can depend on previous exposure to stress (Walter et al. 2013; Li et al. 2023). Evidence from past studies led us to expect that exposure to water limitation in the first year would achieve drought-adaptive phenotypes that remain expressed through the second growth year (Moler and Nelson 2021). We furthermore expected that water limitation imposed only during the second year would not promote drought-adaptive phenotypes as effectively as water limitation imposed during the first year because growth patterns of older plants tend to be shaped by structural and allocational constraints established during a younger age (Weinig and Delph 2001). We also suspected that water-limited cultivation across the first and second growth years may produce unique phenotypes not achieved through other cultivation regimes as plant stress response can be mediated by previous stress exposure (Walter et al. 2013). But we expected that consecutive water-limited cultivation across the first and second growth years would result in diminished drought-adaptive morphological or physiological traits relative to water limitation during only a single year because insufficient water availability in year two would restrict expression of desirable patterns of biomass allocation and drought-responsive water regulation physiology, as in Rook (1973). Specifically, we posed the following two hypotheses:

1. A greater number of seedling traits will vary with the age at which seedlings are exposed to water limitation than whether seedlings experience consecutive water limitation.
2. Seedling water potential during the drought pulse trial will vary among factorial combinations of water limitation history, and the lowest plant moisture stress will be achieved by water-limited cultivation only in the first year, coinciding with the greatest production of new root tissue in seedlings exposed to water limitation only in the first year.

Methods

Plant material and first-year growth conditions in a greenhouse

Seeds were collected in August 2016 from a single maternal tree genotype (half-sib seed) at the Kalamalka Research Station and Seed Orchard (Vernon, British Columbia, Canada). Seeds were sown on April 15, 2021, into 91/130 Styroblock® containers with cavity volumes of 130 ml (Beaver Plastics, Alberta, Canada) in the Franklin H. Pitkin Forest Nursery greenhouse in Moscow, Idaho, USA (46.73° N, 117.00° W). Container cavities were filled with Berger© BM8 growing media (Saint-Modeste, Quebec, Canada) amended with 7.9 g slow-release Osmocote® fertilizer ($N = 15\%$, $P = 9\%$, $K = 12\%$) per liter of soil media. Cavities were then topped with TARGET® Forestry Sand (no. 9992002; Burnaby, BC, Canada).

After seed sowing, containers were well-watered for 6.5 weeks to allow time for seed germination before water limitation treatments commenced at the beginning of the seedling rapid growth phase. 120 seedling trays (8 seed sources × 3 watering treatments × 5 tray replicates), with 91 seedlings per tray, were arranged in a split-plot experimental design; the whole plot factor was the watering treatment and the sub-plot factor included several seed sources as part of a larger study. A single seed source was included in the second year of cultivation for the present study (see next section for the second-year cultivation experimental design). Seedlings were grown under natural photoperiod during the day and were supplemented with intermittent night disruption with photoperiod lights set for five minutes on and 15 min off throughout the night. Photoperiod lights were on from the time of germination until July 1, 2021. Greenhouse temperature averaged 21.7 °C throughout the experiment and relative humidity averaged 66%. Daytime temperatures during active growth were maintained between 21.1 °C and 29.4 °C during the day and set to 21.1 °C during the night. Water limitation treatments were initiated 6.5 weeks after seed sowing and maintained through the seedling hardening phase. The low-watering treatment achieved 50%–65% saturated container weight, and the high-watering treatment achieved 75%–100% saturated container weight. Selected saturated container weight values translate to gravimetric soil water contents of 32–42% and 48–65%, respectively. The saturated container weight range of 50%–65% was found to be just above the level at which wilting occurred for *L. occidentalis* (pers. obs.), and the range of 75%–100% saturated container weight is the standard irrigation protocol in use for *L. occidentalis* production at the Franklin H. Pitkin Forest Nursery. Saturated container weights were established by measuring tray weights after watering planted Styroblock® containers with a boom irrigation system until tray weight did not increase after further watering, and after gravitational water drained from trays for 2 h (following MacDonald et al. 2012). New saturated container weights were calculated monthly to adjust for increases in seedling mass, at which time full saturation of containers was expected to help stabilize nutrient availability across watering treatments. Desired percentages of saturated container weights were maintained by weighing containers daily and irrigating trays when tray weights reached the lowest weight permitted for the low and high soil moisture content treatments. Container weights were based on the average weight across five trays per soil moisture treatment. Bud set was hastened using a 12-day reduced day-length treatment (10.5 h light, 13.5 h dark) applied 9.5 weeks after initiating drought preconditioning. See Moler and Nelson (2021) for further description of the first-year treatment. Seedling predawn (Ψ_{pd}) and midday (Ψ_{mid}) stem water potential were measured on five seedlings per watering group (conducted by randomly selecting and destructively harvesting one seedling per each of five trays per treatment) at approximately 0100–0400 h and 1200–1400 h, respectively, using a Scholander pressure chamber (PMS Model 1505D, PMS Instrument Company, Albany, OR, USA) to ensure the gravimetric-based watering treatments achieved distinct levels of plant moisture stress. Water potential measures averaged across July-September were: $\Psi_{pd} = -0.38$ (± 0.17(SD) and $\Psi_{mid} = -0.51$ (± 0.19) for seedlings in the high-watering treatment, and $\Psi_{pd} = -0.53$ (± 0.15) and $\Psi_{mid} = -1.50$ (± 0.40) for seedlings in the low-watering treatment. Each treatment regime was applied for six months using an overhead boom irrigation system. Seedlings were removed from containers eight months after seed sowing and stored at −2.2 °C in a freezer for one month.

Second-year growth conditions and reciprocal watering treatment

In May 2021, 24 seedlings from the first-year high watering treatment and 24 seedlings from the first-year low watering treatment were planted individually in five-gallon (18.9 L) pots, filled with moistened Berger© BM8 growing media, and placed on pallets approximately 15 cm above a paved ground surface outdoors at the Franklin H. Pitkin Forest Nursery for three months of growth. Seedlings were positioned on pallets in a completely randomized design with random assignment of seedlings to two watering levels (high or low), resulting in reciprocal watering treatments consisting of $n = 12$ seedlings in each of four treatments: (1) ample water during the first and second year (high-high), (2) ample water during the first year and water-limited in the second year (high-low), (3) water-limited in the first year and ample water in the second year (low-high), and (4) water-limited in year one and two (low-low). High and low watering treatments were maintained by monitoring volumetric soil water contents (VWC) individually for each seedling pot each day for the duration of the treatment using a handheld VWC meter with 12 cm long sensor rods (Hydrosense II, Campbell Scientific, Logan, UT). The low watering treatment was achieved by irrigating pots when mean VWC across all pots dropped to 50%, at which time pots were watered to saturation by applying water with an automated overhead irrigation boom for 30 min. The irrigation time of 30 min was found to achieve soil saturation as further watering past 30 min did not increase pot mass. The high watering treatment was implemented in the same manner when mean VWC across all pots dropped to 75%. For both watering treatments, pots scheduled for watering were moved out of their completely randomized positions to an empty pallet for watering with the boom irrigation system. Pots were then moved back to their randomized positions after watering. Berger© BM8 growing media has gravimetric soil water content of 0.462 kg/kg at the permanent wilting point (soil-media water potential of − 1.5 MPa; Moler and Nelson 2021). With a bulk density of 192.4 kg/m^3, permanent wilting point using Berger© BM8 growing media corresponds to a VWC of 8.9%, which is well below the minimum target water contents of 50% and 75% VWC in second-year watering treatments. Pots were systematically rotated to a new position twice weekly throughout the duration of the experiment to ensure seedlings occupied each position in the experimental area at least once to minimize microsite effects.

Drought pulse trial

During the final month of the study (July) all seedlings were subjected to two periods of non-lethal water withholding (hereafter *drought pulse trial*) during which seedlings did not receive irrigation and were sheltered from potential precipitation. Seedling water status and recovery were compared across watering treatments before, during, and after the drought pulse trial. We intended to complete two drought pulse periods separated by three days of drought recovery (achieved by watering pots to saturation daily), with each water withholding period lasting ten days. However, logistical constraints restricted the duration of the second drought pulse period to five days (Fig. 1), and we restricted our analyses to measurement events surrounding the single ten-day drought pulse period. Mean maximum daily high and low temperatures during the drought pulse trial were 31.3˚C and 8.3˚C, respectively. Five seedlings from each reciprocal watering treatment combination were randomly selected and measured for Ψ_{pd} and Ψ_{mid} to compare water status across treatments before,

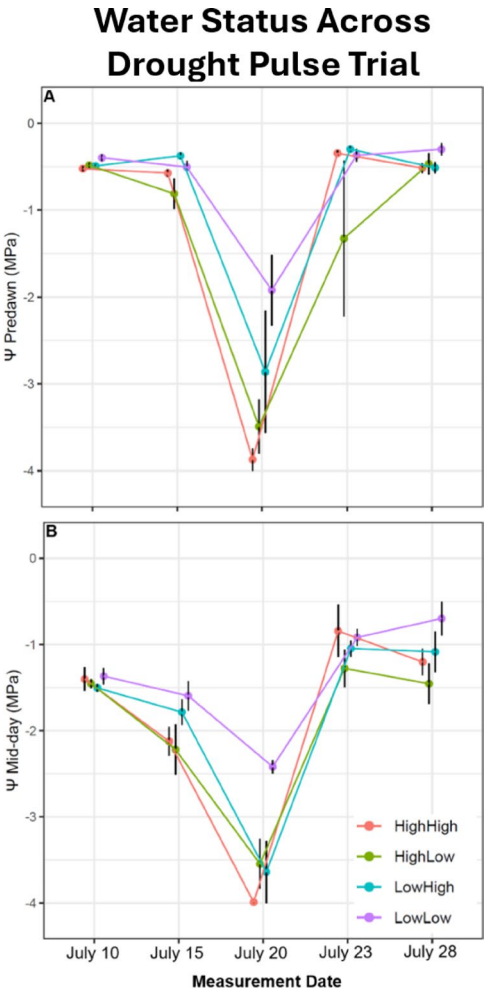

Fig. 1 Ψ (stem water potential, MPa) measured across a drought pulse trial applied to *Larix occidentalis* seedlings during the second year of growth. Pre-dawn stem water potential (2 **A**). Mid-day stem water potential (2 **B**). Measurement timepoints include: July 10 (immediately following final saturation watering at the beginning of the ten-day water withholding period); July 15 (middle of the ten-day water withholding period); July 20 (end of the ten-day water withholding period); July 23 (three days after daily watering to saturation, beginning of the five-day water withholding period); July 28 (end of the five-day water withholding period). Filled circles are means; error bars are standard error of the mean. Means offset horizontally for visibility

during, and after the drought pulse trial (Fig. 1). Specifically, predawn and midday water status were measured one day after watering all pots to saturation on the day that water withholding commenced (July 10, 2021), five days after water withholding commenced (July 15), and 10 days after water withholding commenced (July 20); predawn and midday water status were measured again after seedlings were watered to saturation daily for three days (July 23), and measured again at the end of the truncated five-day drought pulse before destructive harvest (July 28). After 10 days of water withholding, the average stem water potential value of each treatment group reached at least $\Psi_{pd} = -1.5$ MPa. The drydown target of $\Psi_{pd} = -1.5$ MPa was selected based on Harayama et al. (2021), who demonstrated that the shoot water potential at turgor loss point (TLP) was −2 MPa for Japanese larch (*Larix kaempferi*), the species most closely related to *L. occidentalis* for which Ψ at TLP is available. Because water withholding was applied until the average stem water potential value of each treatment group reached at least $\Psi_{pd} = -1.5$ MPa, treatment groups exhibited a wide range of average Ψ_{pd} values, as follows: high-high = −3.87 MPa, high-low = −3.49 MPa,

low-high = −2.86 MPa, low-low = −1.92 MPa. Two seedlings from the high-high treatment failed to recover from the drought pulse treatment; these two seedlings were not part of the group of seedlings monitored for Ψ_{pd} and Ψ_{mid} before, during, and after the 10-day drought pulse.

Seedling morphology

Ten foliar, stem, and root traits were destructively measured immediately after ending the drought pulse trial on July 28 (Table 1). Seedling root collar diameter (RCD) was measured with calipers at the soil level to the nearest 0.01 mm. Seedling height was measured from the soil level to the top of the tallest woody tissue to the nearest 0.1 cm. Aboveground and belowground plant material was separated at the root collar upon harvest; roots were cleaned of soil media and were separated into three sections per seedling: (1) taproots, (2) lateral roots within the first-year root plug, and (3) roots that egressed from the root plug (i.e. extended beyond the dense 130 ml volume of roots and potting soil formed during the first growth season), and each section was bagged and labeled accordingly. Aboveground

Table 1 Statistical results of the effects of year 1 and year 2 watering level and their interaction on *Larix occidentalis* seedling morphological and physiological traits, where watering level is a comparison between high versus low watering levels

Tissue	Trait	Year 1 watering treatment	Estimate (±SD)	Year 2 watering treatment	Estimate (±SD)	Cross-year watering treatment interaction	Estimate (±SE)
Foliar	$\delta^{13}C$	***	0.72 (±0.20)	***	0.98 (±0.19)	NS	–
	total N	NS	–	NS	–	NS	–
	dry foliar mass	NS	–	NS	–	*	−1.19 (±0.55)
	LMA	NS	–	NS	–	NS	–
Stem	aboveground woody mass	NS	–	NS	–	*	1.29 (±0.50)
	final RCD	NS	–	**	−0.53 (±0.18)	NS	–
	final seedling height	NS	–	NS	–	*	7.50 (±3.13)
Root	total mass belowground	NS	–	NS	–	NS	–
	egressed root mass	*	0.33 (±0.10)	NS	–	NS	–
	lateral root mass	NS	–	NS	–	NS	–
	taproot mass	***	−0.37 (±0.08)	*	−0.18 (±0.08)	NS	–
	root branch nodes	*	0.16 (±0.06)	*	−0.15 (±0.06)	NS	–
Biomass ratio	egressed root: new foliage	NS	–	**	−0.33 (±0.52)	NS	–
	root: shoot	NS	–	NS	–	NS	–

Estimates with standard deviations (SD) or standard errors (SE) and significance levels are reported for each trait. *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$; NS: $p>0.05$; –: no effect detected

growth was separated into foliar and woody biomass sections for each seedling. Foliar tissue was scanned and analyzed using WinSEEDLE™ (Regent Instruments Inc., QC, Canada) to measure projected surface area prior to drying. All plant material was then dried at 65 °C in a force-draft oven until plant material reached constant mass (72 h) before weighing dried masses to the nearest 10^{-4} gram using a Veritas® M214I balance (H & C Weighing Systems™). Total mass belowground was the sum of dry masses of taproots, lateral roots, and roots egressed from the root plug. Aboveground woody mass was measured as the sum of stem and branch tissue mass without leaves. Leaves separated from stems were measured for dry leaf mass. Leaf mass per area (LMA, g m^{-2}) was measured as the average total dry leaf mass divided by the projected area of all fresh leaves per plant, following Poorter et al. (2009). The number of root branch nodes was measured manually for each seedling as the sum of all root branching points including first-order laterals extending from taproots to all higher order roots located across taproots. The ratio of egressed root biomass to new foliage was calculated as the dry mass of egressed roots divided by dry mass of foliage. Root-to-shoot ratio was calculated as the dry mass of belowground tissue divided by the dry mass of aboveground tissue (including woody aboveground tissue plus foliage).

Total nitrogen and stable carbon isotope analyses

Total carbon and nitrogen content, and stable carbon isotope discrimination ($\delta^{13}C$) of foliar tissue was measured on $n = 12$ seedlings per treatment using isotope ratio mass-spectrometry on samples consisting of 10 homogenized leaves randomly sampled from each seedling following the drought pulse trial. Foliar $\delta^{13}C$ represents the ratio of ^{13}C to ^{12}C isotopes in foliar tissue, which varies with the extent to which plants regulate water loss through stomatal closure when exposed to water limitation, providing an estimate of water-use efficiency integrated across the period of tissue development (iWUE; Farquhar 1989). Larger $\delta^{13}C$ values indicate increased stomatal closure in response to water limitation (Plantevin et al. 2022), which can aid avoidance of hydraulic failure under drought. Samples were prepared by packing 4 × 6 mm tins with four milligrams of oven-dried and powderized seedling foliar tissue using a precision analytical balance (Mettler-Toledo©). Seedling tissue was powderized by hand using liquid nitrogen in a new sterile mortar and pestle for each sample. Samples were analyzed on an elemental analyzer-isotope ratio mass spectrometer (EA-IRMS) system at the UC Davis Stable Isotope Facility by combusting samples at 950 °C in a reactor packed with chromium oxide and silvered copper oxide, ensuring complete combustion. The resulting CO_2 and N_2 gases were separated by a gas chromatography column in a Sercon Elemental Analyzer (EA) and by an adsorption trap in an Elementar EA, yielding sample content of carbon and nitrogen. Analyte gases were carried to the IRMS for measurement by calibrating in-house standards against international standards to create a two-point calibration curve to correct raw isotope measures following Ueda et al. (2011).

Statistical analyses

At the end of the second season of growth, seedling water use efficiency ($\delta^{13}C$) and morphological traits on 48 seedlings total ($n = 12$ seedlings from each watering treatment) were assessed as a function of seedling watering treatment history using linear and generalized linear models of the form:

$$Y \sim \beta_o + \beta_1(\text{year 1 treatment}) \times \beta_2(\text{year 2 treatment}) + \beta_3(\text{total seedling mass}) + \varepsilon$$

Model results were first called using type III sum of squares ANOVA to assess main effects in the presence of interactions (Garofalo et al. 2022; *car* package). In cases where interaction terms did not pass a filter of $p<0.05$, interaction terms were replaced with additive terms for each model covariate using type II sum of squares ANOVA. The variable *total seedling mass at harvest* (which did not vary among treatments and was not a statistically significant predictor of seedling biomass tradeoffs; data not shown) was retained in all models as a covariate to account for random variation in plant size and ensure accurate attribution of trait variation to the effects of the timing of watering treatment and not differences in seedling development rate among treatments (Coleman et al. 1994).

The discrete numeric variable *number of root branch nodes* was assessed as a response variable using the glm.nb() function (*MASS* package, R statistical environment), assuming a discrete probability distribution with a variance that may exceed the mean (Bolker 2008). Mass ratios of root-to-shoot (*root: shoot*) and egressed roots-to-new-foliage (*egressed root: new foliage*) were modeled using beta distributions with the betareg() function in R, which assumes a continuous probability distribution with a range of zero to one (Bolker 2008). All other response variables were assessed using lm(). Significance testing of omnibus models with interaction terms were tested using type III ANOVA. Where interaction terms were non-significant ($p>0.05$), additive models were tested using type II ANOVA. Quantile-quantile plots were assessed visually to evaluate distribution appropriateness and to test the assumption of model residual normal distribution. Dispersion was checked using standard procedures.

The influence of watering treatment history on seedling Ψ_{mid} was assessed using separate models for data from each of three stages of the drought pulse trial: (1) before drought pulse, (2) at the peak of the drought pulse, and (3) after three days of post-drought recovery watering following the ten-day water withholding drought pulse. 20 seedlings ($n=5$ seedlings of each watering treatment) were measured for each of the three independent analyses of watering treatment effects on seedling water relations. Before-after effects of the drought pulse were not assessed due to a strict focus on elucidating the water status associated with each watering treatment, which required use of a separate model for each measurement timepoint instead of repeated measures in a single model. Ψ_{mid} among watering treatments was compared using linear models and pairwise comparisons using Tukey HSD for post-hoc contrasts using the *emmeans* package in R. All tests were conducted on untransformed data after confirming that diagnostic plots supported assumptions of normality and homogeneity of variance. Estimates and standard deviations for each model were extracted using the tidy function in the R package *broom*.

Biplot graphing of principal components analysis (PCA) was conducted to visually assess traits that varied most strongly among watering treatments including only the nine traits that varied significantly with water limitation in univariate models described above (*foliar mass*, *aboveground woody tissue mass*, *taproot mass*, *egressed root mass*, *number of root branch nodes*, *seedling height*, *root collar diameter*, $\delta^{13}C$, and *dry mass of egressed root: new foliage*). PCA was computed using prcomp(), with each variable scaled by converting to a z-score and centered. The function fviz_pca_biplot() (R package *factoextra*) was used to visualize the positioning of seedlings from each treatment in trait-space across the first two axes of the PCA, and vectors showing the approximate contributions of seedling traits

to each principal component. The R statistical environment (version 4.1.3) was used for all analyses and data visualization.

Results

Summary of trait responses to watering

Four traits varied significantly ($p \leq 0.05$) across watering levels in year one, four traits varied significantly across watering levels in year two, and three traits varied significantly with interactions between watering levels in year one and year two (Table 1). Trait-space ordination of seedling phenotypes across watering treatments showed that traits related to woody biomass allocation and water-use efficiency (height, RCD, woody mass, taproot mass, and $\delta^{13}C$) loaded on PC1 (accounting for 40.0% of variation in the data), with multivariate seedling trait coordinates belonging to high-high and low-low treatment groups located at opposite ends of PC1 (Fig. 2). Foliar mass, egressed root mass, dry mass of egressed roots: foliage, and root branch node number loaded most strongly on PC2 (accounting for 21.0% of variation in the data; Fig. 2). While trait-space ordination demonstrated that trait-

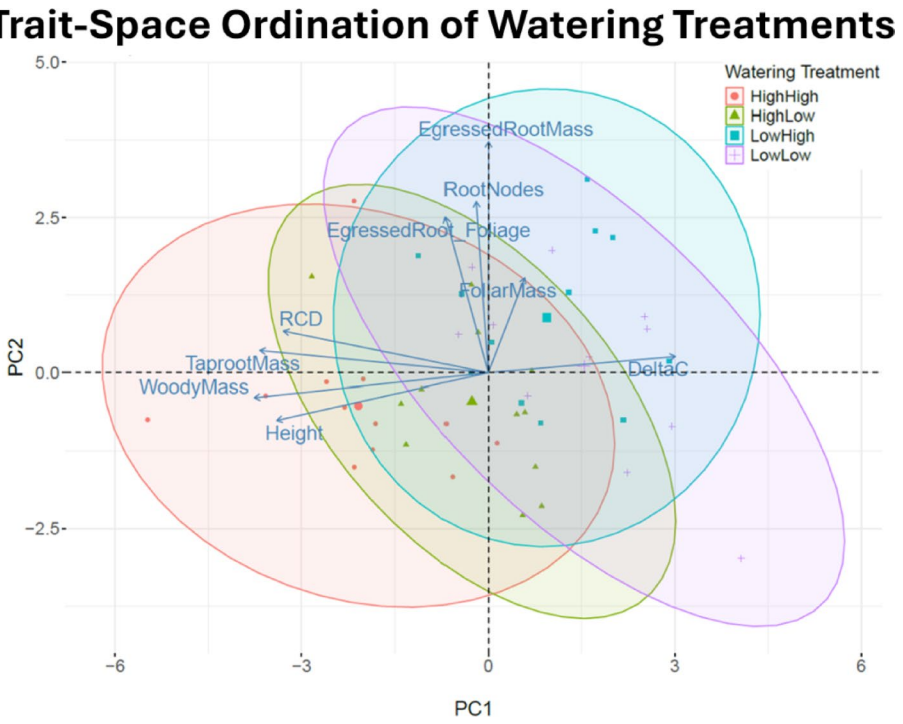

Fig. 2 Biplot PCA of *Larix occidentalis* seedling traits measured at the end of the second growing season. Blue arrows labeled with traits indicate the relationship among individual traits and between traits and the first two principal components based on the eight traits shown. Arrow lengths indicate relative trait contributions to the PCA. Points are measurements of individual seedlings positioned in trait-space. Ellipses are centered around points for each watering treatment for visualization. EgressedRoot_Foliage is the ratio of dry mass of egressed roots to new foliage. RootNodes is the count of root nodes

integrated morphological differentiation was greatest along the axis defining consecutive drought from consecutive high watering, seedlings exposed consecutively to water limitation did not express unique phenotypes, whereas seedling traits varied with seedling age during water limitation exposure.

Aboveground traits

Aboveground dry woody mass varied with an interaction between watering levels in year one and year two ($F_{3,43}$ = 5.0, p=0.013, Table 1). High-high seedlings exhibited mean woody masses approximately 55% heavier than low-low seedlings ($t_{3,44}$ = 8.6, p<0.0001, Fig. 3A). Seedling height varied with an interaction between watering levels in year one and year two ($F_{3,43}$ = 5.7, p=0.021, Table 1), and height showed a maximum treatment effect of 30% greater height in high-high seedlings relative to low-high seedlings ($t_{3,44}$ = 5.7, p<0.001, Fig. 3B). RCD varied with watering level in year two ($F_{1,44}$ = 8.1, p=0.007, Table 1), and showed a maximum treatment effect of 6.0% higher RCD in high-high seedlings relative to high-low seedings ($t_{1,44}$ = 2.9, p<0.032, Fig. 3C). Dry foliar mass varied with an interaction between watering levels in year one and year two ($F_{3,43}$ = 4.7, p=0.036, Table 1) and showed a maximum treatment effect of 68% greater dry foliar mass in low-low seedlings relative to high-high seedlings. ($t_{3,43}$ = −3.5, p<0.0001; Fig. 3E).

Belowground traits

Taproot mass varied with watering levels in each year and showed a maximum treatment effect of 35% less mass in low-low seedlings relative to high-high seedlings ($t_{3,44}$ = 4.7, p=0.0001; Fig. 3F). Root branch node number varied with watering levels in each year and

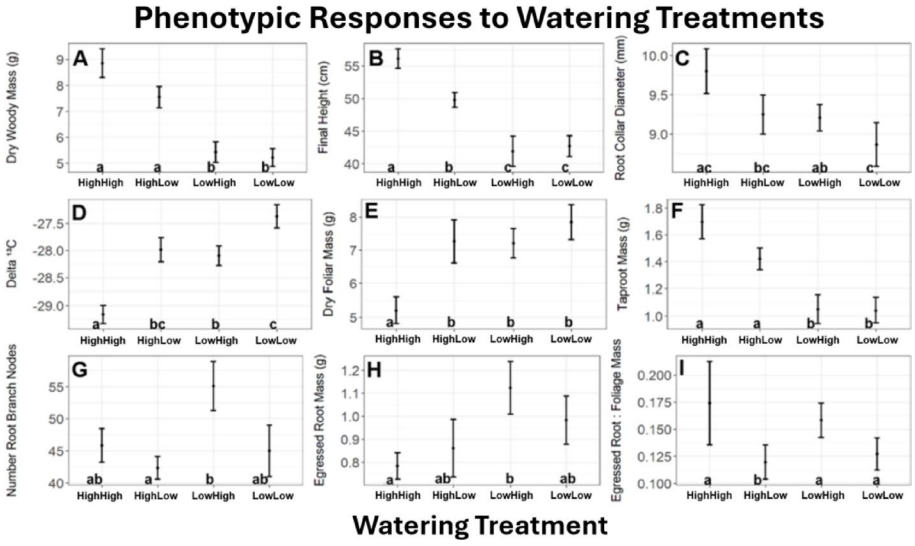

Fig. 3 *Larix occidentalis* seedling traits with statistically significant differences among reciprocal watering treatments as measured at harvest. Filled circles are means and error bars are standard error of the mean. Letters below points at bottom of plots represent statistically significant differences among watering groups at p<0.05

showed a maximum treatment effect of 8% more root branch nodes in low-high seedlings relative to high-low seedlings ($Z_{3,44}$ = 3.5, p=0.002; Fig. 3G). Egressed root mass varied with first-year watering level ($F_{1,44}$ = 12.0, p=0.001, Table 1) and showed a maximum treatment effect of 42% greater mass in seedlings exposed to low-high watering than high-high seedlings ($F_{1,44}$ = −3.469, p=0.006; Fig. 3H).

Ecophysiological and mass ratio traits

$\delta^{13}C$ values varied with first-year ($F_{1,44}$ = 12.7, p=0.001, Table 1) and second-year ($F_{1,44}$ = 25.8, p<0.0001, Table 1) watering level, resulting in high-high seedlings with $\delta^{13}C$ values significantly lower (more negative) than all other treatments and a maximum treatment effect of 6% higher $\delta^{13}C$ values in low-low seedlings relative to high-high seedlings ($t_{3,44}$ = −6.2, p<0.001, Fig. 3D). The mass ratio of egressed roots to foliage that grew during the second year (hereafter egressed root: foliage) varied with second-year watering level ($Z_{1,44}$ = −2.2, p=0.028, Table 1), and showed a maximum treatment effect of 31% greater egressed root: foliage in low-high seedlings relative to that of high-high seedlings ($Z_{1,44}$ = 2.1, p=0.027; Fig. 3I).

Seedling water potential during the drought pulse trial

No significant variation in Ψ_{mid} was observed prior to the ten-day drought pulse (Fig. 4A). After water was withheld for 10 days, low-low seedlings exhibited statistically significantly greater Ψ_{mid} relative to high-high seedlings ($t_{1,15}$ = 4.2, p=0.004, Fig. 4B) and relative to low-high seedlings ($t_{1,15}$ = 3.6, p=0.013, Fig. 4B). High-low seedlings exhibited significantly higher Ψ_{mid} than high-high seedlings ($t_{1,15}$ = 3.6, p=0.013, Fig. 4B). While low-low seedlings expressed the highest mean Ψ_{mid} during the ten-day drought pulse, differences between Ψ_{mid} values of low-low and high-low seedlings were not statistically significant. No differences in plant water status were observed among watering treatments after irrigating to saturation for three days post-drought (Fig. 4C).

Discussion

Seedling responses to water limitation timing versus consecutive water limitation

Divergent trait profiles across watering treatments

Seedling water limitation history influenced *L. occidentalis* seedling trait profiles and ecophysiological responses to the ten-day drought pulse. Specifically, across reciprocal high–low watering combinations spanning two years, variation in seedling morphology was most closely associated with the timing of water limitation (low–high vs. high–low), whereas variation in stomatal regulation and iWUE was best explained by whether seedlings were exposed to water limitation in one versus both years. This work demonstrates that growers can promote distinct seedling phenotypes for *L. occidentalis* depending on the timing of water limitation applications. In support of our first hypothesis, several traits varied with the timing of water limitation (i.e. water limitation during either year one or two), whereas no

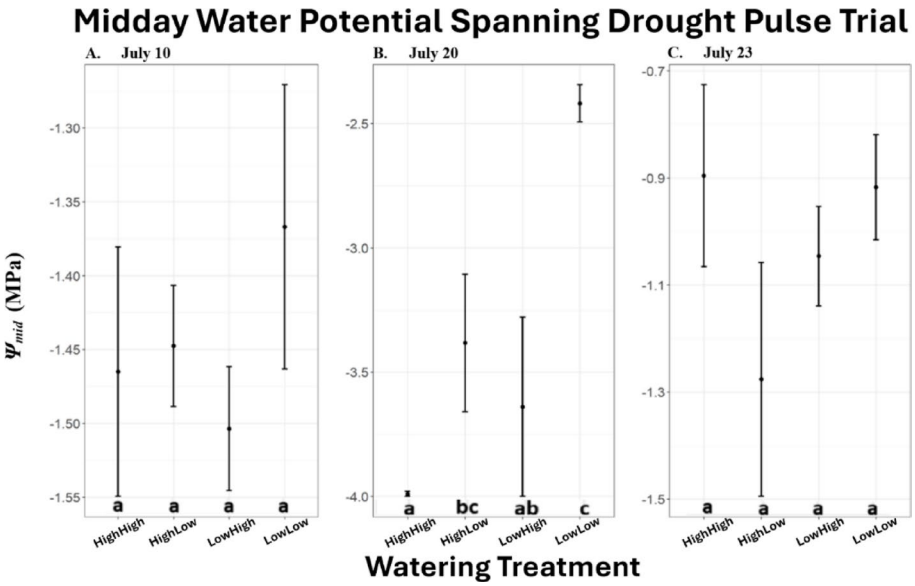

Fig. 4 *Larix occidentalis* seedling Ψ_{mid} (mid-day stem water potential; MPa) before (**A**; July 10), immediately after (**B**; July 20), and following a three-day recovery watering period after (**C**; July 23) the 10-day water withholding period. Letters below points at bottom of plots represent statistically significant differences among watering treatments at $p < 0.05$. Error bars are standard error of the mean

distinct traits resulted from consecutive water limitation (i.e. water limitation applied across both years). For example, low-high seedlings exhibited a distinctly greater number of root branch nodes than high-low seedlings, whereas low-low seedlings did not differ from other treatments in number of root branch nodes, and similar trends were observed for seedling dry woody mass, height, taproot mass, and egressed root: foliage mass. The overall trend of reduced woody tissue (e.g. height, taproot mass, and aboveground woody mass) with water limitation in the first growing season was as expected, but notably, increased foliage production resulted from water limitation in either year. Although we observed significant interactions between watering level in year one and two for height, foliar mass, and aboveground woody mass, seedlings cultivated with limited water in the second year (high-low) and consecutively across both years (low-low) expressed similar traits and performance during the drought pulse trial, suggesting that previous exposure to drought did not moderate seedling responses to subsequent water limitation. We speculate that age-dependent phenotypic responses to water limitation and the lack of effect of early water limitation exposure on subsequent responses to water limitation results from two constraints. Firstly, structural constraints imposed by earlier tissue development likely shape the trajectory of future developmental patterns, as demonstrated by seedling age-based differences in water limitation responses. Secondly, water limitation during postplanting conditions could constrain expression of desirable drought-hardy phenotypes resulting from low water availability and cell turgor precluding cell growth irrespective of drought exposure history (Dodd and Ryan 2016), as described above for *P. radiata* (Rook 1973).

Watering treatment effects visualized in multivariate trait-space revealed coordinated trait responses to water limitation and trait tradeoffs. Partitioning of water limitation effects

in trait-space showed segregation of long-lived woody tissue traits and $\delta^{13}C$ (on the first principal component) from ephemeral tissues including roots and foliage (on the second principal component). Together with trait response patterns exhibited by univariate analyses, multivariate ordination suggested morphological groupings between high-high seedlings and high-low seedlings, and between low-high seedlings and low-low seedlings. High-high and low-low seedlings showed the greatest separation in multivariate trait-space, demonstrating that low-low seedlings produced the greatest phenotypic differences relative to high-high seedlings, and that increased iWUE was achieved at the expense of seedling size. Also notable is that variation in $\delta^{13}C$ and dry foliar mass was especially associated with *whether or not* seedlings were exposed to water limitation, whereas the *timing* of water limitation was associated with a smaller extent of trait variation.

Water limitation effects on aboveground traits

L. occidentalis seedlings from the three treatments that featured water limitation (low-low, low-high, high-low) invested more in foliar tissue than high-high seedlings in terms of foliar biomass (Fig. 3E) and foliar surface area (Fig. S1, S2), suggesting that water limitation produced plants better able to maximize aboveground resource acquisition. Similarly, *Pinus ponderosa* seedlings exposed to experimental drought in the first year before a simulated outplanting trial exhibited increased needle dry mass (Pinto et al. 2023), and drought-exposed potted *Quercus petraea* seedlings exhibited increased growth of new shoots upon rewatering (Turcsán et al. 2016). In a study of nursery-grown *Pinus tabuliformis*, Luo et al. (2021) observed increased shoot mass with drought conditioning of longer duration, but no effect of drought intensity, and this trend persisted after transplanting into a field setting. However, Luo et al. (2021) did not separate foliage from woody stem material, precluding interpretation of drought effects on foliage biomass. In our study, water limitation during the first year of growth resulted in seedlings with relatively long branches low on seedling stems, whereas high-high and high-low treatments promoted short branches clustered near the apical meristem (data not shown). We observed that production of greater foliar tissue under water limitation coincided with increased stomatal regulation, presenting opportunities for future investigation of whether increased foliar biomass together with enhanced stomatal regulation may combat carbon starvation in seedlings exposed to chronic drought by enhancing carbon assimilation during conditions favorable to stomatal conductance (Buckley 2005; McDowell 2011; Ross et al. 2025). And considering that seedling investment in foliage (LMA) did not vary across watering treatments, and *L. occidentalis* foliage is deciduous, there is likely low risk of investing in foliar tissue under dry conditions that may promote xylem hydraulic failure (Chen et al. 2022).

Seedlings exposed to water limitation shifted plant biomass from woody to photosynthetic tissue. Foliar nitrogen content did not vary across watering treatments, thus water-limited seedlings in our study did not appear to upregulate foliar chlorophyll content. Water-limited seedlings were shorter than high-high seedlings. However, water-limited seedlings exhibited greater iWUE and foliar tissue dry mass relative to high-high seedlings, which may enable rapid shoot growth when conditions allow (Zhang et al. 1994). While shorter seedlings may be at a disadvantage where there is an abundance of competing vegetation (Newton et al. 1993), Pinto et al. (2018) found limited differences in survival of outplanted western larch seedlings of several stocktypes and initial seedling sizes. This sug-

gests that limited height at planting may not pose a serious survival risk for *L. occidentalis* if seedlings rapidly grow in height following outplanting, which also aligns with results from another recent study of *L. occidentalis* under water-limited cultivation (Ross et al. 2026). In contrast, seedlings in the present study grown with ample water in the first year were taller and had greater aboveground biomass than low-high and low-low seedlings irrespective of second year watering level.

Water limitation effects on belowground traits

Early exposure to water limitation promoted increased root branching and egressed root growth only when seedlings received ample water in the following year. Specifically, greater egressed root mass was exhibited by low-high seedlings relative to high-high seedlings, and we observed a greater number of root branching nodes in low-high seedlings relative to high-low seedlings. If dry soil restricts root growth for *L. occidentalis* as described for other species (Day and MacGillivray 1975; Robbins and Dinneny 2015), our results may imply that the utility of water limitation for promoting drought hardiness could be limited if outplanting timing does not coincide with periods of ample soil moisture. Such a trend was observed in a study of *L. kaempferi*, in which the effects of drought exposure early in seedling development on egressed root biomass did not persist for seedlings exposed to drought conditions in the second growth year (Kitao et al. 2022). However, trends exhibited by low-high seedlings in the present study suggest that adequate soil moisture at the time of outplanting could result in improved root traits for previously drought-exposed seedlings and enhanced seedling tolerance of subsequent hot and dry summer conditions. Previous work on *L. occidentalis* demonstrated that water limitation applied during the first growth year promoted biomass reallocation from taproot tissue to lateral roots at lower locations in seedling root plugs during the first year of seedling growth (Vale et al. 2021), and promoted root growth under moist conditions in the second year of growth (Moler and Nelson 2021). First year watering regimes in the present study and Moler and Nelson (2021) were identical, but the second year of growth in the present study was conducted in pots for a duration of three months whereas Moler and Nelson (2021) used well-watered conditions in an aeroponic chamber for 21 days of growth. Results from the present study raise the question of whether dry conditions during outplanting may restrict the expression of root trait improvements achieved by prior water limitation. While this contrasts with conclusions from a metanalysis suggesting that the benefits of controlled drought exposure are most apparent when seedlings are planted under arid site conditions (Puértolas et al. 2024), the authors noted that controlled drought exposure was more likely to benefit shrub species than tree species (and their study did not include *Larix* spp.).

Water relations physiology

Water use efficiency (WUE, i.e. carbon gained per unit water lost) has been shown to be strongly negatively correlated with carbon isotope discrimination in plant leaves across numerous conifer species including *L. occidentalis*, enabling indirect assessment of WUE through foliar carbon isotope discrimination (Adiredjo et al. 2014; Zhang et al. 1994). In the present study, cultivation with limited water in either year one or two resulted in a statistically significant increase of iWUE values relative to the high-high treatment. Low-low

seedlings exhibited a further increase of iWUE values relative to low-high seedlings. Similarly, in a reciprocal drought study of *Encelia* spp. in California, the highest iWUE among drought treatments was observed in low-low seedlings, and the lowest iWUE was observed in plants that consistently received ample water (Valliere et al. 2019). And consistent with our results, in a study of *Pinus ponderosa* grown under three levels of water limitation (determined by gravimetric water content), the most water-limited treatment promoted the greatest iWUE (Pinto et al. 2023).

Seedling water potential during drought pulse trial

We found partial support for our second hypothesis that seedling water potential during the drought pulse trial would vary among factorial combinations of water limitation history. But against our expectations, we found that the lowest plant moisture stress during the drought pulse trial was achieved by the low-low treatment, not the low-high treatment. Although the least plant moisture stress was exhibited by low-low seedlings, Ψ_{mid} values during the ten-day drought pulse were indistinct statistically from high-low seedlings, and these experimental groups also exhibited increased iWUE, with low-low seedlings also exhibiting the greatest mean iWUE. We suspected that water limitation would influence water potential due to effects of water limitation on new root tissue development. However, trends in iWUE mirrored trends found for plant moisture stress during the ten-day drought pulse, suggesting that seedling Ψ_{mid} during the ten-day drought pulse coincided better with seedling physiological responses to water limitation than with morphological responses.

Key takeaways and study limitations

Our findings build upon and underscore the importance of the Target Plant Concept, which prescribes the cultivation and use of plant materials that are well-suited, genetically and phenotypically, to planting sites (Dumroese et al. 2016; Davis and Pinto 2021). Our results suggest that water limitation during different periods of seedling development promote different trait profiles that may be useful in distinct planting environments. Although desirable drought hardiness traits (i.e. greater egressed root mass and more root branch nodes) were obtained from low-high seedlings, the greatest gains in iWUE and the least plant moisture stress during the ten-day drought pulse was exhibited by low-low seedlings. Meanwhile, low-low seedlings exhibited less egressed root mass and root branch nodes relative to low-high seedlings. These distinct trait profiles represent a tradeoff between maximum iWUE and maximum root growth, and each may be best suited to a distinct planting environment. For example, maximum iWUE at the expense of rapid growth may be desirable in exceptionally dry planting sites with unpredictable precipitation patterns, where, within reasonable limits, physiological drought avoidance may prevent seedling desiccation until precipitation facilitates growth. And seedling cultivation regimes that maximize root growth potential may be the best strategy for promoting seedling drought avoidance at planting sites where seedlings can be expected to encounter sufficient soil water for root growth to occur. Results described here suggest that cultivation regimes designed to promote root growth after outplanting may enhance seedling establishment when *L. occidentalis* seedlings are planted into moist soil following spring snowmelt. Our findings also suggest a need for

field-based investigations into the value of increased iWUE for promoting long-term seedling establishment under xeric outplanting conditions.

We used only one maternal genotype as the seed source in the present study, and future studies should consider including multiple maternal genotypes and quantifying the extent of phenotypic variation in response to watering treatments across maternal genotypes to explore the generalizability of findings described here. Additionally, findings from the present study should not be considered transferable to other species given that species may express divergent responses to water limitation (Ross et al. 2025). Investigation of water limitation effects across years entails working with greater volumes of soil as seedlings grow, leading us to use different approaches for assessing water availability across years. This poses a limitation for direct comparisons of water limitation across years, and interpretation of the impact of water limitation timing should thus consider that we used different measures of soil water availability in year one versus year two. Plant moisture stress was furthermore assessed using stem water potential measurements in the first year but not the second year, limiting comparisons of water limitation in year one versus year two. While we observed a significant effect of water limitation on taproot mass, we note that the measurement of taproot biomass alone, as was done in this study, does not describe the potential role of taproots in influencing the vertical distribution of absorptive lateral roots. Additionally, roots of seedlings grown in evenly watered pots are expected to behave differently than roots in natural soil environments, and the limited depth of pots used in this study likely constrained natural patterns of root exploration and growth. Finally, despite watering all treatments to saturation monthly, the use of the same quantity of slow-release fertilizer across watering treatments in the first year of cultivation may have resulted in differences in nutrient availability across watering treatments since the slow-release fertilizer is designed to release nutrients in the presence of moisture. This could have led to confounding of watering level with the timing of nutrient availability, the amount of nutrient availability, or both, since the high-watering group likely received nutrients sustained over the course of the experiment whereas the low-watering group likely received nutrients mostly during monthly periods of saturation watering. Nutrients were not supplied in the second year of cultivation.

Conclusion

Departures from historic climate conditions pose challenges to artificial forest regeneration, and this work explores the potential for novel seedling cultivation regimes to promote improved seedling performance under drought. While our results demonstrate that seedlings express age-dependent phenotypic responses to water limitation, water limitation applied during the first year promoted the most desirable patterns of belowground growth among the four watering treatments explored in this study. Desirable patterns of belowground growth achieved by water limitation in the first year were muted when seedlings were subsequently transplanted into a dry environment, suggesting that there are limitations to the extent to which stress exposure may facilitate improved subsequent responses to stress. On the other hand, improved root traits exhibited by low-high seedlings suggest the possibility that outplanting drought-exposed seedlings during periods of adequate soil moisture could result in drought-adaptive root traits that enhance seedling performance under subsequent hot and

dry summer conditions. We encourage further synthesis of plant developmental biology and silviculture to promote improved seedling establishment in challenging field conditions. And we emphasize that the translation of results presented here into practice ultimately requires evaluation of seedling performance in field settings.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11056-026-10198-x.

Acknowledgements We are grateful to the donors who facilitated this study by contributing through the following crowdfund link: https://experiment.com/projects/does-successive-exposure-of-conifer-seedlings-to-drought-prepare-them-for-subsequent-extreme-drought).

Author contributions ERVM and ASN conceived and designed the study. ERVM and AMV conducted the study. ERVM and ASN analyzed and interpreted the data. ERVM wrote the manuscript. All authors read, revised, and approved the manuscript.

Funding Funding was provided by the United States Department of Agriculture, National Institute of Food and Agriculture, grant number 2019-67014-29109 and the National Science Foundation award number 1916699.

Data availability All data generated or analysed during this study are accessible through Open Science Framework at: [https://osf.io/yugtd]. The corresponding author may also be contacted for data access.

Declarations

Competing interests Andrew S. Nelson is an Associate Editor for New Forests.

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