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Research paper

Linking carbon and water relations to drought-induced mortality in *Pinus flexilis* seedlings

Keith Reinhardt^{1,7}, Matthew J. Germino², Lara M. Kueppers³, Jean-Christophe Domec^{4,5} and Jeffry Mitton⁶

¹Department of Biological Sciences, Idaho State University, Pocatello, ID 83209-8007, USA; ²United States Geological Survey, Forest and Rangeland Ecosystem Science Center, Boise, ID 83706, USA; ³Sierra Nevada Research Institute, University of California, Merced, CA 95343, USA; ⁴Bordeaux Sciences Agro, INRA ISPA UMR 1391, 33175 Gradignan, France; ⁵Nicholas School of the Environment, Duke University, Durham, NC 27708, USA; ⁶Department of Ecology and Evolutionary Biology, University of Colorado-Boulder, Boulder, CO 80309, USA; ⁷Corresponding author (reinkeit@isu.edu)

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Survival of tree seedlings at high elevations has been shown to be limited by thermal constraints on carbon balance, but it is unknown if carbon relations also limit seedling survival at lower elevations, where water relations may be more important. We measured and modeled carbon fluxes and water relations in first-year *Pinus flexilis* seedlings in garden plots just beyond the warm edge of their natural range, and compared these with dry-mass gain and survival across two summers. We hypothesized that mortality in these seedlings would be associated with declines in water relations, more so than with carbon-balance limitations. Rather than gradual declines in survivorship across growing seasons, we observed sharp, large-scale mortality episodes that occurred once volumetric soil-moisture content dropped below 10%. By this point, seedling water potentials had decreased below -5 MPa, seedling hydraulic conductivity had decreased by 90% and seedling hydraulic resistance had increased by $>900\%$. Additionally, non-structural carbohydrates accumulated in aboveground tissues at the end of both summers, suggesting impairments in phloem-transport from needles to roots. This resulted in low carbohydrate concentrations in roots, which likely impaired root growth and water uptake at the time of critically low soil moisture. While photosynthesis and respiration on a leaf area basis remained high until critical hydraulic thresholds were exceeded, modeled seedling gross primary productivity declined steadily throughout the summers. At the time of mortality, modeled productivity was insufficient to support seedling biomass-gain rates, metabolism and secondary costs. Thus the large-scale mortality events that we observed near the end of each summer were most directly linked with acute, episodic declines in plant hydraulic function that were linked with important changes in whole-seedling carbon relations.

Keywords: carbon balance, hydraulic resistance, non-structural carbohydrates, productivity, respiration.

Introduction

The first-year seedling life-stage is a key population bottleneck in many forest types due to the high mortality in this developmental phase (often $>90\%$; Cui and Smith 1991, Cuevas 2000, Castro et al. 2004, Castanha et al. 2013). In ecosystems that have high seasonal variability in soil moisture (e.g., semi-arid forests, Mediterranean shrublands; Kolb and Robberecht 1996, Padilla et al. 2007), as well as in mesic systems experiencing

severe episodic drought, seedling survival is constrained by plant–soil water relations (e.g., Robson et al. 2009, Will et al. 2013). However, it remains unclear if seedling mortality is linked more directly with plant water relations, or indirectly through water effects on plant carbon relations. While many studies have investigated the ecophysiology of older seedlings under imposed drought (e.g., Robson et al. 2009, Varela et al. 2010, Galvez et al. 2011, Will et al. 2013), few have focused specifically on the

ecophysiological limitations to survival of first-year seedlings under drought-induced mortality (Kolb and Robberecht 1996, Germaine and McPherson 1999). Understanding the ecophysiological limitations to seedling survival under seasonally dry or droughty conditions is important for predicting ecosystem stability and changes in forest range limits under future climate scenarios because precipitation is expected to become more variable in many ecosystems, and increased temperatures will tend to dry soils more rapidly (IPCC 2013).

Many previous studies on seedling ecophysiology in both field and greenhouse settings have focused on constraints to carbon (C) balance, i.e., the balance of C gain, metabolic C use and investment of C into growth and reserves (e.g., Bansal and Germino 2008, 2010). This is because seedlings have relatively low C reserves for buffering against environmental stresses, and an upset in any of the C-balance components can result in insufficient growth or C reserves for survival past the first year. For example, conifer seedlings in microsites with greater sky exposure had lower photosynthesis and lower survival relative to seedlings in covered microsites at alpine treeline, which was attributed to low-temperature photoinhibition of C uptake following frost nights (Germino and Smith 1999, Maher et al. 2005). In another study, O'Brien et al. (2014) showed that seedlings with low carbohydrate concentrations had lower survival under drought, due to diminished capacity for osmoregulation and hydraulic functioning. On the other hand, Galvez et al. (2011) observed that while photosynthetic C intake was greatly reduced in aspen seedlings subjected to severe drought, this did not result in death or exhaustion of C supplies, but did trigger a switch in C allocation from shoot growth to root storage. These results highlight that in young seedlings, critical relationships exist among growth, physiology and storage.

While C imbalance has been linked to mortality in first-year seedlings, declines in water relations during seasonal drought are also likely a major constraint to seedling survival, either directly or in concert with C imbalance. For example, mortality in first-year *Pinus ponderosa* seedlings growing near lower-tree line in Idaho, USA was associated with severe declines in stomatal conductance that resulted in stem heat-girdling (Kolb and Robberecht 1996). Additionally, numerous investigators have found that photosynthesis and survival of seedlings of various conifer tree species are inhibited at water potentials below -2.0 MPa (Teskey et al. 1986, Cui and Smith 1991, Guehl et al. 1993). The few ecophysiological studies on seedling survival in moisture-limited ecosystems thus suggest that declines in water relations may be at least as consequential to seedling survival as declines in C relations, and that these two processes are linked in many cases. However, little research has directly compared how these processes limit first-year seedling survival.

Herein we report on the C and water limitations to seedling growth and survival of *Pinus flexilis* (limber pine) in a severe drought experiment that typifies the dominant environmental

change (cool/wet to warm/dry) during the few months from germination to initial establishment for trees at lower elevations in semi-arid, montane forests. The setting was a common garden just beyond the warm and dry edge of this species' range limit (i.e., at a lower elevation). To examine the ecophysiological limitations to survival during the first growing season, we measured and modeled C relations (photosynthesis, respiration and carbohydrate concentrations of source, sink and transport tissues) and water relations (water potentials, stomatal conductance and hydraulic conductivity), and compared these with dry-mass gain and survival across two summers. We hypothesized that both seedling water relations and C balance would decline with increasing drought duration, but that mortality would be primarily ascribed to severe declines in hydraulic functioning (i.e., interruption of hydraulic pathway), as opposed to critical declines in C reserves (i.e., trending toward C-reserve exhaustion).

Materials and methods

Plant materials, germination and survival

We conducted two trials at an outdoor garden experiment in a full-sun location on the campus of Idaho State University (Pocatello, ID, USA; $42^{\circ}54'03''\text{N}$, $112^{\circ}25'48''\text{W}$; 1386 m) in 2011 and 2012. We used different seed sources in each of the two summers for our common-garden study, although provenance comparisons were not evaluated in this aspect of the study. Rather, we used the variation in seed sources to confirm the generality of our results. For the first summer (2011), we used seeds obtained from trees at 3295 m on Black Mountain in the Pike National Forest, CO ($38^{\circ}43'409''\text{N}$, $105^{\circ}41'574''\text{W}$) in September 2009. We identified the collected seeds to be from two mitochondrial haplotypes—one was a broad-ranging haplotype, found above and below 3000 m, and throughout the western USA, while the other haplotype has been found only above 3000 m (Mitton et al. 2000; see Supplementary Data at *Tree Physiology* Online). For the second summer (2012), we used seeds from trees at two different sites (site 1: between 3370 and 3450 m; site 2: at ~ 3360 m) located at Niwot Ridge, CO ($40^{\circ}3'\text{N}$, $105^{\circ}36'\text{W}$), collected between 2009 and 2011 for both elevations (Castanha et al. 2013). Thus, our combined seed-source pool consisted of two identified mitochondrial haplotypes, and six provenance-year combinations (3 years $\times$ 2 elevations), whose mother trees occupied a >400 m elevation range. This constitutes a wide range of genetic material for our study.

We prepared seeds as follows: we rinsed seeds for 10 min in a 3% hydrogen peroxide solution, imbibed in running water for 24 h, and then cold-stratified for 30 days at 4°C according to procedures described in Schopmeyer (1974) beginning in late spring (April–May) both years of the study. Following cold-stratification, we then planted seeds into 12-cm Ray Leach cone-tainers, using a 50 : 50 planting soil : sand mixture. This

mixture was generally representative of the highly variable soil texture and organic matter content of soils found at local lower-tree line sites (e.g., range 17–63% sand and 6–31% organic matter). We randomly arranged cone-tainers into cone-tainer-stands, placed in a greenhouse, and watered daily until germination (approximately 7–14 days after sowing). As soon as all seedlings had germinated, we transferred the cone-tainer-stands outside the greenhouse to a raised garden plot filled with sand. We submerged the cone-tainer-stands into the sand (tops of cone-tainers level with the sand surface). We watered seedlings every day for 1 week after being placed into the garden, after which we provided no supplemental water (only ambient precipitation). We determined survivorship approximately biweekly throughout both summers by visual surveys; dead seedlings had 100% brown needles and exhibited shrunken stems (e.g., Figure S1 available as Supplementary Data at *Tree Physiology* Online). Furthermore, we confirmed lack of metabolism in dead seedlings by measuring dark respiration (see details below); respiration of live seedlings was typically $>2 \mu\text{mol m}^{-2} \text{s}^{-1}$, and was $\sim 0 \mu\text{mol m}^{-2} \text{s}^{-1}$ for dead seedlings.

Microclimate

We measured air temperature at 5-cm height (shielded from solar radiation) and soil-surface temperature every 15 min using type-T thermocouple wires connected a CR-23X datalogger (Campbell Scientific, Inc., Logan, UT, USA). We measured soil volumetric water content (VWC, in $\text{m}^3 \text{m}^{-3}$) at 0–5 cm depth (and at 30 cm depth in 2012) hourly using EC-5 soil-moisture probes (Decagon Devices, Pullman, WA, USA), connected to the datalogger. We temperature-corrected soil-moisture probe data according to procedures provided by Decagon Devices (Decagon 2011).

Gas exchange

We measured instantaneous gas exchange (photosynthesis [A_{net}], transpiration [E] stomatal conductance of H_2O [g_s]) using a portable photosynthesis system (model LI-6400, LI-COR Biosciences, Lincoln, NE, USA) equipped with a CO_2 -controller and a red-blue LED chamber (model LI-6400-02b). We measured gas exchange variables on entire seedlings and, for area-based measurements, report on a silhouette leaf area basis according to Smith et al. (1991). We determined silhouette leaf area by digitally photographing the seedling and objects of known size (for calibration) from the angle of the LI-6400 light source, and then quantified using image processing software (ImageJ, Scion Co., Fredrick, MD, USA). We measured gas exchange of 5–15 seedlings between 12:00 and 14:00 h local time approximately monthly (2011) and approximately biweekly (2012). During all gas exchange measurements, we set CO_2 gas concentration to 385 ppm, and matched relative humidity and temperature to ambient conditions. Mean leaf temperature during measurements was $32.3 \pm 1.5^\circ\text{C}$ for all measurements. We measured

light-saturated A_{net} with the chamber light source set at an intensity of $1200 \mu\text{mol m}^{-2} \text{s}^{-1}$. We then measured dark respiration (R_d) by darkening the chamber (light intensity of $0 \mu\text{mol m}^{-2} \text{s}^{-1}$) and waiting until gas exchange fluxes stabilized (total coefficient of variation of CO_2 and H_2O fluxes in time $<5\%$). R_d under sunlit conditions may be less than in darkness (Krömer 1995). However, we assumed that differences in R_d in the light compared with dark were similar between seedlings, and emphasized relative changes in R_d .

Water potentials and hydraulic conductivity

In 2011 and 2012, we measured predawn (04:00–06:00 h) seedling water potentials (Ψ) the morning after gas exchange measurements (on the same seedlings as gas exchange measurements) using a Scholander type pressure chamber (model PMS-1000, PMS Instruments, Corvallis, OR, USA). In 2012, we quantified declines in hydraulic conductivity (K_{shoot}) of above-ground shoots (stems + needles) using the evaporative flux method (described in Sack et al. 2002) on 3–5 seedlings at 10, 40 and 70 days after emergence (approximately early June, July and August). Briefly, we cut seedling stems under water (needles intact with stems), then measured transpiration with a LI-6400 portable photosynthesis machine with the chamber PAR set to $1200 \mu\text{mol m}^{-2} \text{s}^{-1}$. Once steady-state transpiration was achieved (typically between 15 and 20 min), we removed the shoot and then immediately measured seedling Ψ . We calculated K_{shoot} as $E_{\text{steady state}}/\Psi$, and we report seasonal declines in K_{shoot} as percent loss of conductivity (PLC) of early-season measurements (when stems were green and succulent, and we assumed that early-season K_{shoot} was approximately equal to maximum K_{shoot} values, i.e., $K_{\text{shoot_max}}$).

Plant growth, survival, allometry and non-structural carbohydrates

On each measurement date, we harvested seedlings on which we had measured gas exchange. On these seedlings we measured stem and root lengths, needle area and dry mass of these tissues. For dry-mass determinations, we dried tissues at 70°C for 48 h and weighed them with a precision of $\pm 0.01 \text{ mg}$. Following measurements of dry mass, we ground plant tissues (needles, stem, roots) to a fine powder and weighed to $\pm 0.1 \text{ mg}$ in order to measure non-structural carbohydrates (NSC). Non-structural carbohydrates are herein defined as starch plus soluble sugars (glucose, fructose and sucrose). Our protocol followed Bansal and Germino (2009). To measure soluble sugars, we added 2 ml water to each sample and heated to 100°C with steam for 30 min to extract soluble sugars into aqueous solution, then we enzymatically treated a $200 \mu\text{l}$ aliquot of each sample with invertase, phosphoisomerase and glucose hexokinase to convert sucrose, fructose and glucose to 6-phosphogluconate (Sigma Diagnostics, St Louis, MO, USA). Oxidation of the soluble sugars to 6-phosphogluconate resulted in an equimolar reduction of

NAD to NADH, increasing absorbance of the solution at 340 nm, which we measured using a spectrophotometer (Synergy Microplate Reader, Biotek Instruments, Winooski, VT, USA), and was directly proportional to soluble sugar concentrations. To obtain total NSC (starch plus soluble sugars), we treated the original sample (powder plus water) with a high activity fungal α -amylase from *Aspergillus oryzae* (Clarase G-Plus, Genecore International, Rochester, NY, USA) to metabolize starch to glucose and re-analyzed for total NSC with the procedure described previously. We calculated starch as total NSC minus soluble sugars. We normalized all NSC data for dry mass of needle tissue. Sampling time-of-day does not significantly affect NSC concentrations in conifer seedlings (Bansal and Germino 2009).

Analysis

We assessed differences in response variables (dry mass, leaf area, gas exchange variables, pressure–volume variables, water potentials, carbohydrate concentrations and seedling hydraulic conductivity) using two-way ANOVA, with study year and sampling date as main factors (JMP v8.0.1, SAS Institute, Inc., Cary, NC, USA). We evaluated all data sets to ensure statistical assumptions of normality and homoscedasticity were met before analysis. Effects were considered significant at the $P = 0.05$ level. We used the Tukey's HSD multiple-comparisons method for post hoc evaluation of differences between individual means.

Modeling

The soil–plant atmosphere (SPA) model simulates plant photosynthesis, water use and stand water balance at short temporal scales (30 min time-step) with, if needed, multiple canopy and soil layers (see Williams et al. 1996, 2001 for a full description). The scale of parameterization (leaf, root and plant levels) and prediction (plant and canopy-level) of this model were designed for scaling up leaf-level processes to canopy and landscape scales. The version of SPA used in this study (Way et al. 2013) has been upgraded as it now accounts for plant loss of hydraulic conductivity (cavitation function). The SPA model was forced with the measured microclimate data and parameterized for the seedlings using the experimental potting soil properties and seedling physiological characteristics. In the model, the maximum flux rate of water through seedlings is determined by the difference between soil and leaf water potential and is controlled by whole-plant hydraulic conductance (K_{tree}). The root components of K_{tree} were assumed to equal K_{shoot} and thus K_{tree} was taken as half K_{shoot} (Sperry et al. 1998, Williams et al. 2001, Domec et al. 2009). Leaf-to-air energy, water and CO_2 exchange consists of a coupled photosynthesis model (Farquhar model) with a stomatal conductance model that balances atmospheric demand for water with rates of water uptake and supply from the soil. Stomatal conductance is varied by increments so water loss prevents seedling Ψ from falling below a critical value that would induce cavitation of the hydraulic system. Allometric (leaf

area index, plant size, root biomass), hydraulic and leaf nitrogen content used to drive the model were directly measured and are presented throughout the results. Soil particle size analysis (% sand, silt and clay) was calculated based on the 50 : 50 planting soil : sand mixture used in the cone-tainers using the hydrometer method (Gee and Bauder 1986). We tested model outputs against measurements of leaf-level A_{net} , g_s and Ψ , and then used the model to predict seedling water use, carbon exchange, gross primary productivity [GPP] and NSC dynamics in roots and shoots. Simulations were done for 2011 and 2012, and commenced on Day 0 after emergence and were terminated on Day 100 when the (remaining dead) seedlings were harvested. All output variables (transpiration, GPP, respiration, NSC) were expressed on a per ground area basis, which is the default model output. For C balance calculations at the organism scale, model outputs (g C ground^{-1}) were converted to g C seedling^{-1} by multiplying model outputs $\times$ seedling leaf area index (projected leaf area/cone-tainer ground area) $\times$ seedling total leaf area. Leaf area index and leaf area of seedlings did not change appreciably over the growing seasons.

Specifically for this study, SPA outputs were coupled with a single-substrate tree pool model (Ogée et al. 2009) to estimate daily fluctuation in NSC. This pool of carbohydrates comprised shoot and root carbohydrates and was filled by A_{net} in the proportion (α) of the mass of each organ to the total seedling mass. These sugar pools were then used as substrate for maintenance and growth respiration of each compartment (R_{total}) and for each organ's biomass production ($\text{Root}_{\text{biomass}}$ or $\text{Shoot}_{\text{biomass}}$). In turn, $\text{Root}_{\text{biomass}}$ and $\text{Shoot}_{\text{biomass}}$ were assumed to be source-driven and defined as $\text{Root}_{\text{biomass}} = k_{\text{root}} \text{NSC}_{\text{root}}$ and $\text{Shoot}_{\text{biomass}} = k_{\text{shoot}} \text{NSC}_{\text{shoot}}$, where k represented the pool turnover rates determined for each seedling organ from growth rates (Dewar et al. 1998). For each organ, the carbon budget of the overall pool was then written as:

$$\text{For roots: } \frac{d\text{NSC}_{\text{root}}}{dt} + k_{\text{root}} \text{NSC}_{\text{root}} = \alpha(A_{\text{net}} - R_{\text{total}})$$

$$\text{For shoots: } \frac{d\text{NSC}_{\text{shoot}}}{dt} + k_{\text{shoot}} \text{NSC}_{\text{shoot}} = (1 - \alpha)(A_{\text{net}} - R_{\text{total}}).$$

Carbon pools were initialized using initial measured root and shoot masses and %NSC, and driven by modeled GPP, R_{total} and root and branch growths. Total NSC was then calculated as $\alpha \text{NSC}_{\text{root}} + (1 - \alpha) \text{NSC}_{\text{shoot}}$.

Results

Microclimate

Daily maximum air temperatures at seedling height ranged from ~ 12 to 45°C in 2011, and between 20 and 60°C in 2012 (Figure 1a and c). Daily maximum soil-surface temperatures ranged from 12 to 60°C in 2011 and between 12 and 50°C in 2012. Shallow-soil VWC declined steadily from $>30\%$ to below

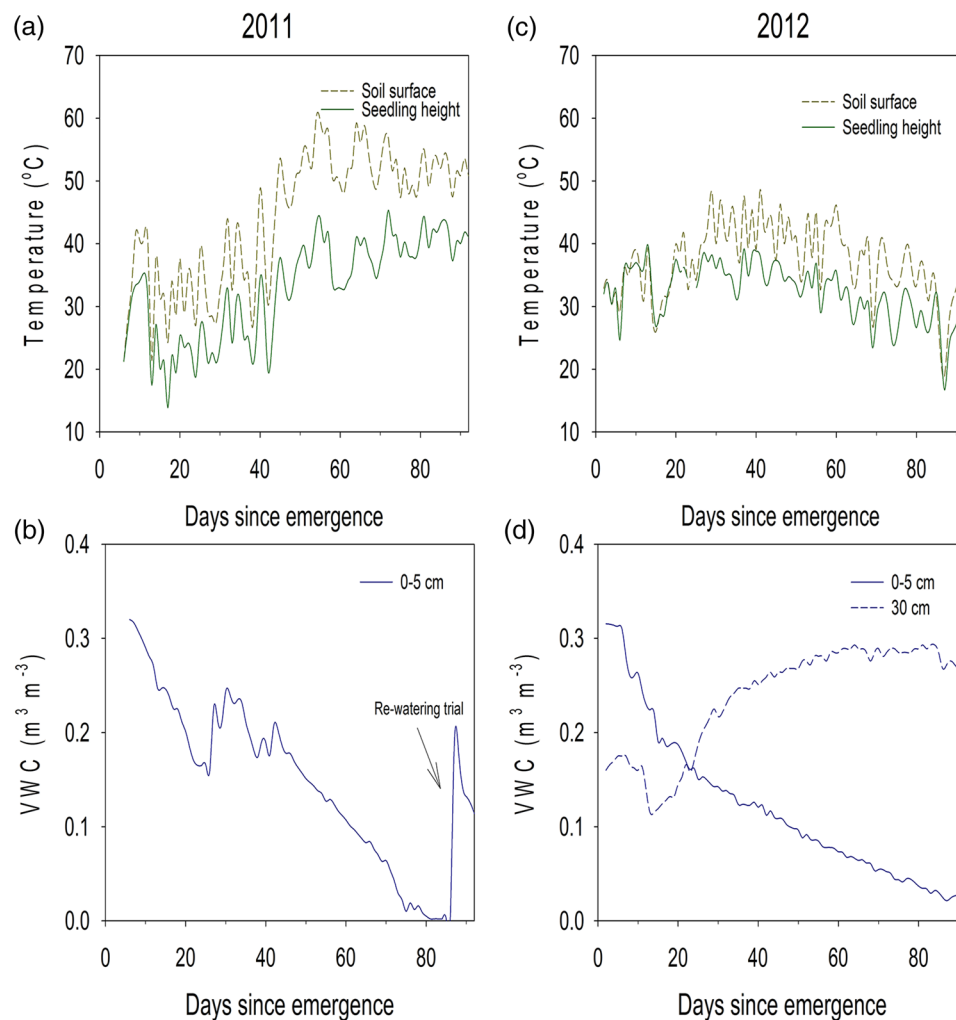


Figure 1. Daily maximum air (at seedling height, ~3 cm) and soil-surface temperatures (a, c), and daily mean volumetric soil water content (VWC, 0–5 cm depth; b, d) of cone-tainer soils during the experiment (soil moisture of garden soil at 30-cm depth in 2012 is also shown). In 2011, the results of a post-experiment watering trial (to see if seedlings recovered, which they did not) are shown. Lines represent the means of three sensors for each measurement.

5% in both years (except for during the end-of-season re-watering trial in 2011). In 2012, deep-soil (30-cm depth) VWC increased at first due to the irrigations early in the experiment, then remained close to 30% through the end of the experiment.

Survival, dry-mass gain, growth allometry and modeled GPP

In both years, survival declined approximately linearly until around Day 60 of the experiment, and then all remaining seedlings died within 7 days (Figure 2a). Visually, seedling appearance went from green and seemingly healthy to brown and shriveled within these 7 days; we observed no lesions on stems characteristic of heat girdling. Early in the experiment (both years), there was relatively little dry-mass gain (i.e., 'growth') in shoots and roots between sampling dates (Figure 2b). Later in the experiment, dry-mass gain between sampling dates was much greater, and appeared to be increasing even as seedling mortality rates were increasing. In both

years, aboveground seedling-height did not increase after the initial germination 'flush' (as is typical in seedlings of many conifer species; data not shown but see Figure S1 available as Supplementary Data at [Tree Physiology Online](http://www.treephys.oxfordjournals.org)). In both years, modeled daily GPP (expressed per m^2 ground area) decreased approximately linearly, while cumulative GPP (expressed per seedling) increased logarithmically (Figure 2c and d). The observed onsets of tree mortality (Days 60 and 75 since emergence in 2011 and 2012, respectively) corresponded to a 70–75% reduction in daily GPP. Cumulative GPP on Day 70 (when all seedlings had perished) was 76 and 73 $\text{g C m}^{-2} \text{ ground}^{-1}$ in 2011 and 2012, respectively (both corresponding to 2.3 $\text{g C g}^{-1} \text{ dry-mass}^{-1}$ or $\sim 0.09 \text{ g C seedling}^{-1}$).

Physiology—carbon relations

In both years, midday $A_{\text{net_area}}$ remained at maximal levels until the final sampling date, when $A_{\text{net_area}}$ dropped to $\sim 0 \mu\text{mol m}^{-2} \text{ s}^{-1}$ (corresponding to when VWC was <10%) (Figure 3a). $A_{\text{net_area}}$

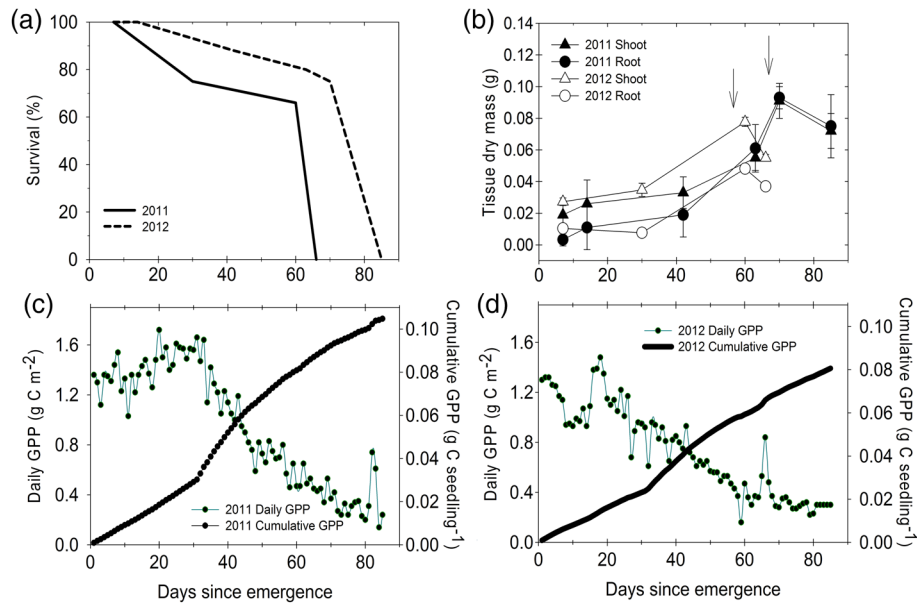


Figure 2. Survival (a), dry-mass gain (b) and modeled gross primary productivity (GPP) per day and accumulated across the 2011 and 2012 growing seasons (c and d, respectively). Mass mortality of seedlings is indicated by breaks in survival slopes in (a), as well as the arrows in (b). Data in (b) are reported as means $\pm$ SE.

was greater in 2012 than in 2011 throughout the experiment (Figure 3a; Table 1). R_{d_area} increased slightly in mid-experiment (both years), then declined to $\sim 1 \mu\text{mol m}^{-2} \text{s}^{-1}$, and was generally less in 2012 than in 2011 (Figure 3b; Table 1). $A_{net_area} : R_{d_area}$ remained >1.0 (i.e., C-flux photosynthetic inputs exceeded respiratory C-flux losses) in both years (but was greater in 2012), until the end of the experiment when $A_{net_area} : R_{d_area}$ was <1 (Figure 3c; Table 1). A_{net_mass} and R_{d_mass} both declined throughout both experiments, and were close to 0 and $5 \text{ nmol g}^{-1} \text{s}^{-1}$, respectively, at the end of the experiment (Figure 3d and e). $A_{net_mass} : R_{d_mass}$ remained above 1.0 until the end of the experiment, and was greater in 2012 than 2011 (Figure 3f; Table 1).

Seasonal patterns of NSC differed between the two years. In 2011, NSC (both soluble sugars and starch) initially decreased by nearly 80% in both shoot and root tissues, but increased later in the summer, especially in shoot tissues (and in stem tissues in particular; Table 2; Figure S2 available as Supplementary Data at [Tree Physiology Online](#)). In 2012, soluble sugar concentration increased, and starch concentration decreased across the growing season. Generally, NSC was greater in seedlings in 2012 than in 2011.

Physiology—water relations

Stomatal conductance (g_s) and seedling predawn water potentials (Ψ) varied little throughout most of the experiment in both years, but dropped quickly at the end of both experiments to $\sim 0 \text{ mol m}^{-2} \text{s}^{-1}$ (g_s) and to between -5 and -8 MPa (Ψ) (Figure 4a and b; Table 1). Internal CO_2 (C_i) concentrations did not vary throughout the experiments, except for at the end of the experiment in 2011 (but note large variance; Figure 4c; Table 1). By mid-experiment (Day 40), seedling

hydraulic conductivity (K_{shoot}) was $<10\%$ of maximal values measured earlier in the experiment (Figure 4d; Table 1), in agreement with increased modeled seedling hydraulic resistance (Figure 5).

In both years, modeled daily GPP and NSC values were responsive to plant hydraulic resistance (Figure 5). Seedling hydraulic resistance increased ninefold and induced a 2.5-fold decrease in GPP before seedlings died (Figure 5a). The comparisons of modeled NSC and seedling hydraulic dynamics indicated that seedling hydraulic resistance increased very rapidly, inducing a steep initial drop in NSC to approximately half of starting NSC concentrations (Figure 5b). Doubling the hydraulic resistance (100% increase) was sufficient to decrease NSC by more than threefold (vertical dashed line in Figure 5b). At the lower concentrations of modeled NSC (Day 47 in 2011 and Day 58 in 2012), seedling hydraulic resistance had already increased by 400% (fivefold increase). The modeling outputs also showed a recovery in NSC at high resistance, corresponding to the end of the drying cycle just before plants died (see arrows in Figure 5b). At that threshold, seedling hydraulic resistance had increased to $\sim 1.0 \text{ m}^2 \text{s MPa mmol}^{-1}$, or by ~ 900 – 1500% . Modeled NSC values were in strong agreement with measured values (Figure 6).

Discussion

We observed complete (100%) mortality in first-year *P. flexilis* seedlings, which mostly occurred during a 1-week episode in each summer (as compared with gradually throughout the

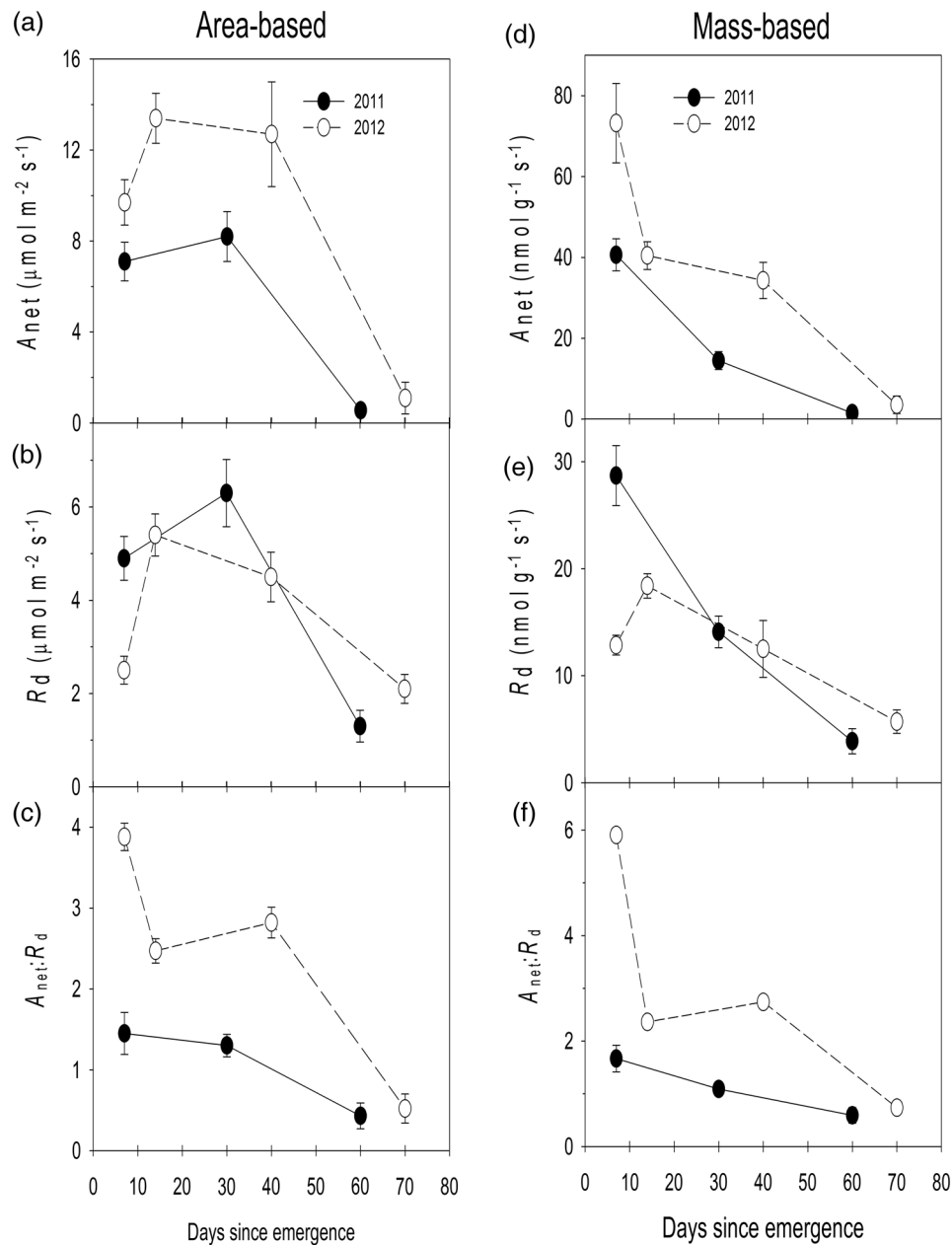


Figure 3. Seasonal variation in net photosynthesis (A_{net} , a and d), dark respiration (R_d , b and e) and $A_{\text{net}}:R_d$ (c and f). Measurements are presented based on leaf area (left column) and on needle mass (right column). All data are reported as means $\pm$ SE.

growing seasons) (Figure 2; Figure S1 available as Supplementary Data at *Tree Physiology* Online). This happened in both years with seedlings from different seed sources. Mortality occurred when soil water content dropped below 10% (Figure 1), when seedling water potentials dropped below -2 MPa and when seedling hydraulic resistance had increased >15 -fold (from 0.07 to over 1.15 MPa $\text{mmol}^{-1} \text{s}^{-1} \text{m}^{-2}$) (Figure 5). This large increase in water transport resistance corresponded to a 90–95% reduction in both shoot hydraulic conductivity (Figure 4d) and daily GPP (Figure 5a). Our data therefore suggest that there are important linkages between hydraulics and whole-plant C relations that impact seedling survival.

Episodic seedling mortality: linkages between hydraulic dysfunction and C relations

The most likely explanation for the large-scale, sharp mortality episodes we observed was acute exceedance of lethal soil–plant hydraulic thresholds, and drought-induced catastrophic embolism (i.e., irreversible hydraulic failure) may have been the mechanism most closely associated with death. At the end of the experiments (both years), seedling predawn Ψ was less than -5 MPa. This is well below the water potential at which there is a 50% loss in hydraulic conductivity (Ψ_{50} ; approximately -1.5 to -2.0 MPa, extrapolated from Figure 4b and d; similar to Ψ_{50} reported in other conifer seedlings in Kavanagh

Table 1. Summary ANOVA table for the effects of year and days-since-germination on response variables. A_{net} , net photosynthesis; R_d , respiration; Ψ , seedling water potential; g_s , stomatal conductance; C_i , internal CO_2 concentration; K_{shoot} , shoot hydraulic conductance; NSC, non-structural carbohydrates. Numbers in bold indicate statistical significance at the $P < 0.05$ level, while numbers in bold italics indicate marginal significance ($0.05 > P > 0.10$).

	Year			Days since emergence			Year $\times$ days		
	dF	F	P	dF	F	P	dF	F	P
Shoot dry mass	1, 105	24.38	<0.0001	3, 105	34.09	<0.0001	2, 105	15.1	<0.0001
Root dry mass	1, 105	3.67	0.06	3, 105	45.5	<0.0001	2, 105	16.48	<0.0001
A_{net} (area based)	1, 102	13.73	0.0009	3, 102	18.34	<0.0001	2, 102	17.82	<0.0001
R_d (area based)	1, 79	9.31	0.003	3, 79	16.59	<0.0001	2, 79	8.65	0.0004
$A_{\text{net}} : R_d$ (area based)	1, 79	40.78	<0.0001	3, 79	8.88	<0.0001	2, 79	0.75	0.47
A_{net} (mass based)	1, 102	68.31	<0.0001	3, 102	66.77	<0.0001	2, 102	0.92	0.4
R_d (mass based)	1, 79	0.06	0.8	3, 79	44.27	<0.0001	2, 79	12.8	<0.0001
$A_{\text{net}} : R_d$ (mass based)	1, 102	133.21	<0.0001	3, 79	6.55	0.0005	2, 102	16.42	0.0001
g_s	1, 1	0.67	0.41	3, 2	2.58	0.08	2, 2	9.3	0.0002
C_i	1, 1	0.38	0.54	3, 2	2.58	0.08	2, 2	9.3	0.0002
K_{shoot}^1				2, 10	25.57	0.0001			
Shoot NSC	1, 7			3, 7	49.14	<0.0001	2, 7	7.89	<0.0001
Root NSC	1, 7			3, 7	11.68	0.0009	2, 7	3.08	0.0066

¹2012 data only (year effects do not apply).

Table 2. Soluble sugars (glucose + fructose + sucrose, % dry mass) and starch concentrations (% dry mass) in root and shoot (stem + needles) tissues. Values are means, and numbers in parentheses are SE. Harvest times for each summer ('early', 'mid' and 'late') correspond to days 7, 30 and 60 in 2011 and days 7, 40 and 70 in 2012 since emergence.

Harvest	Tissue	2011		2012	
		Soluble sugars	Starch	Soluble sugars	Starch
Early	Root	3.4 (0.5)	4.8 (0.7)	¹	¹
	Shoot	4.3 (0.7)	7.6 (1.4)	5.0 (0.4)	2.7 (1.1)
Mid	Root	2.7 (0.9)	0.4 (0.1)	5.0 (0.5)	4.3 (0.4)
	Shoot	1.9 (0.4)	0.3 (0.1)	11.5 (0.3)	3.1 (0.5)
Late	Root	2.0 (0.3)	1.3 (0.5)	6.9 (0.8)	1.7 (0.4)
	Shoot	7.2 (0.8)	3.5 (0.1)	10.0 (0.8)	1.8 (0.2)

¹In 2012, early seedling roots were too small to analyze, so only shoot data are presented.

et al. 1999). Similarly, seedling mortality has been shown to increase sharply as water potentials drop below -3 MPa (e.g., Cui and Smith 1991, Kolb and Robberecht 1996).

Additionally, we observed large declines in K_{shoot} ($<15\%$ of early-season values; Figure 4d) and sharp increases in modeled seedling hydraulic resistance (Figure 5) that preceded the acute mortality episodes. This seemingly did not have immediate consequences for gas exchange or biomass-gain rates, which were maintained (or increased in the case of biomass-gain rate) despite increasing soil-moisture deficit, until an apparent soil-moisture threshold was crossed (although modeled GPP did decrease; see Discussion below). Observationally, we determined the critical soil-moisture threshold to be $\sim 10\%$ (approximately -6 MPa). This compares well with other studies in which similar soil-water thresholds were observed to limit survival and photosynthesis in pine seedlings (Kolb and Robberecht 1996,

Moyes et al. 2013), although our study is unique in that we observed a sharp mortality event (all seedlings dying in <1 week) as opposed to a wave of mortality lasting weeks to months.

Another symptom of lethal hydraulic failure that we observed was possible interruption of phloem transport of NSC. In both summers, NSC increased near the end of the experiments, especially in aboveground tissues (Table 2; Figure 5b). In 2011, we were able to determine that the increase in NSC was specifically in stems, with lower concentrations in needles and roots (see Figure S2 available as Supplementary Data at *Tree Physiology* Online). Stems are not typically considered carbohydrate storage tissue, as ^{14}C tracer studies suggest for pine (Hansen and Beck 1994). Therefore, we speculate that the carbohydrate accumulation in stems was symptomatic of phloem translocation limitations. This interpretation is consistent with Mitchell et al. (2014), who ascribed increases in NSC in aboveground tissues to translocation problems in *Eucalyptus globulus* subjected to chronic drought. Also, Woodruff (2014) found that phloem sieve cell conductivity declined by $>60\%$ as xylem water potentials declined from approximately -1.2 to -1.8 MPa in 2-m tall Douglas-fir trees. Slowing or interruption of the translocation of soluble sugars from needles to roots may have reduced root growth, which has been shown to be the primary C sink later in the summer in first-year conifer seedlings (Bansal and Germino 2010). As a consequence of this reduced root-uptake capacity, whole-plant water transport may have also been impaired during time, when moisture of near-surface soils was approaching limiting levels. Furthermore, seedling roots are more vulnerable to embolism than shoots (i.e., less negative Ψ_{50} ; Kavanagh et al. 1999), and thus declines in root-water transport most likely compounded decreases in root-water uptake. Meanwhile deeper

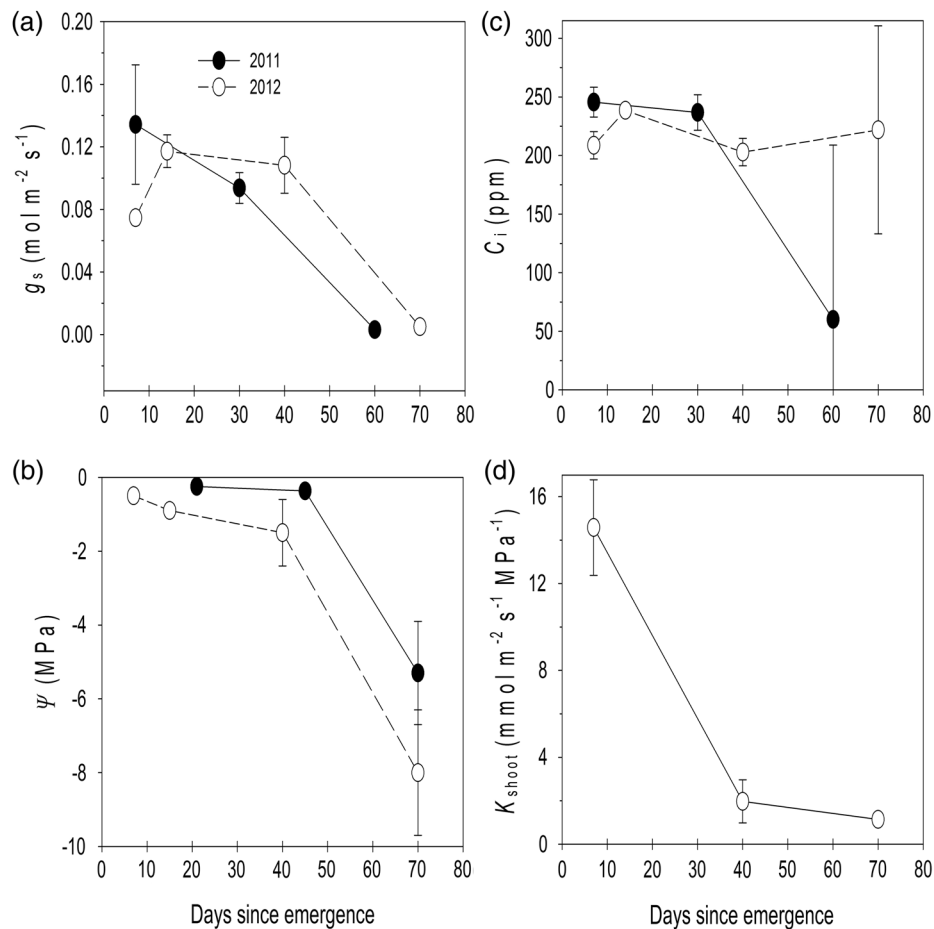


Figure 4. Seasonal variation in stomatal conductance to water (g_s ; a), shoot water potentials (Ψ ; b), internal CO_2 concentrations (C_i ; c) and hydraulic conductivity of shoots (2012 only; K_{shoot} ; d). All data are reported as means $\pm$ SE.

soils, where older plants typically have roots (and do not perish under seasonal drought), had much greater water supplies. Soil moisture remained at 30% at 30-cm depth in our garden (Figure 1d), while maximum root elongation was only 15–18 cm in both years (data not shown, but see Figure S1C available as Supplementary Data at *Tree Physiology* Online). Thus, seedling roots were not able to escape the soil drying-front and tap into the deep-soil water reservoir. Previous investigators have proposed that environmental factors such as fine-textured soils that inhibit root extension from reaching deeper soil-moisture limit seedling establishment (Daubenmire 1943, Patten 1963, Padilla et al. 2007). We propose that problems in the delivery or use of carbohydrate in roots may have contributed partly to an inability of seedling roots to outpace the seasonal soil-drying front, ultimately leading to episodic hydraulic failure.

Another indicator of hydraulic-C relation linkages is the association between GPP and seedling hydraulic resistance. GPP declined steadily throughout the summers, and was associated with exponential increases in hydraulic resistance (Figure 5a). GPP was quite low by the end of each experiment (Figures 2c,d and 5a; less than $\sim 0.6 \text{ g C m}^{-2}$ at the time of the mortality epi-

sodes, corresponding to less than $\sim 1.0 \text{ mg C seedling}^{-1} \text{ day}^{-1}$; whole-seedling values extrapolated from the depicted area-based values). While this might have been sufficient to support daily dry-mass gain ($\sim 0.3 \text{ mg C seedling}^{-1} \text{ day}^{-1}$; Figure 2b) and modeled metabolic respiration demands ($\sim 0.6 \text{ mg C seedling}^{-1} \text{ day}^{-1}$; data not shown), ‘leftover’ C reserves would have been minimal. This would have negatively affected seedlings’ ability to use NSC for osmotic repair of embolisms (e.g., O’Brien et al. 2014), among other complications. GPP declined despite seemingly sufficient C uptake and C pools when compared with in situ (non-drought-specific) studies on first-year conifer seedlings within their natural ranges. In previous studies of first-year seedlings of *P. flexilis* and related *Pinus* species, maximum $A_{\text{net,area}}$ was 2–12 $\mu\text{mol m}^{-2} \text{s}^{-1}$, and $R_{\text{d,area}}$ was 2–6 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (Maher and Germino 2006, Bansal et al. 2010, Reinhardt et al. 2011, Moyes et al. 2013), similar to fluxes we report (Figure 3). $A_{\text{net,area}}$ and $R_{\text{d,area}}$ did gradually decline across the experiments, but this was not due to failing photosynthetic C inputs (as evidenced by the sustained area-based fluxes throughout most of both summers in our experiment, until the point when hydraulic thresholds were passed). Instead these declines were most likely due

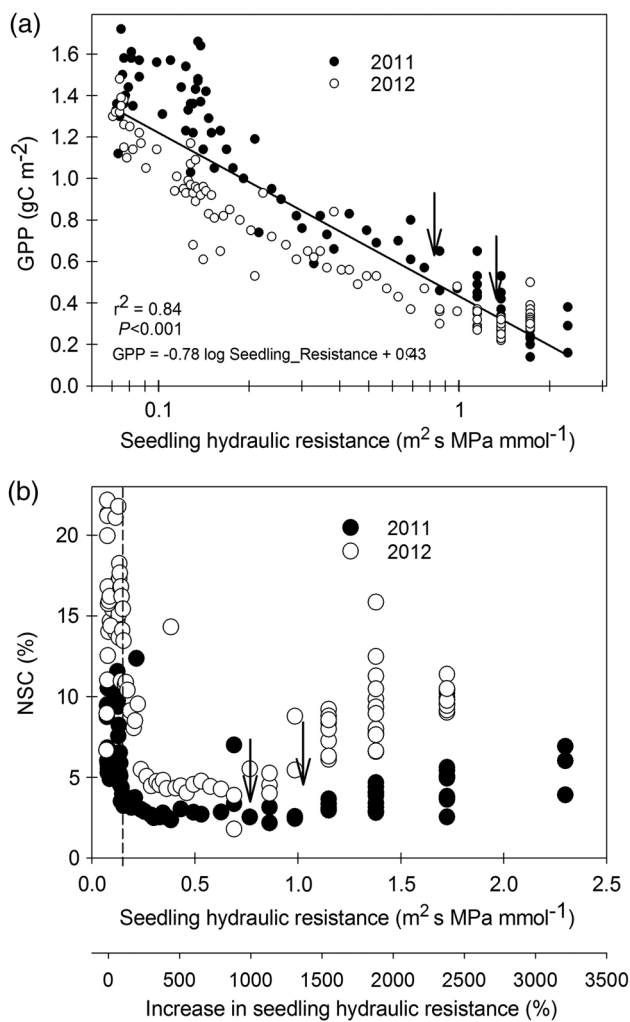


Figure 5. Modeled daily GPP (a) and NSC (b) as a function of modeled seedling hydraulic resistance across the 2011 and 2012 growing seasons. In (a), note the 2.5-fold decrease in GPP that is associated with a 10-fold increase in hydraulic resistance over the drying period. In (b), the percent increase in seedling hydraulic resistance is also shown. The vertical dashed line represents a 100% increase (doubling) in plant resistance. In both panels, arrows represent the seedling mortality episodes depicted in Figure 2a (Days 60 and 70 for 2011 and 2012, respectively).

to increasing tissue density (greater biomass area⁻¹), and possibly decreasing mesophyll conductance with leaf development (Scartazza et al. 1998, Grassi and Magnani 2005). As was the case with C fluxes, NSC concentrations were not lower than in studies reporting NSC in non-droughted first-year conifer seedlings (e.g., Bansal and Germino 2008, Bansal et al. 2010, Reinhardt et al. 2011), and had no clearly diminishing trend (i.e., towards 'C-reserve exhaustion'). However, in model scenarios NSC concentrations sharply decreased as seedling hydraulic resistance increased early in the experiment, which might have set the stage for chronically low C reserves during drought-stressed growing seasons.

We predicted that C-flux balance (herein defined as the ratio of photosynthesis over respiration) would decrease during the

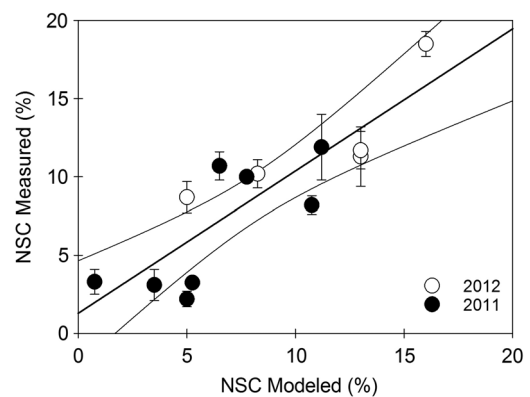


Figure 6. Comparisons of measured versus modeled NSC. Measured NSC values are also represented in Table 2.

experiments, negatively impacting C balance. However, we observed in both years that patterns in R_d mirrored those of photosynthesis and did not increase relative to A_{net} (Figure 3; Figure S3 available as Supplementary Data at [Tree Physiology Online](#)), despite increasing air and soil-surface temperatures. Thus any decreases in the balance between C input and C output fluxes (i.e., $A_{net} : R_d$ [Figure 3c and f] or $A_{gross} - R_{total}$ [Figure S3 available as Supplementary Data at [Tree Physiology Online](#)]), which dropped below 1.0 ($A_{net} : R_d$) or 0.0 ($A_{gross} - R_{total}$) only at the end of each experiment, were due to abrupt declines in A_{gross} , and not gradual increases in R_d (Figure S3 available as Supplementary Data at [Tree Physiology Online](#)). It is likely that our seedlings were acclimated to the ambient temperature regimes (daily T_{min} and T_{max} range often 30–40 °C), and thus were able to maintain a relatively constant ratio of $A_{gross} : R_d$ throughout most of the summer. This hypothesis is consistent with other studies that demonstrated acclimation of R_d to higher temperatures in conifer seedlings, which partially offset declines in whole-plant C balance (e.g., Way and Sage 2008). This physiological strategy would be helpful for *P. flexilis* seedlings, which are known to colonize hot, dry sites after disturbances (Rebertus et al. 1991). Decreases in A_{net} at the end of each experiment were due to strong stomatal limitations on photosynthesis (Figure 4), which would not have affected R_d in the short term because respiration is not as diffusion-limited by stomatal regulation as photosynthesis (Flexas et al. 2006).

Supplementary data

Supplementary data for this article are available at [Tree Physiology Online](#).

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Conflict of interest

None declared.

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