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Research paper

Willow oak (*Quercus phellos*) seedling roots continue respiration and growth during fall and winter in a soil temperature-dependent manner

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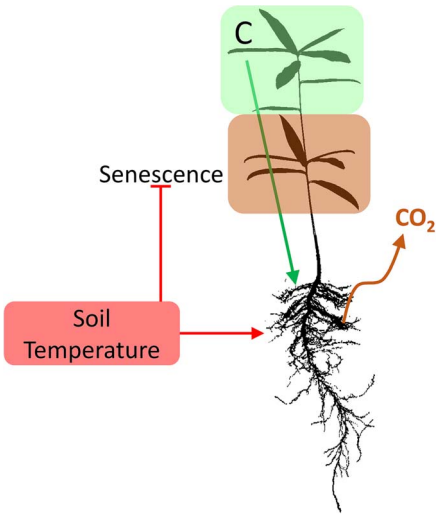
Many greentree reservoirs (GTRs) and other bottomland hardwood forests have experienced a shift in tree species composition away from desired red oaks (*Quercus* section *Lobatae*), like willow oak (*Quercus phellos* L.), due to flood stress mortality. Trees experience flood stress primarily through their root system, so it is surmised that GTR flooding may be occurring before root systems have reduced their activity entering the winter. Because soils buffer seasonal temperature changes, we hypothesized that root activity would respond to the belowground environment rather than the aboveground environment. To investigate whether cold soil temperatures reduce root growth and respiration in willow oak during winter, soil temperatures for container seedlings were either held at 15 °C or transitioned to 10 or 5 °C in the late fall. Root elongation was measured in seedlings grown in rhizotron pots by analyzing repeated images of roots during the fall–winter transition period. Root respiration, measured at soil temperature levels, was used as an indicator of root energetic expenses. Also, root respiration was measured at 15 and 5 °C to determine Q_{10} values to test for acclimation to low soil temperature. Root elongation continued in winter, even after stem elongation stopped in soil temperatures ≥5 °C, a condition usually met throughout most of the native range of willow oak. Both root elongation and respiration rates decreased in cooler soil temperatures. However, Q_{10} values were unaffected by soil temperature treatment. These findings do not support root dormancy or cold acclimation of root respiratory activity but indicate that temperature directly and reversibly affected root respiration rate. Root elongation may have been dependent on photoassimilates produced by green leaves that were retained through much of winter. Overall, our results suggest that willow oak roots may continue a high rate of growth throughout winter, unlike most temperate species measured to date, and that soil temperature has a major influence over their growth and respiration rates.

Graphical Abstract



Influences on winter root growth and respiration. During winter, elevated soil temperature increases root growth and respiration while delaying leaf senescence of willow oak seedlings. Potential photosynthesis conducted by semipersistent leaves fuels root growth and metabolic activity, soil temperatures permitting.

Keywords: bottomland hardwood forest, greentree reservoir, *Quercus* seedling, rhizosphere temperature, root phenology, winter dormancy.

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Introduction

Greentree reservoirs (GTRs) are areas of bottomland broadleaf forests that are artificially flooded during the late fall and through the winter to provide wintering habitat for migratory waterfowl and incidentally provide habitat for diverse migratory birds and wildlife species. Willow oak (*Quercus phellos* L.), a species preferred in GTRs for its acorn production (Cypert and Webster 1948, Francis 1983), has declined under current GTR management in the Lower Mississippi Alluvial Valley (LMAV). This is likely tied to the timing of artificial flooding in the fall (Sample and Babst 2020). While various mature tree species can survive intermittent growing season floods over multiple years, seedlings may be severely stressed or killed if long-term flooding occurs during the growing season, including fall and spring (Broadfoot 1967, Broadfoot and Williston 1973, Sample and Babst 2020, Sample et al. 2023). Flooding initiates root stress in mesophytes by decreasing oxygen availability in the rhizosphere, and under hypoxia, roots must switch from aerobic respiration to more inefficient anaerobic respiration, which may also result in accumulation of toxic byproducts (Kozlowski and Pallardy 1984, Armstrong and Drew 2002, Kreuzwieser et al. 2004, Parent et al. 2008, Voesenek and Bailey-Serres 2013, Beamer et al. 2021, but also see Sauter 2013). Oak species that have some flood tolerance can form adventitious roots to a limited extent and hypertrophied lenticels, but they do not develop aerenchyma or similar structures to transport oxygen to roots. Instead, many mesophytes, including oaks, adopt a quiescence strategy to tolerate flood-induced root hypoxia, reducing growth and metabolic activity to use oxygen efficiently, conserve carbohydrates and limit inefficient expenditure of sugars by anaerobic respiration (Pezeshki and Anderson 1996, Gravatt and Kirby 1998, Pezeshki et al. 1999, Crawford 2003, Parelle et al. 2006, Bailey-Serres et al. 2012, Bourgeade et al. 2018, Fujita and Tange 2020). As such, oak and other deciduous tree seedlings and saplings are thought to be more tolerant of flooding that occurs during the winter months when their shoots are dormant (King and Fredrickson 1998, Miao et al. 2017, Wang et al. 2017, Domisch et al. 2018, Sample et al. 2023). However, it is not clear if willow oak roots enter dormancy or stay active during the winter because temperature below the soil surface is buffered from daily minimums of air temperature and soil rarely freezes in the LMAV. A better understanding of the factors that influence winter root activity, i.e., respiration and growth, could inform management for improved willow oak seedling survival in GTRs. In general, tree root activity may be influenced by factors including soil temperature, sink competition and anything affecting photoassimilate supply, such as leaf senescence.

Multiple reports have shown some evidence of tree root growth during shoot dormancy, suggesting that the roots of many tree species do not appear to enter dormancy (Radville et al. 2016). However, there have been several studies in which trees had no winter root production or elongation (Burke and Raynal 1994, Contador et al. 2015). Soil temperature in temperate forests decreases during the winter, leading to very slow root growth (e.g., Kuhns et al. 1985, Eissenstat et al. 2006, Alvarez-Uria and Körner 2007, McCormack et al. 2014), and root growth may cease when soil temperatures are too cold (Reich et al. 1980). However, most research focused on winter root activity of trees has been conducted at higher latitudes than the LMAV. For example, in white oak (*Q. alba* L.) seedlings, soil temperature below 17 °C was limiting to root growth, and roots of black walnut (*Juglans nigra* L.) trees ceased growing when soil temperature was below 5 °C (Teskey and Hinckley 1981, Kuhns et al. 1985). Soil temperatures in the LMAV only occasionally approach 5 °C or below (NRCS SCAN 2021). In the few studies of warmer temperate systems (i.e., longleaf pine-wiregrass ecosystem, loblolly pine, cherimoya trees), root growth was very low during winter relative to peak root growth rates (Ibacache et al. 1999, Pritchard et al. 2008, McCormack et al. 2010). In more tropical systems, where winter shoot dormancy does not occur, root growth may be continuous throughout the year, influenced primarily by soil temperature and competition with other sinks such as growing shoots and developing fruits (Marler and Willis 1996, Broschat 1998, Mickelbart et al. 2012, but see Bevington and Castle 1985), or root growth may be dependent on soil moisture (Janos et al. 2008).

Suberization tends to increase during reduced root growth, which might also increase tolerance to flooding. New developing root tips are white and fleshy when they are young and unsuberized. Basal to the tips, the region of the root that has elongated and begun to differentiate turns brown as suberization begins, first in the endodermis, forming the Casparian strip, and later in the phellem (Machado et al. 2013, Campilho et al. 2020). Generally, suberized roots would be less susceptible to stresses such as low soil moisture, nutrient toxicity, salt stress and flood stress than unsuberized roots (Enstone et al. 2002). A suberized barrier to radial oxygen loss may be induced under hypoxia and associated with flood tolerance (Le Provost et al. 2016, Watanabe et al. 2017, Colmer et al. 2019, Ejiri et al. 2020, Pedersen et al. 2021), including in pedunculate oak (*Q. robur*) (Bourgeade et al. 2018). Thus, as differentiation and suberization tend to occur closer to the tips (Enstone et al. 2002), reduced root growth during winter may decrease the vulnerability of roots to flood stress.

Decreased root growth may be driven by decreased rates of biochemical processes, such as respiration. Respiration provides most of the energy for cellular reactions and so indicates the sum of most energy-dependent processes, including growth-related processes. Root energy expenditures for aspen (*Populus tremuloides* Michx.) fluctuated with temperature during winter, but aspen roots also had lower respiration during winter than summer when moved to the same temperature during measurements, indicating that aspen roots acclimated to winter soil temperatures by reducing overall respiration (Desrochers et al. 2002). However, this species ranges far north and can frequently experience freezing soil conditions for which acclimation or root dormancy may be necessary to protect root tissues from damage. On the contrary, sugar maple (*Acer saccharum* Marsh.) root respiration showed no sign of acclimation to soil temperature according to temperature coefficients (Q_{10}), the quotient of respiration values measured over a 10 °C temperature span; rather, sugar maple root respiration appeared to be driven by direct effects of seasonal soil temperature on the rate of reactions (Zogg et al. 1996, Burton and Pregitzer 2003). There has been little research on winter root activity in willow oak or other lowland tree species of the southern USA Given that willow oak is unlikely to experience freezing soil temperatures for sustained periods

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(NRCS SCAN 2021), acclimation of respiration rates seems doubtful, and we suspect root respiration and growth track soil temperature during the fall and winter, driven by the direct effect of temperature on reaction rates.

Decreased winter root activity may be associated with the termination of photosynthate translocation after autumn leaf senescence in many species. Autumn leaf senescence is an organized process that includes carbohydrate translocation to the lower stem and roots, and nutrient translocation from leaves to stem rays and bark. Autumn leaf senescence is accompanied by cessation of aboveground growth and reduced metabolic activity in stems, which help preserve resources and cellular structure, and allow for continued tissue maintenance even under freezing conditions (Sauter et al. 1996, Rinne et al. 1999, Wisniewski et al. 2003, Li et al. 2020). Whether or not roots enter dormancy, leaf senescence halts carbohydrate translocation to roots coincident with the winter reduction in root activity, which may be caused by the termination of photoassimilate transport when leaf senescence occurs, decreasing soil temperatures or possibly shoot-to-root senescence-related signaling (Romberger 1963, Desrochers et al. 2002). Reduced root respiration and growth in response to leaf senescence and cool soil temperatures may incidentally help conserve carbohydrate reserves through winter flooding. However, willow oak seedlings often have semi-persistent leaves, characterized by delayed leaf senescence, and seedlings may even retain leaves throughout winter. If willow oak seedlings retain leaves, root activity may continue during winter with rates influenced primarily by soil temperature and sink competition, similar to trees that do not enter winter dormancy. It is unclear how soil temperature and sink competition might interact in the case of willow oak seedlings. Although leaves may be retained, stem elongation stops, which would make the roots the major carbohydrate sink during late fall and winter. Thus, unlike most temperate trees that severely reduce root growth during winter, it is possible that willow oak seedling root growth may continue at high rates or even increase during winter if soil temperatures permit. In the absence of strong competing sinks, cold soil temperatures may be the primary limit on root sink strength and growth.

Assuming that leaves would be retained during winter, we hypothesized that willow oak root respiration would decrease with decreasing soil temperature but without an indication of dormancy or acclimation. Additionally, we hypothesized that soil temperature below 15 °C would reduce root growth and that root elongation would not occur in 5 °C soil. To test these hypotheses, we established a controlled greenhouse experiment to study willow oak root respiration and growth during the late fall and winter when soil temperature was either kept at 15 °C or lowered to winter temperature levels of 10 or 5 °C. In addition to studying root respiration at soil temperature, we also measured respiration at two fixed temperatures, 5 and 15 °C, to determine whether Q_{10} changed during the transition to winter. Root elongation was simultaneously studied by imaging roots visible in rhizotron pots and tracking root elongation rates and mortality.

Materials and methods

Plant material

Willow oak seed used to produce seedlings for this study was collected at Cut-Off Creek Wildlife Management Area (WMA), Drew County, AR, USA (33° 26′ N, 91° 32′ W) under at least 10 different parent trees in November and December 2018. Acorns were cleaned, soaked in 4 °C water overnight, and then stored at 1.6 °C in polyethylene bags. Immediately prior to sowing, which occurred on 29–30 August 2019, acorns were sorted (those with insect or fungal damage were removed and the lot was prioritized based on radicle emergence) and soaked in reverse osmosis (RO) water at 1.6 °C for 24 h.

The two different foci of this study, root respiration and root elongation, required that seedlings be raised in different container types. Seedlings produced for root respiration sampling were raised in 1.0 L plastic Deepot containers (35.6 cm tall × 6.8 cm diameter top) (Steuwe and Sons Inc., Tangent, OR, USA) fitted with a patch of landscape fabric at the base to prevent loss of potting medium. Seedlings produced for root elongation sampling were raised in 1.4 L 'rhizotron pots' constructed from 35.6 cm tall × 7 cm diameter clear plastic tubing. Rhizotron pots were painted black, leaving a 6 cm wide × 29 cm long clear viewing window for root elongation measurements.

Both container types were filled with a soil-less medium, Profile® Greens Grade™ (Profile Products, Buffalo Grove, IL, USA), which is a silica-based, fine ceramic particulate that has 74% porosity and is easily removed from harvested roots with gentle washing. Slow release, 15:6:12 NPK fertilizer with micronutrients (Osmocote Smart-Release Plant Food Plus, The Scotts Miracle-Gro Company, Marysville, OH, USA) was mixed into the potting medium prior to filling containers (5 g Osmocote to 1 kg Profile Greens). A single acorn was sown in each container, 5100 for the respiration focus and 240 for the root elongation focus of the experiment, on the dates noted above. All containers were placed in a greenhouse in Stoneville, MS, USA (33° 25′ N, 90° 54′ W) under 60% shade cloth and maintained at ambient temperature. Rhizotron pots were placed in racks such that they were held at a 20° angle from vertical with the viewing window covered and facing down to promote root growth against the window. Potting medium was maintained at field capacity by misting with RO water multiple times a day during initial germination. Then, after seedlings established an initial shoot flush, soil moisture was monitored daily and watered as needed to maintain field capacity.

In early November 2019, we selected 3240 plants for use in the respiration focus of the experiment, including only single-stemmed seedlings that had completed a second shoot flush and had entered the 2-lag stage of shoot development (Hanson et al. 1986). Likewise, 108 seedlings raised in rhizotron pots were initially selected for inclusion in the root elongation focus of the experiment, but within these seedlings, analysis was limited to 41 seedlings that still had roots visible against the window at the initial root imaging date. These seedlings met the same criteria as those selected for the respiration focus and, additionally, part of their root system could be observed through the viewing window of the pot.

Experimental design

Eighteen open-topped rectangular tanks of 1890 L volume (122 cm wide × 183 cm long × 61 cm tall) were organized in three blocks of six tanks in a greenhouse at the same location as above. Blocks were established parallel to the wall of greenhouse exhaust fans, differing in the distance from the wall to account for an air temperature gradient created by

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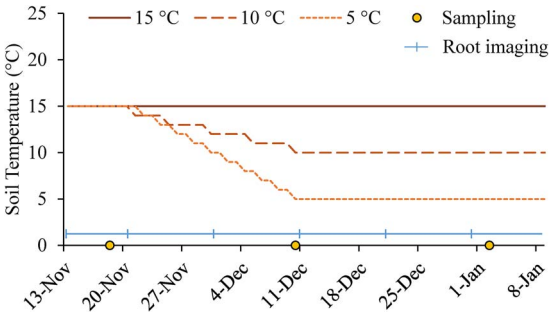

Figure 1. Timing of soil temperature treatment levels over the span of the experiment. Starting date of root respiration sampling periods are indicated on the timeline as yellow dots, and root imaging dates are indicated by the blue hash marks.

airflow through the greenhouse. Tanks were lined with black polyethylene sheeting, filled with RO water and fitted with a commercial-grade aquaculture chiller/heater (D1–100, Frigid Units Inc., Toledo, OH, USA). The aquaculture unit circulated water and maintained it at ±0.5 °C of a set temperature, effectively creating a controlled temperature water bath. Seedlings were randomly assigned to a tank (experimental unit) on 10 November 2019; 180 seedlings per tank for the respiration focus of the experiment and 12 seedlings per tank for the root elongation focus. Containers were held in racks placed in each tank such that the surface of potting medium in containers was level with the surface of water in the tank. To prevent tank water from flooding seedling containers, each container was placed in a 76.2 μm thick polyethylene bag (15.2 × 50.8 cm) prior to being moved to its assigned tank. Water in all tanks was maintained at 15 °C, which is a temperature consistent with soil temperature in the field (Cut-off Creek WMA) at that time of year, for a 10-day acclimation period.

Each of the three tanks in a block was randomly assigned a winter temperature level (15, 10 or 5 °C). Tanks assigned 10 or 5 °C received a gradual reduction of temperature over about 20 days (Figure 1). Temperature was lowered 1 °C every 5 days for tanks assigned 10 and 1 °C every 2 days for tanks assigned 5 °C, and both temperature levels reached targeted winter temperature on 10 December. These temperatures were maintained for 30 days to the conclusion of this phase of the experiment (Figure 1).

Measurements

Air temperature, relative humidity and photosynthetically active radiation (PAR) were recorded once every 15 min using a LI-1400 data logger (LI-COR Biosciences, Inc., Lincoln, NE, USA) equipped with a temperature thermocouple, humidity sensor and quantum sensor. Observations on seedling phenology were recorded daily by tank for the duration of the experiment. Leaf color and progression of senescence were categorized by proportion of seedlings in a tank that met one of six condition classes from fully green to fully senesced. The stem length (cm) and root-collar diameter (mm) of each seedling were measured on 20 November 2019 (immediately before winter temperature levels were initiated), 11 December 2019 (once winter soil temperatures were reached) and 9 January 2020 (after 30 days at winter temperature levels).

Measurements to quantify CO_2 efflux of roots at soil temperature and Q_{10} values were conducted during three periods of this phase of the experiment (Figure 1). The first sample period was after 8 days of seedling acclimation to 15 °C soil (18 November 2019). During this sample period, nine pre-senescence seedlings (three per temperature level) were randomly selected for root respiration measurements that captured data for late in the growing season. The second sample period began on 10 December 2019 when soil temperatures reached target winter levels. Five plants from each tank were sampled during this period. The third sample period began on 2 January 2020, and three plants were collected from each tank for this mid-winter time point.

To prepare roots for respiration measurements, the root system of each sample seedling was removed from potting medium, and it was gently and thoroughly cleaned in RO water. The root systems were typically dominated by a long primary root or tap root (>10 cm), with much shorter secondary roots or lateral roots branching off from the tap root (0.1–5 cm). In turn, the secondary roots had many very short lateral roots branching from them (<1 cm). For fixed temperature measurements and Q_{10} determination, two secondary roots of average size were detached from the tap root, leaving all roots branching from the secondary root intact, in each of three zones: just below the root collar, the middle and near the tap root apex. The tap root and all remaining lateral roots were used for CO_2 efflux measurements.

Two Oxytherm+R instruments and OxyTrace+ software (Oxytherm+R, Hansatech Instruments Ltd, Pentney, UK) were used to obtain data to calculate a Q_{10} value for each sample plant. One instrument was programmed to measure O_2 consumption rate (OCR) at 15 °C and the other at 5 °C. On each sample day, both instruments received two-point calibration with O_2-saturated water and anoxic water, which was produced by sparging deionized water with N_2 gas. Water in Oxytherm+R chambers was replaced with 2 mL of fresh O_2-saturated deionized water prior to measuring each root sample. Three lateral roots, one from each zone of the root system, were placed into each chamber, one for measurement at 15 °C and the other for measurement at 5 °C. Sample data were recorded for 50 min using a live rate of μmol 10 min^{-1}. Because the OCR required up to 15 min to stabilize, a representative value for a plant was calculated by averaging measurements recorded between 20 and 40 min. After measurement, root tissues were dried at 70 °C and weighed so that OCR values could be converted to dry mass basis. Values of Q_{10} were calculated for each seedling by dividing the OCR at 15 °C by the OCR at 5 °C ($Q_{10} = OCR_{15°C} \div OCR_{5°C}$).

A LiCor 6400XT gas analyzer (LI-COR Biosciences, Lincoln, NE, USA) fitted with a conifer chamber was used to measure CO_2 efflux from roots at the soil temperature in which the roots had been growing. Root tissue samples were separated from the plant, bundled and wrapped in a thin paper wipe. The paper wrap was moistened with RO water, the bundle was placed in the cuvette and CO_2 efflux was recorded every 15 s for 40 min using the LiCor Insect Respiration configuration, which determines gas exchange per gram of sample, assuming the sample is 1 g. Afterward, all samples were dried at 70 °C and weighed. Stability of CO_2 efflux was typically reached in less than 10 min, so measurements between 10 and 30 min were averaged to calculate a representative CO_2 efflux rate for each seedling. This value was corrected by dividing it by the actual dry weight of the sample.

Imaging rhizotron pots for root elongation and mortality began on 14 November 2019 (Figure 1). Each rhizotron pot

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was removed from its assigned tank, the viewing window was uncovered and dried and the pot was placed upright in a photography light box that had uniform brightness and angle of lighting, with the pot a fixed distance from the camera, the camera and pot at fixed heights and the viewing window of the pot facing directly toward the camera for every photo. The entire length and width of viewing window were included in each image. A D3500 camera (Nikon Inc., Tokyo, Japan) was used to capture a digital image of the viewing window. Each pot was re-sampled on 21 November 2019 and on a 10-day interval thereafter through 31 December 2019. WinRhizoTRON 2020c (Regent Instruments Inc., Quebec, Canada) software was used to measure length of roots visible in images—length of the visible portion of unsuberized roots (white), suberized roots (brown) and dead roots (black) were recorded for each pot. The WinRHIZO TRON software color analysis feature was used to recognize root colors based on color ranges that we selected for each category. We manually verified that the color analysis results were accurate. For each tank, we calculated the average unsuberized root length, average suberized root length, average dead root length and average total live root length. Root elongation was calculated for each plant by subtracting root length totals measured in the preceding sample period from those of the current sample period and growth was converted to a rate by dividing it by the number of days since the preceding sample. Growth rates were averaged for each tank for analyses.

Statistical analyses

All analyses were conducted with SAS 9.4 (SAS Institute Inc.) software with significance defined at $\alpha = 0.05$. General linear model (GLM) diagnostic plots were coded for winter temperature against each response variable to test for normality and homogeneity of variance. Proc Mixed was used to analyze tank means for stem length, stem diameter, root elongation rate and changes in root lengths, according to a randomized block design with repeated measures such that time (date of measurement), soil temperature and time × temperature were source effects and seedlings were nested within block as a random effect in the following model (Eq. (1)).

$$V_R = \beta_0 + \beta_1 \text{Temp}_i + \beta_2 \text{Time}_j + \beta_3 (\text{Temp} \times \text{Time})_{ij} + u_k + \epsilon_{ijk} \quad (1)$$

where V_R is the response variable, Temp_i represents the fixed effect of temperature, Time_j represents the fixed effect of the date of measurement, $(\text{Temp} \times \text{Time})_{ij}$ is the interaction between temperature and date of measurement, u_k is the random effect of the kth block and ϵ_{ijk} is the residual error term. For respiration, Proc GLM was used to analyze tank means within each sampling date, such that soil temperature, measurement temperature and interactions of soil and measurement temperature were source effects in the following model (Eq. (2)).

$$\text{Resp} = \beta_0 + \beta_1 \text{Temp}_{\text{soil}} + \beta_2 \text{Temp}_{\text{meas}} + \beta_3 \left(\text{Temp}_{\text{soil}} \times \text{Temp}_{\text{meas}}\right) + \epsilon \quad (2)$$

where Resp is the respiration, $\text{Temp}_{\text{soil}}$ represents the effect of soil temperature, $\text{Temp}_{\text{meas}}$ represents the effect of the temperature at which the roots were held during measurement, $(\text{Temp}_{\text{soil}} \times \text{Temp}_{\text{meas}})$ is the interaction between soil temperature and temperature at which the measurement was conducted, and ϵ is the residual error term. For leaf senescence, Proc GLM was used to analyze soil temperature effects on mean dates that tanks reached each senescence rating. Where interactions or main effects were statistically significant, pairwise comparisons were made with Tukey's post hoc correction.

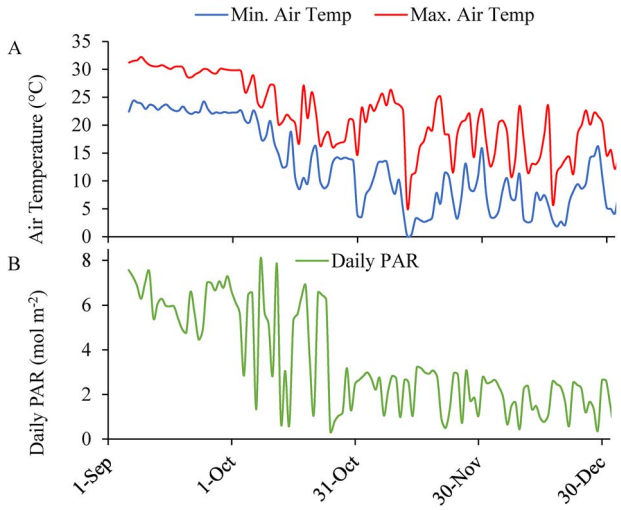

Figure 2. Greenhouse conditions over the course of the experiment. (A) Maximum and minimum daily air temperatures. (B) Daily sum of PAR.

Results

Greenhouse environment and seedling phenology

Nightly minimum air temperatures decreased below 5 °C on 31 October, and thereafter remained mostly in the cold to cool range (5–10 °C), occasionally rising above 15 °C (Figure 2A). During the winter temperature period, which began on 11 December, the daily maximum air temperature averaged 15.9 °C and the minimum averaged 7.1 °C. Air temperature approached but did not drop below freezing in the greenhouse (Figure 2A). The average daily maximum PAR during the same period was 114 μmol m^{-2} s^{-1} and the average total daily PAR was 1.7 mol m^{-2}, both of which were considerably lower than in September (339 μmol m^{-2} s^{-1} and 6.3 mol m^{-2}, respectively) (Figure 2B).

Leaf senescence was incomplete for most seedlings, with many plants retaining green leaves through the measurement period, especially on the second (younger) flush. This semi-persistence of leaves has also been observed in willow oak seedlings growing under natural field conditions (personal observation, Kressuk and Babst). First flush leaves senesced by late December for most seedlings assigned to the 5 °C treatment level, but not for those assigned to 10 or 15 °C. Cool soil temperature hastened leaf senescence. On 20 December, one-quarter of the seedlings experiencing the 5 or 10 °C soil treatment level had noticeable color change, with seedlings in the 15 °C level reaching this mark on 25 December ($P = 0.006$) (Table 1). Similarly, one-half of seedlings in 5 or 10 °C soils had begun changing color earlier than in 15 °C soil ($P = 0.009$). Color change peaked on 2 January for seedlings assigned the 5 °C treatment level, 6

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Table 1. Effect of soil temperature on date of leaf color change in willow oak seedlings. Where temperature effects were significant, like letters in a column indicate means that are not different.

Soil temperature (°C)	Proportion of seedlings with leaves changing color		
	1/4	1/2	3/4
15	25 December 2019 a	10 January 2020 a	15 January 2020 a
10	20 December 2019 b	28 December 2019 b	6 January 2020 ab
5	20 December 2019 b	23 December 2019 b	2 January 2020 b

January for seedlings assigned the 10 °C level and 15 January for those assigned the 15 °C level ($P = 0.02$) (Table 1). Three-quarters of the seedlings for each treatment level showed color change at those respective peaks.

Stem length was not influenced by time and temperature interactions ($P = 0.09$). It did not change between the November and January ($P = 0.68$) sample periods, indicating that stem elongation had ceased by 18 November (Figure 3). Likewise, soil temperature did not have a statistically significant effect on stem length ($P = 0.08$). Root collar diameter also did not show a time × temperature interaction ($P = 0.47$). Time, but not soil temperature, affected root-collar diameter ($P_{\text{Time}} = 0.003$, $P_{\text{Temperature}} = 0.13$); it did not change from November to December ($P = 0.98$) but increased about 5% from December to January ($P < 0.01$).

Root elongation rate

Adherence to the previously described sampling protocol resulted in 14 seedlings acceptable for root elongation sampling in the 15 °C treatment level, 12 seedlings in the 10 °C level and 15 seedlings in the 5 °C level. Time and temperature did not interact to influence root elongation rate ($P = 0.79$). Root elongation rates were not influenced by time ($P = 0.29$) but were affected by soil temperature ($P = 0.03$) (Figure 4). Root elongation rate variability was high among seedlings, but the average was stable and relatively high from late November through December for seedlings experiencing 15 °C soil. Seedlings assigned 10 or 5 °C soil had root elongation rates 59% and 55% lower, respectively, than those in 15 °C. After 10 December 2019, when winter soil temperatures were reached, the average root elongation rates were 73% lower for 10 °C and 81% lower for 5 °C than for 15 °C ($P = 0.01$). Time and temperature did not interact to influence the proportion of seedlings with roots growing ($P = 0.84$), nor was there a temperature main effect ($P = 0.15$). The main effect of time was not quite significant ($P = 0.09$), and pairwise comparisons indicated no date was significantly different from any other date (lowest pairwise $P = 0.20$). After 10 December 2019, when winter soil temperatures were reached, there was still no significance of the interaction of time and temperature ($P = 0.92$), nor temperature or time main effects ($P = 0.14$, $P = 0.77$, respectively).

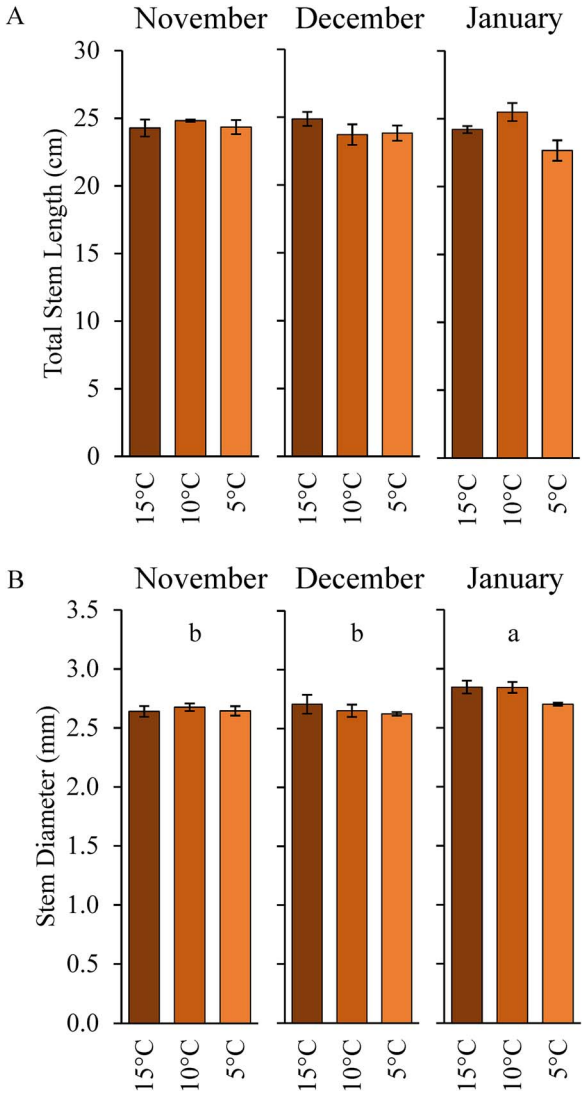

Figure 3. Soil temperature effect on (A) stem length and (B) diameter in November, December and January. November measurements were immediately before, and January measurements were 30 days after soil temperature cool down for winter. Bars represent means ± SE. Where there was a significant effect of time, same letters at the tops of graphs indicate monthly means that are not different.

New root lengths

There was not a significant interactive effect of time and temperature on new length of total live roots ($P = 0.63$). Total live root length increased over time ($P = 0.004$), and was significantly affected by soil temperature level ($P = 0.008$), with 15 °C being significantly greater than 5 °C and 10 °C in pairwise comparisons ($P_{15 \text{ vs } 10} = 0.01$, $P_{15 \text{ vs } 5} = 0.04$, $P_{10 \text{ vs } 5} = 0.80$) (Figure 5A). There was not a significant interaction of time × temperature on the amount of suberized (brown) root length ($P = 0.79$), but the suberized root length increased over time ($P = 0.001$). Also, high soil temperature increased suberized root length ($P = 0.03$), particularly relative to the 5 °C level ($P_{15 \text{ vs } 10} = 0.99$, $P_{15 \text{ vs } 5} = 0.04$, $P_{10 \text{ vs } 5} = 0.053$). Length of unsuberized roots was not affected by the time × temperature interaction ($P = 1.0$),

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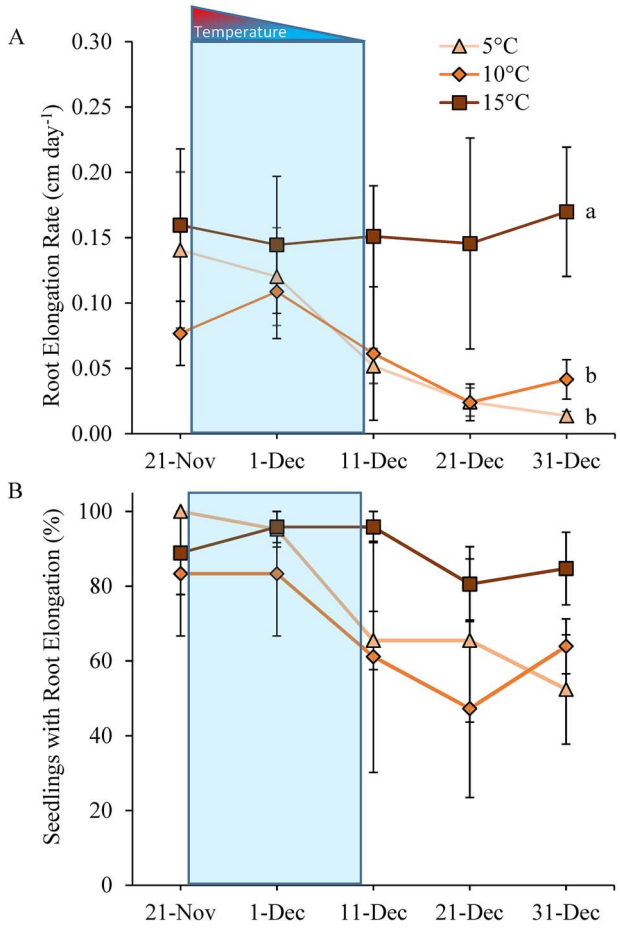

Figure 4. Soil temperature effects on root elongation rate during the transition from fall to winter. (A) Root elongation rate and (B) proportion of seedlings with growing roots. Blue shading indicates the period during which soil temperatures were decreased. Symbols and error bars represent means ± SE. Where there was a significant soil temperature effect, like letters next to symbols indicate means that are not different for the analysis excluding pre-chilling dates.

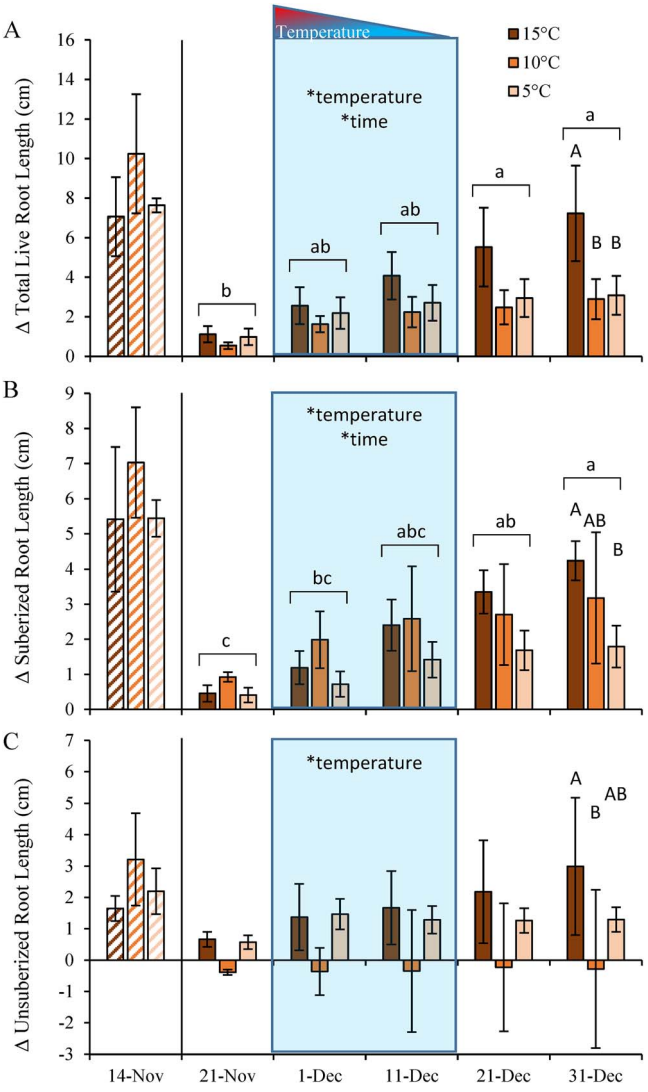

Figure 5. Change (Δ) in root lengths for each soil temperature level at imaging time points for (A) total live root length, (B) suberized root length (brown roots) and (C) unsuberized root length (white roots). Hatched bars show initial lengths visible in the rhizotron windows. Blue shading indicates the period during which soil temperatures were decreased. Bars represent means ± SE. Main effects that were significant are listed at the top of the graph with an asterisk. Where there were significant effects of time, like letters above brackets indicate times that are not significantly different for Tukey's pairwise comparison. Since there were no temperature × time interactions, where temperature main effects were significant like letters above bars at the final date indicate temperature levels that are not significantly different for Tukey's pairwise comparison.

nor by time main effects ($P = 0.90$), but the temperature main effect was significant ($P = 0.048$) (Figure 5C). The unsuberized roots in 10 °C soil temperature had less new length than in 15 °C ($P_{15\text{ vs }10} = 0.04$, $P_{15\text{ vs }5} = 0.75$, $P_{10\text{ vs }5} = 0.19$).

Root respiration at soil temperature

Root respiration was similarly high (0.30 μmol CO_2 g^{-1} s^{-1}) across soil temperature treatment groups in November ($P = 0.76$), when all seedlings experienced 15 °C soil. However, after winter soil temperatures were imposed, respiration ranged from about 0.04 to 0.15 μmol CO_2 g^{-1} s^{-1}, and was significantly affected by soil temperature in December and January ($P = 0.001$ and $P = 0.002$, respectively). During this time, root respiration rates were 27% and 64% lower in 10 and 5 °C, respectively, than in 15 °C soil.

Root respiration at fixed temperature

In addition to the above respiration measurements, roots from all soil temperatures were excised and measured at both 5 and 15 °C to compare them under like conditions. There was no interactive influence of the soil temperature level from which roots were sampled and the temperature at which roots were held during measurement of root respiration when measured at fixed temperatures in November, December and January ($P = 0.52$, $P = 0.81$, $P = 0.92$). The temperature level of the soil from which roots were taken had no effect on root respiration ($P_{Nov} = 0.25$, $P_{Dec} = 0.84$, $P_{Jan} = 0.93$). Instead, root respiration was completely dependent on the temperature roots experienced immediately during the measurement, and respiration was 44–55% lower when measured at 5 °C than at 15 °C ($P_{Nov} = 0.006$, $P_{Dec} < 0.0001$, $P_{Jan} < 0.0001$) (Figure 6B and C). Soil temperature did not affect Q_{10} values ($P_{Nov} = 0.21$, $P_{Dec} = 0.28$, $P_{Jan} = 0.89$) (Figure 6D).

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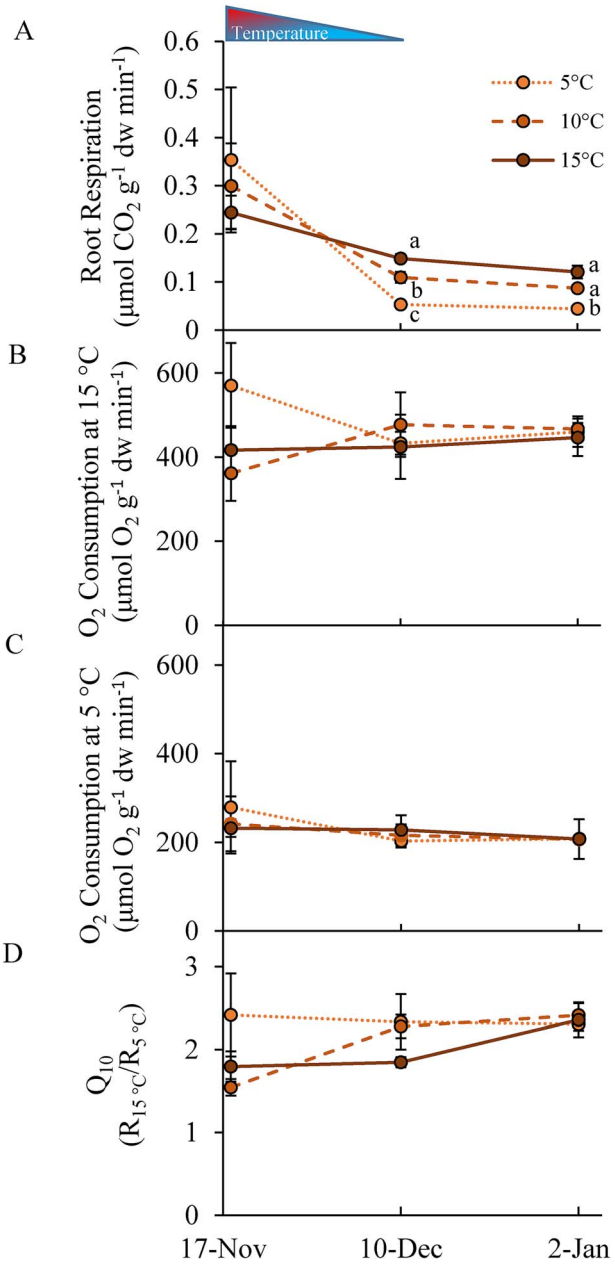

Figure 6. Willow oak seedling root respiration and Q_{10} values during the transition from fall to winter. (A) Respiration of tap and lateral root tissue measured at treatment soil temperature as CO_2 efflux. Lateral root respiration measured as O_2 consumption at (B) 15 °C and (C) 5 °C regardless of soil temperature level. (D) Q_{10} values calculated from O_2 consumption at 15 and 5 °C before (18 November) and after (10 December, 2 January) soil temperatures were ramped down. The blue triangle shows the duration of the ramp-down period. For a given date, significance between temperature levels is indicated by different letters next to symbols.

Discussion

Growth of willow oak seedling roots appeared to respond directly to soil temperature rather than aboveground cues, but may have been dependent on continued photosynthesis. Although stem elongation stopped by 18 November, some root elongation continued in about 50–85% of seedlings through December. The 5 and 10 °C soil temperatures resulted in reduced root elongation compared with 15 °C. During the period when soil temperatures had reached their winter minimums, root elongation rate in 5 °C was 19% of that observed in 15 °C soil, and root elongation rate in 10 °C soil was 27% of that observed in 15 °C soil. Previous work reported temperature thresholds for root growth between 2 and 8 °C depending on species (Teskey and Hinckley 1981, Kuhns et al. 1985, Lopushinsky and Max 1990, Repo et al. 2021). Records from the native range of willow oak in the LMAV over the past 5 years indicate a winter month (December to February) average soil temperature of around 10 °C (NRCS SCAN 2021). Because roots continued to grow at 10 °C and even 5 °C in our study, it is likely that willow oak seedling roots in a natural setting in the LMAV will continue to grow to some degree during winter, assuming ideal moisture conditions.

Photoperiod and air temperature did not appear to be primary factors influencing root elongation during the transition from fall to winter. Previous studies have shown that shortening photoperiod, followed by decreasing air temperatures, initiates dormancy induction in shoots (Romberger 1963, Downs and Bevington 1981, Olsen 2010), including leaf senescence-related signaling, chromatin remodeling and major metabolic shifts that lead to protein degradation and decreasing photosynthetic performance beginning days or even multiple weeks before leaf chlorophyll degradation is visibly apparent (Sample and Babst 2019, Koller et al. 2020, Li et al. 2020, Lu et al. 2020, Fataftah et al. 2021, H.-L.Wang et al. 2021, Lihavainen et al. 2023). However, elongation of willow oak roots that were kept at a constant 15 °C soil temperature did not decline in late fall and early winter, despite a short photoperiod and low air temperatures. Rather, reduced root elongation in 10 °C soil indicated that root elongation was soil temperature-limited below 15 °C. The soil environment in a forest is somewhat uncoupled from the aboveground microclimate, in that soil temperatures tend to cool more slowly in the fall and are buffered from the diurnal extremes that are prevalent in the atmosphere. Studies of potted plants with no soil temperature control might not give an accurate depiction of the physiology of roots that are growing outdoors in soil, because the temperature of soil in pots probably tracks closer to air temperature than to soil temperature found in nature. Although photoperiod and air temperature are key regulators of shoot dormancy, our results suggest that these factors alone are not good predictors of root elongation in willow oak seedlings when air and soil temperatures are uncoupled, as they are in nature.

Root growth during winter was likely supported by carbohydrates translocated from leaves, because most seedlings (95%) retained at least some green leaves. Water oak (*Q. nigra* L.), which is found in similar forest types as willow oak, maintains photosynthesis until leaves senesce in late winter (Goodman et al. 2009). Thus, green willow oak leaves persisting through our study were likely still photosynthetically active. Seedlings growing in 15 °C soil maintained a constant rapid root elongation rate through December, but root growth was limited by soil temperatures below 15 °C. The reduced root elongation rates in 10 and 5 °C were apparent by 10 December, 10 days before the seedlings showed early signs of leaf senescence on 20 December. Even in early January only the first flush of leaves senesced for most seedlings, while the second flush of leaves stayed green. This further supports that photoassimilate supply to roots was sustained, and that the factor limiting root elongation was soil temperature. The constant growth at 15 °C may seem surprising if root

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elongation is dependent on continued supply of photoassimilates from leaves, because photosynthesis is expected to decrease in response to cold air temperatures and lower light during winter months (Kolari et al. 2007). However, the temperature and light environments changed little (less than 15%) between late November and late December in our study. Therefore, it is likely that photosynthesis changed very little between late November and late December, and the sustained photoassimilate supply maintained root elongation rates. Alternatively, root carbohydrate reserves could have been utilized to partially support continued root elongation in 15 °C soil. In older deciduous trees, root growth may continue after leaves senesce, but their growth rates tend to decrease (Kuhns et al. 1985, Tierney et al. 2001, Radville et al. 2016, Moorberg et al. 2017), presumably influenced by a decreasing carbohydrate influx and decreasing soil temperatures that often coincide with or follow soon after leaf fall. In unusually cold years when willow oak seedlings lose all leaves to senescence or frost kill, root growth will likely decrease as it does in older trees. Conversely, in winters that are unusually warm, willow oak seedlings may have greater than normal root growth.

Actively growing root tips are unsuberized (i.e., white tips) and the normal developmental program includes suberization in root tissues basal to the elongation zone (Machado et al. 2013, Campilho et al. 2020). As such, the increase in suberized root length that we observed over time indicates that suberization continued during winter in willow oak seedlings. Under certain environmental stressors, suberization may occur more quickly and closer to root tips (Enstone et al. 2002), which is associated with tolerance to stresses, including osmotic stress and hypoxia that is associated with flooding (Enstone et al. 2002, Le Provost et al. 2016). If suberization occurs closer to root tips, there should be a decrease in unsuberized root length. However, the abundance of unsuberized root tissue (i.e., white roots) in willow oak seedlings did not decrease as we hypothesized, and it did not track tightly with growth rates. For example, unsuberized root length remained the same in the 10 °C soil, but increased for seedlings grown in 5 or 15 °C soil, even though root elongation rates were lowered by both the 5 and 10 °C soil temperatures. White fleshy root tissues may be present during winter even in the absence of detectable root growth (Romberger 1963). Our results that willow oak seedling roots do not become suberized closer to the tips (i.e., that unsuberized root length did not decrease) suggests that there may be no gain of suberin-based resistance to stress near root tips during winter, and there may even be a greater portion of the root tip length that is unsuberized. Nonetheless, induction of other stress resistance mechanisms during winter cannot be ruled out.

Similar to root growth, willow oak seedling root respiration declined during winter when soil temperature was decreased, and the evidence suggests that this was not active acclimation. Metabolic acclimation of roots cannot be determined from respiration measurements collected over a range of ambient soil temperatures because sampling of this nature could be confounded by direct effects of temperature on biochemical reaction rates. However, acclimation of respiration rate can be identified if roots are removed from their environment and respiration is measured at controlled high and low temperatures. In our study, a lack of root acclimation to winter soil temperatures was supported by observations that root respiration and Q_{10} values, when measured at controlled temperatures (5 and 15 °C), were similar for seedlings taken from each soil temperature level (5, 10 and 15 °C). Similarly, sugar maple root respiration is driven by soil temperature and shows no acclimation as temperatures decrease into the winter (Zogg et al. 1996, Burton and Pregitzer 2003). In aspen, which ranges farther north, however, roots appear to acclimate to winter, with lower root respiration rates measured at the same temperatures when shoots are dormant compared with when shoots are growing (Desrochers et al. 2002). This difference may be due to the fact that aspen grows where temperature beneath the soil surface can routinely fall below freezing for 100 or more days (NRCS SCAN 2021), whereas sugar maple typically experiences short periods of near-freezing sub-surface soil temperatures only occasionally in northern limits of its range (NRCS SCAN 2021). It is possible that acclimation may not be necessary unless roots have to survive prolonged freezing temperatures. Because soils in the LMAV rarely experience sub-freezing temperatures (NRCS SCAN 2021), acclimation or dormancy may be unnecessary for willow oak roots to survive winter. Although willow oak seedling roots exhibit neither dormancy nor acclimation of respiration, the decreased growth rate and respiration rate of roots in cool temperature soils might reduce the susceptibility of willow oak roots to winter flooding by reducing O_2 demand and energy demands, which may help to avoid depletion of carbohydrate reserves, and also minimize accumulation of the toxic byproducts of anaerobic respiration.

Finally, soil temperature appeared to influence leaf senescence. Seedlings in relatively cooler soil showed visible signs of senescence earlier than those in warmer soil. This is interesting because our current understanding is that the environmental drivers of autumn senescence are primarily aboveground factors, including decreasing photoperiod and air temperature. First, decreasing photoperiod acts via the photoreceptor phytochrome to initiate bud set and pro-senescence (Olsen et al. 1997), and differences in timing appear to be under genetic control (Lee et al. 2003, Fracheboud et al. 2009, Wang et al. 2019, Lu et al. 2020). Then, declining air temperatures can accelerate senescence via a complex interaction of signaling mechanisms including the abscisic acid and ethylene signaling pathways, triggered by a transient decrease of free salicylic acid concentration and downregulation of salicylic acid signaling (Fracheboud et al. 2009, Fataftah et al. 2021, Lihavainen et al. 2023, Wang et al. 2023, but also see Lee et al. 2020, Zhang et al. 2020, C.Wang et al. 2021, Huang et al. 2022, Ito et al. 2022). In addition to phytohormones, elevated sugars can stimulate senescence (Wingler et al. 2006, 2009, 2012, M.Wang et al. 2021). Eliminating roots as sinks by girdling stems can also accelerate leaf senescence (Lihavainen et al. 2021). The decrease in willow oak root elongation and respiration that we observed in the two coldest soils may have reduced sink strength and, consequently, sugar translocation to roots. Therefore, we hypothesize that decreased transport of sugars from leaves to roots at a time when stems were not growing and could not act as an alternate sink may have resulted in perturbation of leaf carbohydrate homeostasis and downregulation of photosynthesis (Sloan et al. 2016), both of which may stimulate leaf senescence (Wojciechowska et al. 2018, M.Wang et al. 2021, Lihavainen et al. 2023). This would provide a possible mechanism by which soil temperature may have influenced leaf senescence. It follows logically that leaf senescence, in turn, might feed forward to further reduce root elongation.

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Conclusions

Measurements of willow oak seedlings exposed to winter soil temperatures typical of the native range for this species did not yield evidence of root dormancy. In fact, most seedlings exhibited root growth through December after soil temperatures were reduced to their lowest levels, although cooler soil temperatures slowed root elongation. Sustaining root elongation during winter may be dependent on the ready supply of carbohydrates from photosynthesis because willow oak seedlings retain photosynthetically active leaves during the winter. Leaf retention during the winter is common in willow oak seedlings but not saplings or mature trees. An unexpected finding of this work that warrants additional research was that leaf senescence progressed quickest for seedlings experiencing the coldest soil and slowest for those experiencing the warmest soil. This suggests that the role of soil temperature may be often overlooked in physiological studies of leaf senescence.

Like root elongation, root respiration rates declined in relatively cold soil, apparently due to the direct effect of temperature on the rate of biochemical reactions. We found no evidence that respiration rates decreased because of acclimation to cold soil, as is the case for species ranging to higher latitudes that experience extended periods of freezing temperatures below the soil surface. This finding, coupled with our observations of root growth, suggests that winter root dormancy and/or acclimation of root respiration is not required for deciduous tree species ranging through mid- to lower-latitudes of the temperate zone. We propose that root elongation of willow oak seedlings in their native range will continue during the winter months, varying with soil temperature, if soil temperature is at least 5 °C and soil moisture is conducive to growth. If seasonal flooding begins before soil temperatures cool and root growth rates decline, it seems likely that willow oak seedlings may experience greater damage from flooding. Furthermore, we put forward that ongoing root elongation during winter may have other physiological and ecological benefits or consequences for the seedling that should be explored in future studies.

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Author contributions

B.A.B. and E.S.G. conceived and developed the study. J.M.K. performed and analyzed the respiration measurements, J.T.C. performed and analyzed the rhizotron pot growth study and E.S.G. collected stem morphology and meteorological data. B.A.B., J.M.K. and J.T.C. conducted statistical analyses with input from M.M.B. J.M.K., J.T.C., B.A.B. and E.S.G. contributed to interpretation. J.M.K. and J.T.C. contributed to the first draft. J.M.K., B.A.B. and E.S.G. revised the manuscript, with input from all authors.

Conflict of interest

None declared.

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Data availability

All data generated or analyzed for this study are included in this published article.

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