Physiological responses of germinant *Pinus palustris* and *P. taeda* seedlings to water stress and the significance of the grass-stage

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Abstract

Loblolly pine (*Pinus taeda* L.) has gradually replaced longleaf pine (*P. palustris* Mill.) across much of the coastal southeastern United States. Given recent interest in the regeneration of longleaf pine ecosystems, understanding how these two species will respond to drier conditions associated with climate change will be critically important. We conducted a progressive water-stress experiment on germinants of *P. palustris* and *P. taeda* over the course of 9 weeks, measuring gas exchange, allometry, water relations, and tracheid anatomy. *P. palustris* developed more overall biomass, had wider tracheids, allocated more of its biomass into leaf tissue, and maintained a narrower safety margin against hydraulic failure than *P. taeda*. Both species reduced leaf gas exchange as water stress increased, though *P. palustris* experienced earlier and greater declines in photosynthetic rates than *P. taeda*. The greater aboveground growth and narrower hydraulic safety margin observed in *P. palustris* represents a trade-off between growth and hydraulic safety and is likely related to the species' defensive strategy against fire. We suggest that this trade-off may limit *P. palustris* germinant regeneration on sandier, xeric sites as climate change intensifies.

Keywords: Longleaf pine savanna; *Pinus palustris*; *P. taeda*; drought; restoration; leaf gas exchange; hydraulic safety margins

1. Introduction

Longleaf pine (*Pinus palustris* Mill.) once ranged across much of the southeastern United States, historically covering a vast landscape along the Atlantic and Gulf Coastal Plains, from Virginia to eastern Texas (Frost, 1993). However, land clearing for lumber and agricultural production has left less than 3% of longleaf ecosystems remaining, with old growth stands largely found in isolated fragments (Frost, 1993; Kush and Meldahl, 2000). In many logged areas, longleaf pine stands have been removed and replaced with loblolly pine (*P. taeda* L.), which is faster-growing, more shade-tolerant, easier to regenerate, and therefore a more economically viable crop than *P. palustris* (Landers et al., 1995). The replacement of *P. palustris* with *P. taeda* has also been aided by the suppression of fire, which once maintained the open understory of longleaf pine savannas and promoted successful longleaf seedling regeneration (Kush et al., 2004). Together, these trends have resulted in a substantially changed landscape.

Considering most of the historic longleaf pine savanna range has been lost, as well as the unique understory assemblages housed within and ecosystem services provided by these ecosystems (Walker and Peet, 1983), natural regeneration of longleaf pine is a primary interest of both conservationists and ecologists. However, rising temperatures and changing precipitation patterns associated with global climate change might create new challenges for longleaf pine conservation and regeneration. On a global scale, heat waves are becoming more intense, frequent, and long-lasting (Meehl and Tebaldi, 2004). Regionally, the southeastern United States has experienced higher interannual rainfall variability over the last 30 years, including both extremely wet and dry summers, as well as three major drought events (Wang et al., 2010). Drought stress is the leading cause of germinant mortality in pine forests of the southern United States. Mortality rates often exceed 90% among germinants when exposed to drought, though drought-related mortality diminishes as seedlings age beyond the first year (Germino et al., 2002; Conlisk et al., 2017).

Though mature *P. palustris* is generally considered to be more drought-tolerant than *P. taeda*, whether this is also true of recent germinants is unclear. Immature *P. palustris* exhibit a unique "grass stage" in which the seedling does not develop a stem, but rather accumulates a dense assemblage of true needles that surround and physically protect the apical bud from fire (Figure S1, Outcalt, 2000). With longleaf pine savannas having an average fire interval of about two years (Stambaugh et al., 2011), this strategy allows *P. palustris* to cope with frequent fire at early life stages (Shappell and Koontz, 2015). However, this growth strategy may concomitantly increase the susceptibility of *P. palustris* germinants to drought because greater leaf area could increase the hydraulic demand of the plant (i.e., increased whole-plant transpiration) relative to water supply (i.e., root area, McDowell et al. 2008). To increase water supply for such large investments into leaf tissue, *P. palustris* seedlings (≥ 5 years old) in the grass stage typically have high root to shoot ratios compared to mature trees (Samuelson et al. 2017). However, it is unknown whether *P. palustris* germinants follow this strategy, as emergent seedlings are often shallow-rooted and exhibit unique physiological responses to their environment (Brodersen et al. 2019). For instance, germinants often prioritize rapid growth over structural integrity and stress tolerance in order to ensure successful establishment, even under stressful conditions such as drought (Bond, 2000). Indeed, germinants of other pine species have been found to be both especially vulnerable to drought and to maintain photosynthesis as water potentials decline (Augustine and Reinhardt, 2019). This response is atypical of mature pines, which typically reduce photosynthesis via stomatal closure to maintain high water potentials (West et al., 2007).

With this, the unique physiology and growth patterns of germinants may reduce their regeneration during drought conditions. Examining variation in germinant responses to changes in water availability among different pine species can therefore better inform *P. palustris* regeneration efforts and help predict how longleaf ecosystems will respond to a changing climate. In this study, we compared the physiological responses of *P. palustris* and *P. taeda* seedlings under increasing water stress in order to better understand how each species may respond to drought. Specifically, we tested how gas exchange, water relations, allometry, and tracheid anatomy are affected by progressively lower levels of soil moisture. We chose *P. taeda* for comparison due to the high rate at which it has replaced *P. palustris* in forests of the southeastern United States. Given its unique grass stage, in which rapid accumulation of leaf area is prioritized (Outcalt, 2000), we predicted that *P. palustris* would display higher physiological functioning and greater relative growth rates than *P. taeda* when grown under well-watered conditions. Along with this, due to the potential hydraulic demands associated with greater investment in leaf area (McDowell et al. 2008), we predicted that *P. taeda* will show greater tolerance of water stress than *P. palustris*, as exhibited by continued physiological activity under reduced water availability.

2. Materials and Methods

2.1. Plant materials and microclimate

We conducted this study in a greenhouse at the University of Wisconsin – Madison over the course of 9 weeks during September-December 2018. *P. taeda* and *P. palustris* seeds were obtained from Sheffield's Seed Company (Locke, NY, USA). Before planting, the seeds were rinsed for 10 minutes in a 3% hydrogen peroxide solution, soaked in water for 24 hours, then cold stratified for 28 (*P. taeda*) and 21 (*P. palustris*) days at 4 °C. Immediately following stratification, seeds were planted in 175 ml Ray Leach cone-tainers (Stuewe and Sons Inc, Tangent, OR, USA) using a 50:50 peat moss and vermiculite medium. Cone-tainers were randomly separated into water-stressed and control (well-watered) treatment groups for both species, with 196 individuals of each species in the two treatments. Cone-tainers were placed into cone-tainer racks and arranged in a randomized block design, in which cone-tainer racks were divided into groups depending on both species and watering treatment. The seeds germinated after approximately seven days and were then watered to field capacity for an additional two weeks prior to the start of the water-stress treatment. Throughout the experiment, germinants were exposed to a 16 h photoperiod. Five randomly selected individuals from each species and treatment combination were sampled each week.

The water-stress treatment consisted of a step-wise dry-down, with the germinants being exposed to five sequential two-week-long watering regiments where the germinants received 80, 60, 40, 20, and 0% of the water lost through soil evaporation during the previous 24 hours, respectively. The water lost through soil evaporation was calculated once a week by weighing five randomly selected cone-tainers after being watered to field capacity and then again 24 hours later. These five cone-tainers were filled with soil but did not have germinants. Control individuals were watered to field capacity daily. Sampling began 16 days after planting. Water-stressed individuals were not measured until the third week of sampling, at which point they had been exposed to the water-stress treatment for a week.

2.2. Gas exchange

Leaf gas exchange (photosynthesis, transpiration, and stomatal conductance) was measured using a portable photosynthesis system (model LI-6400XT, Li-Cor Biosciences, Lincoln, NE, USA) with a 2 cm^2 fluorometer chamber. The ambient CO_2 concentration was set to 400 ppm, the flow rate to 300 $\mu mol\ s^{-1}$, and the chamber light source to 1000 $\mu mol\ m^{-2}\ s^{-1}$ for all measurements. We collected measurements between 08:30 h -12:00 h each week. We chose seedlings (n = 5) with sufficient leaf area to fit into the fluorometer chamber. Multiple needles were allowed to stabilize in the chamber for approximately five minutes before recording a single measurement. All measurements are reported on a silhouette leaf area basis (Smith et al., 1991), which was determined by processing digital photographs of leaves within the chamber using ImageJ (Scion Co., Frederick, MD, USA). Instantaneous water-use efficiency (WUE) was calculated by dividing photosynthesis by transpiration.

2.3. Water relations

Immediately after gas exchange measurements were recorded, germinants were removed from cone-tainers and cut at the base of the shoot. They were then placed in plastic bags with damp paper towels and stored in a dark cooler, allowing the whole-seedling (shoot + needles) water potential to equilibrate for approximately 30 minutes. Germinants were then brought to the lab, where each individual was placed in a Scholander pressure chamber (PMS Instrument Company, Albany, OR) and processed for midday whole-seedling water potential (Ψ_{md}).

Pressure-volume curves were generated to determine the turgor loss point (Ψ_{tlp}) of five individuals from each species. The Ψ_{tlp} was calculated based on the methods described by Hammel and Tyree (1972). Roughly two months after sampling for gas exchange had finished, healthy individuals of both species grown under well-watered conditions were randomly selected for sampling. Individuals were watered and covered with plastic bags to suppress nighttime water loss the night before sampling to allow them to hydrate. The following morning, each germinant was cut at the base of its shoot and stem water potential was measured with a Scholander pressure chamber. The mass of the sample was measured immediately after the water potential was determined. This process of recording paired mass and water potential values was repeated over approximately two hours for each germinant as it bench-dried. The germinants were not rehydrated prior to the analysis to prevent any potential artefacts that may arrive from the rehydration of the shoots (Meinzer et al. 2014), but initial water potential values were all greater than -0.2 MPa. To assess the physiological relevance of the Ψ_{md}, hydraulic safety margins (Ψ_{sm}) were calculated for each species by subtracting Ψ_{tlp} from the mean Ψ_{md} measured on each sampling date throughout the dry-down experiment. We acknowledge that using Ψ_{tlp} values from older control germinants may not represent the true hydraulic safety margins of the earliest stages of ontogeny and the drought treatment, though ontogenetic variations in cell-water relations have yet to be explored and pine seedlings have been shown to show little plasticity in Ψ_{tlp} when exposed to drought (Kerr et al. 2015).

2.4. Allometry and growth

After weekly midday water potentials were recorded, the roots, stems, and leaves of each germinant were separated and weighed to determine wet mass. Needles were also photographed and processed using ImageJ to determine leaf area. After the wet mass was obtained, roots and leaves were placed in separate coin envelopes, dried at 68˚ C for at least 24 hours, and weighed again to determine dry mass. From these measurements, the leaf mass ratio (the percent of the seedling's biomass that comes from leaf tissue) was calculated. Stems were placed in 50% ethanol and stored for anatomical measurements. The relative growth rate (RGR) for the whole experiment was calculated using Equation 1: $RGR = (\overline{\ln(m_9)} - \overline{\ln(m_3)})/6$, where $\overline{\ln(m_9)}$ and $\overline{\ln(m_3)}$ are the mean of the natural log transformed plant masses at week 9 and 3, respectively (i.e., the beginning and end of the water-stress treatment period; Hoffman and Poorter, 2002).

2.5. Tracheid and sapwood anatomy

Tracheid mean hydraulic diameter, double wall thickness, wall reinforcement, and sapwood area were measured on 5 stems from each species*treatment combination collected during the last week of the dry-down experiment. Five cross-sections were taken from each stem and mounted on microscope slides using glycerin. Images of the cross-sections were taken using a Nikon Eclipse Ci microscope with a Nikon DS-Fi2 camera (Nikon Inc., Melville, NY, USA). Three groups of tracheids from separate cross sections were analyzed, with a total of 100 tracheids from each seedling. Sections were located near the cambium. We used ImageJ to measure the area of individual tracheids, which was then used to calculate diameter assuming the conduits were square. Mean hydraulic diameter (b) was calculated for each segment using Equation 2: $b = \Sigma d^5/\Sigma d^4$, where d is the diameter of an individual conduit. Wall thickness was measured on 30 tracheids of each stem whose diameter measured within 10% of the mean tracheid hydraulic diameter. Cell wall reinforcement was calculated based on the methods described by Hacke et al. (2001). Stem area was calculated by measuring the diameter of the stem using a digital electronic caliper (Tritan Products Inc., North York, ON, Canada), and was then used to calculate leaf area to stem area ratios. For *P. palustris*, the stem was considered to be the portion of the shoot just above the root collar.

2.6. Statistics

Linear mixed models were used to analyze the direct and interactive relationships between the fixed effects (watering treatment, species, and day of experiment) and the physiological and anatomical response variables that were measured throughout the entire experiment. In these models, the cone-tainer block was included as a random factor. Because part of the analysis was a time series analysis, model analysis was performed to test for autocorrelation, and models were selected by minimizing the Akaike's Information Criterion metric. A two-way analysis of variance (ANOVA) was used to analyze the individual and interactive effects of treatment and species on mean tracheid hydraulic diameter, wall thickness, wall reinforcement, stem area, and leaf area to stem area ratios, and a one-way ANOVA was used to analyze the effect of species on turgor loss point.

All statistical analyses were conducted in R version 3.5.0 (R Core Team 2018). Mixed models were performed using the 'nlme' package (Pinheiro et al. 2018) in which the 'acf' function was used to test for weekly autocorrelation. For all data analyzed using the mixed models, post hoc pairwise comparisons (Tukey HSD test, 'lsmeans' package, Lenth and Love 2017) were performed to identify differences between treatment pairs and the week of drought. For all data analyzed using the mixed model, post hoc pairwise comparisons (Tukey HSD test, 'lsmeans' package, Lenth and Love, 2017) were performed to identify differences between treatment pairs and the week of the treatment. An α value of less than 0.05 was considered statistically significant, but we also discuss biological trends throughout the Results and Discussion where $\alpha < 0.10$.

Table 1. Mixed model and ANOVA results showing the effects of species (*P. palustris* and *P. taeda*), treatment (control and water-stressed), week, and their interactions on photosynthesis (A_{net}), stomatal conductance (g_s), transpiration (E), water-use efficiency (WUE), leaf dry mass, root dry mass, leaf mass ratio, leaf area, leaf mass per area (LMA), midday water potential (Ψ_{md}) and hydraulic safety margins, turgor loss points (Ψ_{md}), double wall thickness (t), hydraulic diameter (b), conduit reinforcement ($(t/b)^2$), cone-tainer water content, stem cross-sectional area (SA), and leaf area to stem area ratio (LA:SA). Shown are degrees of freedom (df), F-statistics

(F), and P-values (P) for each parameter. Bold text signifies significance at an $\alpha = 0.05$. Italic text signifies significance at an $\alpha = 0.10$.

	A_{net}			g_s			E		
Effects	df	F	P	df	F	P	df	F	P
Treatment	1	0.80	0.372	1	6.26	**0.012**	1	7.43	**0.006**
Species	1	10.37	**0.001**	1	24.83	**<0.001**	1	3.57	*0.059*
Week	1	35.66	**<0.001**	1	62.98	**<0.001**	1	39.10	**<0.001**
Treatment:Species	1	2.77	*0.096*	1	5.91	**0.015**	1	3.73	*0.053*
Treatment:Week	1	2.32	0.127	1	23.58	**<0.001**	1	6.01	**0.014**
Species:Week	1	1.05	0.305	1	0.04	0.836	1	0.75	0.385
Treatment:Species:Week	1	0.58	0.448	1	0.2142	0.643	1	0.35	0.556
	WUE			Leaf Dry Mass			Root Dry Mass		
Effects	df	F	P	df	F	P	df	F	P
Treatment	1	13.10	**<0.001**	1	0.90	0.344	1	4.13	**0.042**
Species	1	1.83	0.176	1	2349.82	**<0.001**	1	98.62	**<0.001**
Week	1	14.74	**<0.001**	1	1342.16	**<0.001**	1	201.14	**<0.001**
Treatment:Species	1	3.17	*0.075*	1	1.97	0.160	1	1.61	0.204
Treatment:Week	1	33.42	**<0.001**	1	0.42	0.515	1	7.52	**0.006**
Species:Week	1	2.48	0.115	1	439.03	**<0.001**	1	19.56	**<0.001**
Treatment:Species:Week	1	0.1799	0.671	1	0.65	0.422	1	0.66	0.418
	Leaf Mass Ratio			Leaf Area			LMA		
Effects	df	F	P	df	F	P	df	F	P
Treatment	1	0.202	0.653	1	0.3431	0.558	1	0.149	0.699
Species	1	69.241	**<0.001**	1	172.805	**<0.001**	1	9.657	**0.002**
Week	1	1.9728	0.160	1	163.111	**<0.001**	1	0.0027	0.958
Treatment:Species	1	0.1801	0.671	1	0.02	0.890	1	0.8013	0.371
Treatment:Week	1	2.0044	0.157	1	1.6436	0.200	1	0.0802	0.777
Species:Week	1	1.4632	0.226	1	45.7399	**<0.001**	1	8.3406	**0.004**
Treatment:Species:Week	1	0.0738	0.786	1	0.0049	0.944	1	0.2593	0.611
	ψ_{md}			Safety Margins			ψ_{tlp}		
Effects	df	F	P	df	F	P	df	F	P
Treatment	1	25.436	**<0.001**	1	9.72	**0.002**	1	15.23	**<0.001**
Species	1	12.402	**<0.001**	1	178.71	**<0.001**			
Week	1	15.599	**<0.001**	1	4.05	**0.044**			
Treatment:Species	1	5.304	**0.021**	1	2.47	0.116			
Treatment:Week	1	29.01	**<0.001**	1	8.36	**0.004**			
Species:Week	1	0.2486	0.618	1	0.00	0.974			
Treatment:Species:Week	1	2.0544	0.152	1	14.87	**<0.001**			
	t			b			$(t/b)^2$		
Effects	df	F	P	df	F	P	df	F	P
Treatment	1	12.39	**<0.001**	1	34.33	**<0.001**	1	35.11	**<0.001**
Species	1	1.44	0.25	1	0.29	0.60	1	0.84	0.37

Week									
Treatment:Species	1	7.47	**0.01**	1	2.37	0.14	1	0.74	0.40
Treatment:Week									
Species:Week									
Treatment:Species:Week									

	Cone-tainer Water Content			*SA*			*LA:SA*		
Effects	df	F	P	df	F	P	df	F	P
Treatment				1	199.85	**<0.001**	1	24.61	**<0.001**
Species	1	39.70	**<0.001**	1	41.23	**<0.001**	1	10.99	**<0.001**
Week	1	45.69	**<0.001**						
Treatment:Species				1	45.15	**<0.001**	1	26.82	**<0.001**
Treatment:Week									
Species:Week	1	29.07	**<0.001**						
Treatment:Species:Week									

3. Results

3.1. Gas exchange

The water-stress treatment affected the two species differently, as we found interactions between treatment and species across all four main gas exchange variables measured ($0.015 < p < 0.097$, Table 1). *P. palustris* showed reductions in gas exchange parameters and an increase in WUE a week prior to *P. taeda* in the water-stress treatment (Figure 1a-h), tracking declines in soil water content (Figure S2). Conversely, gas exchange measured on seedlings in the control treatment remained relatively stable through time (Figure 1a-h). Along with the treatment effect, there was a significant difference between the two species in most gas exchange parameters, with *P. palustris* having higher photosynthesis, stomatal conductance, and transpiration than *P. taeda* (Table 1, Figure 1a-c,e-g).

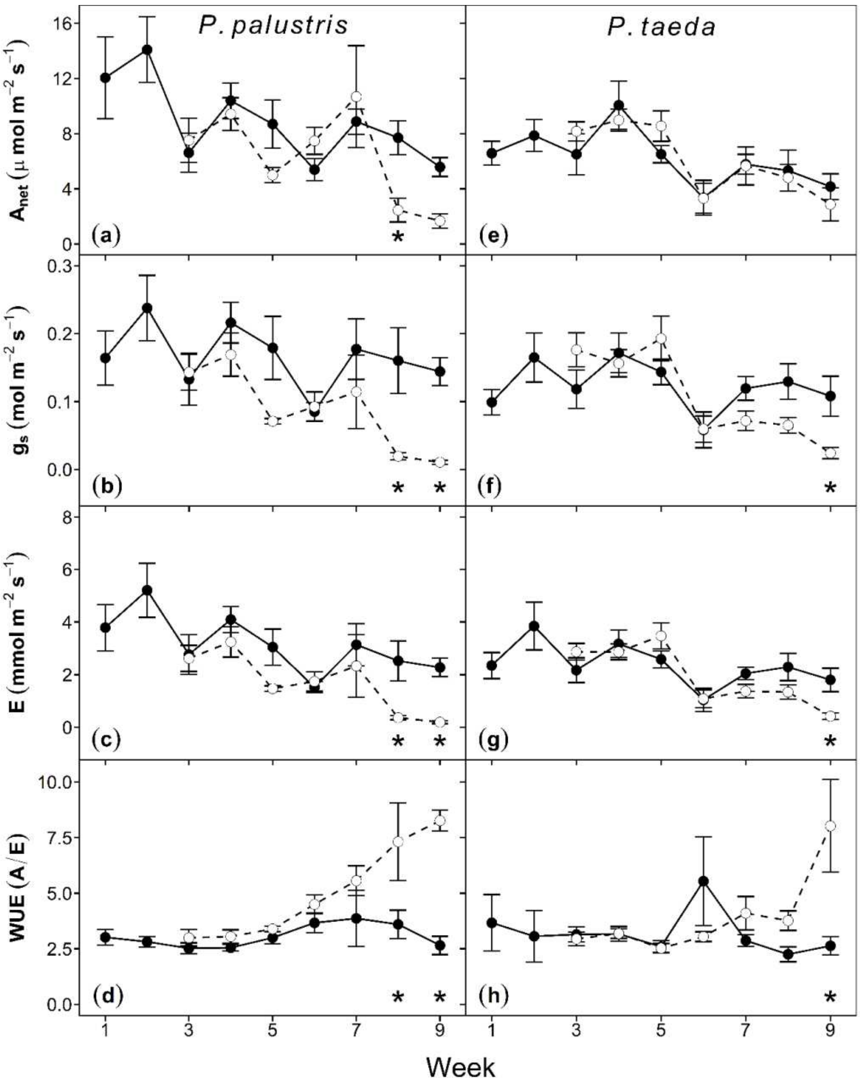

Figure 1. Comparison of area-based gas exchange variables for control (closed circles, solid line) and water-stressed (open circles, dashed line) *P. palustris* (a-d) and *P. taeda* (e-h) seedlings. Data shown are the arithmetic mean ± SE of photosynthesis (A_{net}; a,e), stomatal conductance (g_s; b,f), leaf transpiration (E; c,g), and water-use efficiency (WUE; d,h) for each sample date. Corresponding statistical results are found in Table 1. Asterisks (*) denote significant differences between the control and water-stressed treatments on individual sampling dates based on pairwise comparisons ($p < 0.05$).

3.2. Water relations

There was a significant species difference in midday shoot water potentials (Ψ_{md}, Table 1, Figure 2a,c). *P. palustris* maintained less negative Ψ_{md} than *P. taeda* in the control (Figure 2a,c). There was also a significant interaction between the treatments and species in Ψ_{md} (Table 1), with water-stressed *P. palustris* showing decreases in Ψ_{md}, compared to the control, much earlier than *P. taeda* (week 6 vs. week 8, Figure 2a,c). However, water-stressed *P. taeda* seedlings had lower Ψ_{md} than *P. palustris* by the end of the experiment, exhibiting considerable declines in the final two weeks (Figure 2a,c). The water potential at the turgor loss point (Ψ_{tlp}) was significantly higher (p < 0.01, Table 1) for *P. palustris* (mean Ψ_{tlp} = -0.66 MPa) than for *P. taeda* (mean Ψ_{tlp} = -1.17). As a result, *P. palustris* displayed smaller safety margins (averaged across the whole experiment) against hydraulic failure in both control (Ψ_{sm} = 0.33 MPa) and water-stressed (Ψ_{sm} = 0.10 MPa) seedlings than *P. taeda* (control Ψ_{sm} = 0.67 MPa, water-stressed Ψ_{sm} = 0.59 MPa, Table 1, Figure 2b,d). By the end of the experiment, *P. palustris* seedlings exposed to the water-stress treatment had water potentials below their Ψ_{tlp} (a negative safety margin, Figure 2). Consistent with this predicted loss of turgor, stomatal conductance was negligible in *P. palustris* in the final two weeks, which suggests the guard cells may have lost turgor.

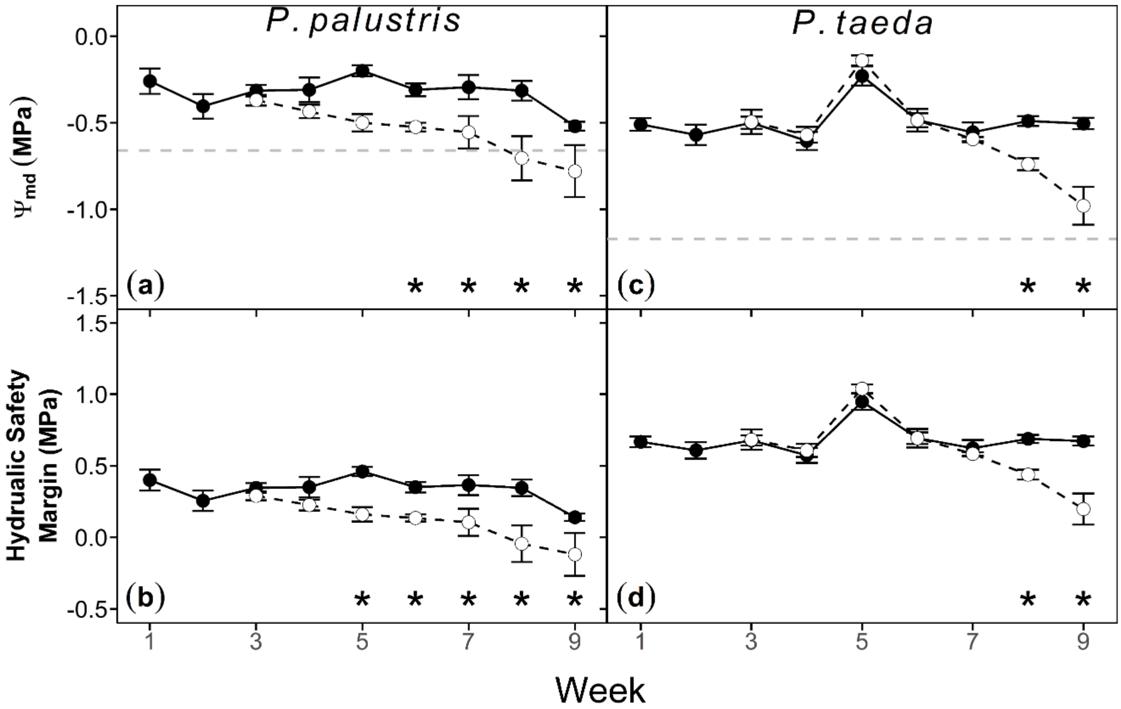

Figure 2. Comparison of water relations for control (closed circles, solid line) and water-stressed (open circles, dashed line) *P. palustris* (a,b) and *P. taeda* (c,d) seedlings. Data shown are the arithmetic mean ± SE of midday whole-seedling water potentials (Ψ_{md}; a,c) and hydraulic safety margins ($\Psi_{md} - \Psi_{tlp}$; b,d) for each sample date. Grey horizontal dashed lines indicate the Ψ_{tlp} of each species. Corresponding statistical results are found in Table 1. Asterisks (*) denote significant differences between the control and water-stressed treatments on individual sampling dates based on pairwise comparisons ($p < 0.05$).

3.3. Allometry and growth

Leaf dry biomass, root dry biomass, and the leaf mass ratio (LMR) were all significantly greater in *P. palustris* than *P. taeda* (all $p < 0.001$, Figure 3a-f, Table 1). Both the leaf dry mass and root dry mass increased in both species and treatments throughout the experiment (Figure 3a,b,d,e). The water-stress treatment did not hinder the addition of leaf dry biomass, but surprisingly reduced root dry biomass (Table 1, Figure 3a,b,d,e). Along with this, both species maintained similar allocation patterns across both treatments, as shown by the lack of an effect of the water-stress treatment on the LMR (Table 1, Figure 3c,f). By the end of the experiment, the mean LMR across both treatments was 66% in *P. taeda*, compared to 80% in *P. palustris* (Figure 3d,h). Relative growth rates (RGR) differed between the species and treatments, with both species having similar RGR in the control (*P. taeda*: 0.036, *P. palustris*: 0.035 g g^{-1} day^{-1}) and *P. palustris* having a higher RGR in the water-stress treatment (*P. palustris*: 0.029 vs. *P. taeda*: 0.025 g g^{-1} day^{-1}). Both species exhibited reduced RGR in the water-stress treatment.

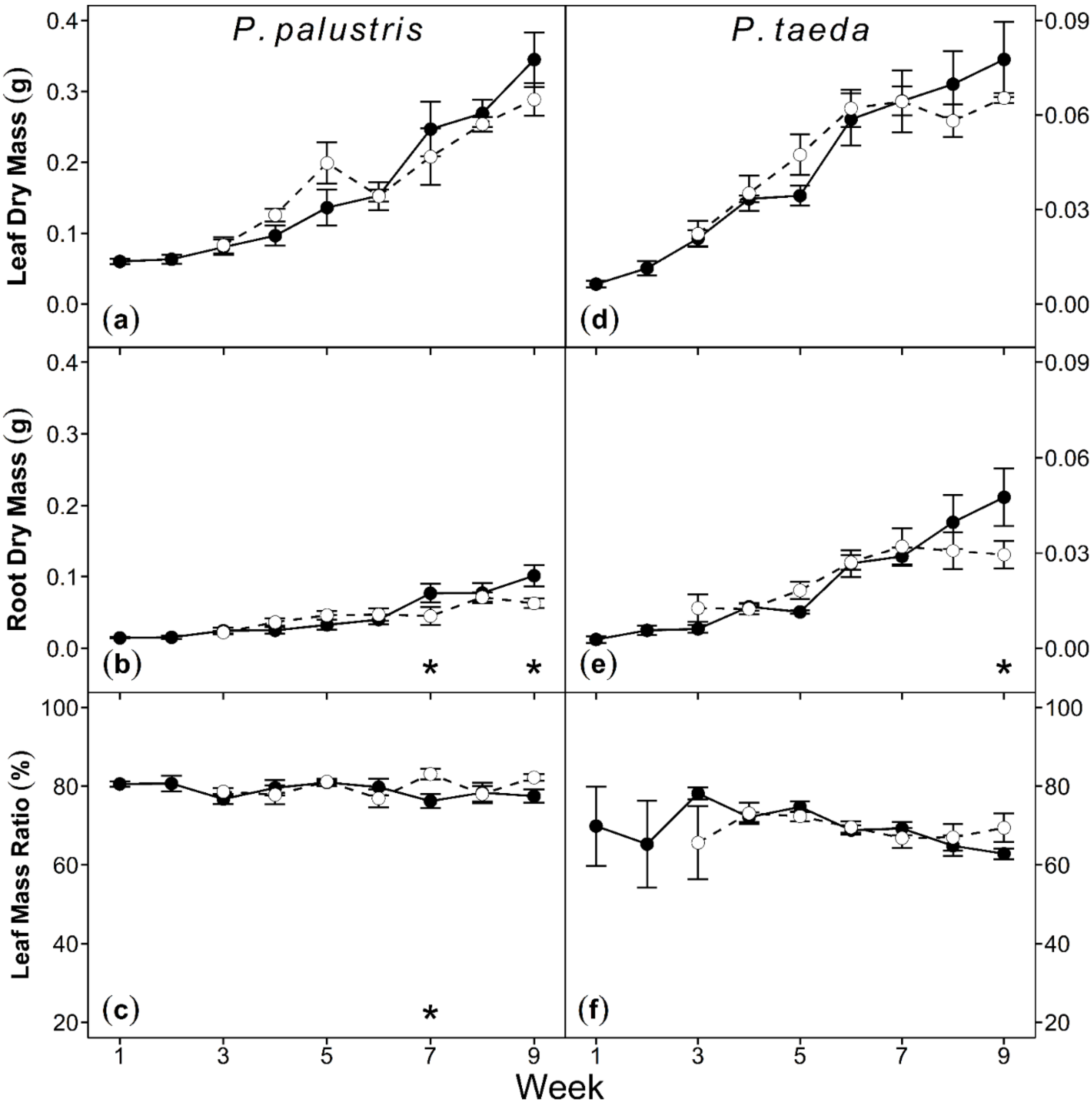

Figure 3. Comparison of biomass variables for control (closed circles, solid line) and water-stressed (open circles, dashed line) *P. palustris* (a-c) and *P. taeda* (d-f) seedlings. Data shown are the arithmetic mean ± SE of leaf dry mass (a,d), root dry mass (b,e), and leaf mass ratio (c,f) for each sample date. Corresponding statistical results are found in Table 1. Asterisks (*) denote significant differences between the control and water-stressed treatments on individual sampling dates based on pairwise comparisons ($p < 0.05$).

Leaf area was significantly greater in *P. palustris* ($p < 0.001$, Table 1), with the final leaf area being 218.9%, and 271.8% greater in *P. palustris* compared to *P. taeda* in the control and water-stress treatment, respectively (Figure 4a,c). The water-stress treatment did not affect leaf area in either species (Table 1, Figure 4a,c). Leaf mass per area (LMA) was significantly greater for *P. palustris* than *P. taeda* ($p < 0.001$, Figure 4b,d, Table 1), but was unaffected by the water-stress treatment in either species (Table 1, Figure 4b,d).

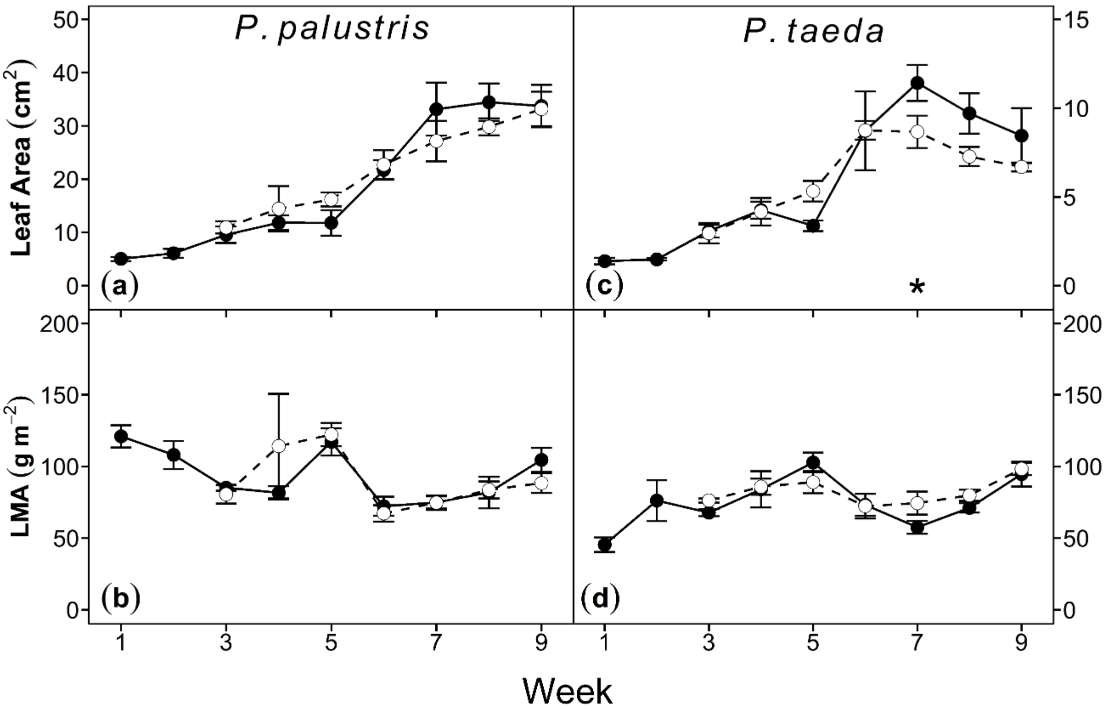

Figure 4. Comparison of leaf variables for control (closed circles, solid line) and water-stressed (open circles, dashed line) *P. palustris* (a,b) and *P. taeda* (c,d) seedlings. Data shown are arithmetic mean ± SE of leaf area (a,c) and leaf mass per area (LMA; b,d) for each sample date. Corresponding statistical results are found in Table 1. Asterisks (*) denote significant differences between the control and water-stressed treatments on individual sampling dates based on pairwise comparisons ($p < 0.05$).

3.4. Anatomy and morphology

Mean tracheid diameter (b) was significantly greater in *P. palustris* (mean = 11.9µm) than in *P. taeda* (mean = 9.6µm, $p < 0.01$, Table 1, 2). Tracheid double wall thickness (t) between conduits, exhibiting approximately the mean hydraulic diameter, differed significantly between species and treatments ($p = 0.01$, Table 1). t was on average 31% greater in control *P. palustris* seedlings than water-stressed ones, but was 8% greater in water-stressed *P. taeda* than control individuals (Table 1, 2). Average conduit reinforcement $(t/b)^2$ was significantly greater in *P. taeda* (mean = 0.37) than *P. palustris* (mean = 0.15, $p < 0.01$, Table 1, 2). *P. palustris* had more stem area than *P. taeda* (roughly 180% more across treatments, $p < 0.01$, Table 1, 2). The total stem area of *P. taeda* was unaffected by the drought, while stem area was 46% less in *P. palustris* in the water-stress treatment ($p < 0.01$, Table 1, 2). Leaf area to stem area ratios (LA:SA) were similar in the control treatment (Table 2), but the water-stress treatment caused species specific responses ($p < 0.01$, Table 1, 2). When exposed to water stress, *P. palustris* had a LA:SA nearly double that of the control, while *P. taeda* showed only a minimal reduction (Table 1, 2).

Table 2. The arithmetic mean ± SE of anatomical traits measured for *P. palustris* and *P. taeda* in control and water-stress treatments. Shown are stem double wall thickness (t), hydraulic diameter (b), conduit reinforcement ($(t/b)^2$), stem area (SA), and leaf area to stem area ratio (LA:SA).

Species	Treatment	t (µm)	b (µm)	$(t/b)^2$	SA (mm^2)	$LA:SA$ ($m^2\ cm^{-2}$)
P. palustris	Control	5.38 ± 0.46	12.18 ± 0.40	0.19 ± 0.02	2.94 ± 0.20	10.66 ± 1.06
	Drought	4.10 ± 0.26	11.78 ± 0.51	0.13 ± 0.04	1.59 ± 0.14	20.78 ± 1.54
P. taeda	Control	5.64 ± 0.18	9.21 ± 0.43	0.38 ± 0.05	0.79 ± 0.07	10.44 ± 1.23

Drought	6.14 ± 0.74	10.04 ± 0.19	0.38 ± 0.05	0.78 ± 0.04	8.64 ± 0.30

4. Discussion

We found that *P. palustris* and *P. taeda* germinants displayed different growth patterns, as well as varying responses to progressive water-stress. Both species showed similar relative growth rates, though *P. palustris* had higher absolute biomass production, allocated proportionately more of its biomass into aboveground tissue, and had higher rates of gas exchange than *P. taeda*. *P. palustris* reduced gas exchange earlier during water-stress and had wider tracheids and a smaller predicted hydraulic safety margin, suggesting that there may be a trade-off between the capacity to grow under water-stressed conditions and growth under optimal conditions in germinants. We suggest that these trade-offs are linked to the broader ecology of these two species and may have important implications for the natural regeneration of longleaf pine savannas.

4.1. Investment in aboveground tissue reduces the growth capacity of P. palustris *when stressed*

Across the experiment and in both treatments, *P. palustris* developed more leaf area and biomass than *P. taeda* and did so at the cost of reduced root growth. With roughly 80% of its carbon allocated to aboveground tissue (compared to 66% in *P. taeda*), *P. palustris* was able to rapidly accumulate leaf biomass when water was not limiting. Such an investment in leaf tissue increases the hydraulic demand of the plant relative to its water supply (McDowell et al. 2008), and consequently requires a proportional increase in the transport efficiency of water conducting tissue (e.g., through wider and/or more numerous conduits, and/or an increase in the total conducting area) in order to maintain leaf water supply while stomata are open (DeLucia et al., 2000; Medhurst and Beadle, 2002). This greater hydraulic efficiency would likely increase photosynthesis (Santiago et al., 2004, Pittermann et al., 2006), but, importantly, could also result in xylem that is more vulnerable to embolism during water stress. Indeed, *P. palustris* had wider tracheid diameters, as well as a less negative Ψ_{tlp} and more narrow safety margins than *P. taeda*, suggesting that *P. palustris* increased water transport efficiency at the cost of more vulnerable xylem (Pittermann et al., 2006). Along with this, the Ψ_{tlp} in both species was much higher than what is found in mature pines and similar trends have been found in other pine germinants (Ψ_{tlp} around -0.8, -0.5, and -1.6 MPa in *P. flexilis*, *P. contorta*, and *P. ponderosa*, respectively, Kerr et al., 2015, Lazarus et al., 2018). These results indicate that a potential trade-off exists between the capacity for growth under optimal and water-stressed conditions in pine germinants, and this tradeoff is amplified in *P. palustris*. *P. palustris* also had over triple the stem area of *P. taeda* in the control treatment, which compensated for its large leaf area and resulted in similar LA:SA across the two species. However, *P. palustris* actually exhibited increased LA:SA when exposed to water stress, which likely exacerbated its hydraulic demands and vulnerability to drought.

The trade-off between rapid growth under optimal conditions and reduced physiological activity when water-stressed observed in *P. palustris* is likely tied to the unusual grass stage that these seedlings remain in for up to 15 years. As an adaptation to the low-intensity fires that historically frequented longleaf pine ecosystems every 1 to 3 years, *P. palustris* develops a dense cluster of needles (the characterizing feature of the unique grass stage), which act as a physical barrier between the meristem and fire (Landers et al., 1995). As fire burns through the understory of longleaf savannas, the outer layer of *P. palustris* seedling needles are destroyed but the inner meristem remains intact. In the time between germination and attainment of sufficient leaf biomass to protect the inner meristem, the plant is vulnerable to fire-induced mortality (Grace and Platt, 1995). Therefore, the greater leaf area and aboveground biomass observed in *P. palustris* is likely an adaptation to overcome this vulnerability as quickly as possible. This adaptation has allowed *P. palustris* to dominate pyritic communities in the southeastern United States but leaves them especially vulnerable to drought and may consequently limit their ability to regenerate in a warmer, drier climate. *P. taeda*, on the other hand, has not historically grown in ecosystems where fire avoidance is necessary, and can consequently produce lower leaf area and aboveground biomass. This strategy may allow *P. taeda* to establish in drier locations than *P. palustris* if fire continues to be excluded in the future.

4.2. Physiological rather than morphological plasticity drives drought responses

Differences in biomass investment and vulnerability to drought resulted in distinct species-specific responses to water stress at the leaf level. *P. palustris* had greater rates of gas exchange during the experiment, but reduced gas exchange parameters earlier than *P. taeda* in the water-stress treatment. *P. palustris* may have reduced stomatal conductance earlier than *P. taeda* to diminish the large amount of water loss associated with its larger leaf area as a drought avoidance strategy. Notably, gas exchange parameters were inhibited in *P. palustris* several weeks after water potentials had begun to decline, while declines in *P. taeda* water potential and gas exchange parameters were tightly linked. These patterns may be associated with the safety margins observed for these species; *P. palustris*, for instance, was characterized by a narrower predicted safety margin than *P. taeda* and may consequently operate closer to its Ψ_{tlp} in order to ensure rapid growth under optimal conditions at the cost of reducing stomatal conductance to avoid drought.

Surprisingly, both species showed little morphological plasticity (e.g., LMR, LMA, tracheid diameter, conduit reinforcement) when water-stressed. We would expect both species, when exposed to water stress, to invest carbon into traits that increase their relative drought tolerance (i.e., decreased LMR, increased LMA, smaller tracheid diameters, increased conduit reinforcement), but these changes were not observed. With *P. palustris* exhibiting significant drops in midday water potential at week 6 (and maintaining similar photosynthetic rates as plants grown under control conditions), shifts in its carbon allocation towards drought tolerance traits should have occurred around this time. Instead, *P. palustris* maintained similar allocation patterns as the control, which may have resulted in its continued drop in water potential below its estimated Ψ_{tlp}. This lack of morphological plasticity may highlight genetic canalization of specific traits in early developmental stages (Valladares et al., 2002), which has been observed in other germinants and 1st-year pine seedlings exposed to water stress including *P. taeda* and *P. echinata* (Bradley and Will, 2017), *P. contorta* and *P. ponderosa* (Augustine and Reinhardt, 2019), and *P. engelmannii, P. leiophylla*, and *P. strobiformis* (Barton and Teeri, 1993). If frequent fire has historically driven *P. palustris* establishment, investing carbon into short-term drought tolerance traits during its grass stage would not convey a long-term advantage in terms of survival. Understanding at which age both *P. palustris* and *P. taeda* begin to exhibit plastic responses to water limitation (Jose et al., 2003; Addington et al., 2006), if at all, may improve restoration efforts of longleaf pine ecosystems, particularly in the context of a changing climate.

4.3. Implications for regeneration

P. palustris once ranged from sandy savannas to riparian areas (Stambaugh et al., 2011) and is often considered to be more drought-tolerant than *P. taeda* due to its growth advantage on sandier sites (Outcalt, 1993) and its ability to maintain less negative water potentials when they coexist (Samuelson et al., 2012). Mature *P. palustris* is characterized by a prominent taproot that can access water at great depths (Heyward, 1933), which suggests that this species utilizes a drought avoidance strategy to persist in drier conditions. Conversely, we show that immature *P. palustris* that have not yet developed their rooting system are unable to tolerate water limitation at the leaf-level, which may inhibit the growth of *P. palustris* seedlings on dry, sandy soils. At what age *P. palustris* seedlings start developing the large root system and belowground carbon reserves that allow them to develop stem tissue (Outcalt, 2000) and avoid seasonal drought is unknown. Determining the relative timing of carbon allocation into above and belowground tissue will provide crucial information regarding the development of *P. palustris* and will allow for more effective restoration efforts in the future. Our results suggest that the early life stages of *P. palustris* will hinder regeneration on sandier, xeric sites, especially as climate change intensifies. Natural regeneration efforts on these sites may be hindered in the future and suggest restoration efforts should plant *P. palustris* at a later life stage.

5. Conclusions

In this study we found contrasting patterns of biomass allocation and water stress responses in *P. palustris* and *P. taeda* germinants. While *P. palustris* developed more overall biomass and allocated more of its biomass into leaf tissue, *P. taeda* was less affected by water limitation, as *P. palustris* reduced leaf gas exchange as water stress increased. These results suggest there may be a trade-off between the capacity to grow under water-stressed conditions and growth under optimal conditions in these two species, which likely result from differing adaptations to fire of both species. The unusual grass stage that *P. palustris* has evolved as an adaptation to low-intensity fires requires that it rapidly develops aboveground biomass in order to protect its meristem from fire. While advantageous for fire-tolerance, our results suggest that greater aboveground biomass is associated with greater vulnerability to water limitation during the first year of development. Although further research into these interactions is needed to inform management decisions, this study may have important implications for the regeneration of *P. palustris* in a changing climate. Because *P. palustris* appears to be less drought tolerant as a germinant than as an adult, successful natural regeneration of *P. palustris* may not occur on sandier, xeric sites, especially as the climate warms.

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