Research paper

Drought responses of two gymnosperm species with contrasting stomatal regulation strategies under elevated [CO$_2$] and temperature

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Future climate regimes characterized by rising [CO$_2$], rising temperatures and associated droughts may differentially affect tree growth and physiology. However, the interactive effects of these three factors are complex because elevated [CO$_2$] and elevated temperature may generate differential physiological responses during drought. To date, the interactive effects of elevated [CO$_2$] and elevated temperature on drought-induced tree mortality remain poorly understood in gymnosperm species that differ in stomatal regulation strategies. Water relations and carbon dynamics were examined in two species with contrasting stomatal regulation strategies: *Pinus radiata* D. Don (relatively isohydric gymnosperm; regulating stomata to maintain leaf water potential above critical thresholds) and *Callitris rhomboidea* R. Br (relatively anisohydric gymnosperm; allowing leaf water potential to decline as the soil dries), to assess response to drought as a function of [CO$_2$] and temperature. Both species were grown in two [CO$_2$] (C_a (ambient, 400 µl l^{-1}) and C_e (elevated, 640 µl l^{-1})) and two temperature (T_a (ambient) and T_e (ambient +4 °C)) treatments in a sun-lit glasshouse under well-watered conditions. Drought plants were then exposed to a progressive drought until mortality. Prior to mortality, extensive xylem cavitation occurred in both species, but significant depletion of non-structural carbohydrates was not observed in either species. T_e resulted in faster mortality in *P. radiata*, but it did not modify the time-to-mortality in *C. rhomboidea*. C_e did not delay the time-to-mortality in either species under drought or T_e treatments. In summary, elevated temperature (+4 °C) had greater influence than elevated [CO$_2$] (+240 µl l^{-1}) on drought responses of the two studied gymnosperm species, while stomatal regulation strategies did not generally affect the relative contributions of hydraulic failure and carbohydrate depletion to mortality under severe drought.

Keywords: drought mortality, hydraulic failure, non-structural carbohydrates, rising [CO$_2$], rising temperature, stomatal regulation.

Introduction

Droughts of increased intensity and frequency are predicted to be more common in the future along with rising atmospheric CO$_2$ concentration ([CO$_2$]) (hereafter referred as '*C*'), increasing global temperatures (hereafter referred as '*T*') and associated increases in vapour pressure deficit (VPD) (Huntington 2006, IPCC 2012, Breshears et al. 2013). Over the past few decades, die-offs observed in forest biomes worldwide have been attributed to extreme drought and rising temperatures (Breshears et al. 2005, Van Mantgem et al. 2009, Allen et al. 2010, Peng et al. 2011, Matusick et al. 2013, Nardini et al. 2013). Previous work suggests that tree response to drought may be related to the wide range of stomatal regulation strategies that occur along the continuum from isohydry to anisohydry (Brodribb and

McAdam 2013, Mitchell et al. 2013, Meinzer et al. 2014). An improved understanding of the mechanisms underpinning tree response to progressive drought in those contrasting species may provide insights into differential time-to-mortality and subsequently, potential shifts in forest community composition under future climates.

During progressive drought, 'isohydric species' will close stomata early to maintain leaf water potentials above critical thresholds and to avoid hydraulic failure (Stocker 1956, Tardieu and Simonneau 1998). However, this strategy exposes these plants to longer periods of negative carbon balance, potentially depleting carbohydrate reserves and leading to carbon starvation (McDowell et al. 2008). Alternatively, 'anisohydric species' have the capacity to maintain stomatal conductance and transpiration over a larger range of leaf water potentials to sustain carbon assimilation, allowing larger declines in leaf water potential as soil dries. However, this strategy risks extensive xylem cavitation and potentially death via hydraulic failure (Brodribb and Cochard 2009, Choat et al. 2012, Choat 2013).

Few studies have experimentally examined the relative contributions of hydraulic failure and carbohydrate depletion during drought-induced mortality among species with contrasting stomatal regulation strategies. Furthermore, existing studies have been inconclusive. For instance, Anderegg and Anderegg (2013) examined saplings of two distinct gymnosperms in the USA (i.e., relatively isohydric *Pinus edulis* Engelm. and relatively anisohydric *Juniperus osteosperma* (Torr.) Little), and found that both species exhibited extensive xylem cavitation, but minimal carbohydrate depletion. In another study, Mitchell et al. (2013) examined the Australian native angiosperm (*Eucalyptus smithii* R. T. Baker; relatively anisohydric) and the North American gymnosperm (*Pinus radiata* D. Don; relatively isohydric), and found that mortality in relatively anisohydric *E. smithii* seedlings was characterized by profligate water use, significant hydraulic failure, and low levels of carbohydrate depletion (~20%). In contrast, the relatively isohydric *P. radiata* tightly regulated water use, survived for a longer period, but exhibited significant carbohydrate depletion (~50%). Mitchell et al. (2013) suggest that despite the primary contribution of hydraulic dysfunction to mortality, the degree of carbohydrate depletion varied between the two contrasting species. Therefore, more manipulative studies are required to investigate how the degree of isohydry would affect hydraulic and carbon processes during drought-induced mortality.

Elevated T (T_e) has often been observed to exacerbate drought stress (e.g., earlier onset of stomatal closure and negative carbon balance) through larger evaporative demand (Will et al. 2013) and higher respiration rates (Adams et al. 2009), leading to faster mortality in tree seedlings (Duan et al. 2014). By contrast, elevated C (C_e) is usually thought to have opposite effects from T_e. Given that C_e often reduces leaf stomatal conductance (g_s) when water is not limiting (Medlyn et al. 2001), it may benefit plants during drought stress through reductions in water use and more favourable leaf water relations (Atwell et al. 2007, Warren et al. 2011). In addition, C_e usually leads to increased carbon assimilation (Wertin et al. 2010, Duan et al. 2013, Franks et al. 2013, Lewis et al. 2013) and higher non-structural carbohydrate (NSC) reserves (Körner 2003, Niinemets 2010, Ayub et al. 2011, Duan et al. 2013) under drought conditions, potentially providing a larger NSC buffer to sustain metabolic activity during drought stress. To date, there remains considerable uncertainty surrounding the interactive effects of C_e and T_e on drought responses of tree species (Wertin et al. 2012*a*, *b*, Zeppel et al. 2012, Duan et al. 2013, 2014, Lewis et al. 2013). In particular, whether C_e would delay the onset of drought stress and drought mortality under T_e conditions is yet to be examined in species with contrasting stomatal regulation strategies.

Pinus radiata (relatively isohydric gymnosperm) and *Callitris rhomboidea* R. Br (relatively anisohydric gymnosperm) are known to have contrasting stomatal regulation strategies (McAdam et al. 2011, Brodribb and McAdam 2013). *Pinus radiata* has a very restricted natural distribution, occurring in three isolated populations in California and Mexico (Moran et al. 1988). However, it is widely planted, relatively fast growing and highly successful as a commercial timber resource around the world (>7 million ha). In contrast, *C. rhomboidea* is widely distributed throughout semi-arid ecosystems across Australia and has greater cavitation-resistant xylem than *P. radiata* (xylem water potential at 50% loss of hydraulic conductivity: $P_{50} = -9.2$ MPa vs -4.4 MPa, respectively) (Brodribb and Cochard 2009, Brodribb et al. 2010, Brodribb and McAdam 2013, Bouche et al. 2014).

In this study, we examined water relations and carbon dynamics under C_e and T_e using potted seedlings of these two contrasting gymnosperm species. This study aimed to examine the relative contributions of hydraulic failure and carbohydrate depletion during drought-induced mortality and determine whether these processes were modified by C_e and T_e. Specifically, we addressed the following hypotheses: (i) while both species would ultimately succumb to hydraulic failure prior to mortality, the relatively isohydric *P. radiata* would exhibit considerable carbohydrate depletion but the relatively anisohydric *C. rhomboidea* would experience limited carbohydrate depletion; (ii) T_e would accelerate the onset of stomatal closure at a given soil volumetric water content (VWC), induce faster declines in leaf water potential, consume greater pools of NSC and hasten mortality compared with ambient T (T_a) in both species; (iii) C_e would delay the onset of stomatal closure at a given soil VWC, induce higher leaf water potential by reducing leaf and whole-plant water use, increase NSC reserves and delay the time-to-mortality in both species compared with ambient C (C_a); and (iv) C_e would ameliorate the negative effects of T_e on plant responses to drought in both species, i.e., the time-to-mortality in C_eT_e is longer than C_aT_e.

Materials and methods

Experimental design

Three-month-old seedlings of *P. radiata* (Pinaceae family) and *C. rhomboidea* (Cupressaceae family) raised in forestry tube stock were obtained from Greening Australia (Sydney) on 13 January 2012. Thereafter, seedlings of each species were transplanted from tube stocks into 80 PVC pots (15 cm diameter × 40 cm height) containing ~10 kg air-dried, native loamy sand soil (86.5% sand, 9.5% clay and 4% silt). Pots contained drainage holes at the bottom to prevent soil waterlogging. Twenty seedlings of each species were then randomly placed in each of the four adjacent, naturally sun-lit and *C/T*-controlled glasshouse compartments (each 3 m (width) × 5 m (length) × 3.5 m (height)), located at the University of Western Sydney (Richmond, NSW, Australia (33°36′40″S, 150°44′26.5″E)). Prior to the drought treatment, seedlings were watered daily to field capacity and fertilized on two occasions (17 February and 19 March) with a commercial fertilizer (N:P:K- 27:2:10; All Purpose, Brunnings, Oakleigh South, VIC, Australia). Seedlings were rotated regularly within and between glasshouse compartments to minimize potential effects of glasshouse compartments on plant performance.

Details of glasshouse design can be found in Ghannoum et al. (2010). In summary, two glasshouse compartments for T_a treatments were set to simulate the 30-year daily average temperature in the growing season (November–May) experienced in Richmond, NSW. Over the course of 24 h, T was changed five times to simulate diurnal temperature cycles in the field. An additional two glasshouse compartments assigned to the T_e treatments were designed to simulate a constant 4 °C increase in temperature relative to ambient daily temperature cycle. The mean growth temperatures for T_a and T_e were 26/16 and 30/20 °C (day/night), respectively. For the two compartments within each T treatment, $[CO_2]$ was automatically regulated to maintain ambient (target 400 µl l^{-1}) and elevated (target 640 µl l^{-1}) C, respectively. C_e was achieved by injecting CO_2 gas (Food grade, AirLiquide, Fairfield, NSW, Australia) from pressurized cylinders through solenoid valves connected to a CO_2 monitor/controller (Lambda T, ADC BioScientific Ltd, Hoddesdon, Herts, UK). The target C and T were continuously monitored through the day and were intended to reflect the projected climate scenarios by the end of 21st century (IPCC 2012, Franks et al. 2013). The four treatments were termed as follows: C_aT_a (400 µl l^{-1}, 26 °C), C_aT_e (400 µl l^{-1}, 30 °C), C_eT_a (640 µl l^{-1}, 26 °C) and C_eT_e (640 µl l^{-1}, 30 °C). According to the primary research objective, we did not separate temperature and VPD effects in this study. Therefore, during the experimental period, VPD was not maintained constant among C and T treatments, reflecting a realistic scenario for future warmer climates. The diurnal VPD varied between 0.1 and 2.9 kPa in T_a treatments (mean value: 0.86 ± 0.01 kPa) and between 0.2 and 4.3 kPa in T_e treatments (mean value: 1.50 ± 0.01 kPa). VPD did not vary between C treatments.

Drought treatment

After 2 months of growth in the C and T treatments, half of the seedlings (i.e., 10 seedlings) within each species were randomly assigned to one of the two water treatments (i.e., *well-watered* or *drought*) in each of the four C and T treatments. The drought treatment commenced on 26 March 2012 (defined as Day 1). Pots were weighed in the morning (between 09:00 and 10:00 h) to determine water loss every second or third day. *Well-watered* seedlings were maintained at field capacity throughout the experimental period by replacing the amount of water lost during the preceding measurement interval. *Drought* seedlings received no water after the commencement of the drought treatment. Four blank pots filled with soil, but without seedlings, in each of the four glasshouse compartments (i.e., two for *well-watered* and two for *drought*) were used to monitor soil water evaporation. Hence, we could determine seedling water use independent of soil water evaporation.

Soil VWC (m^3 m^{-3}) was measured using TDR probes (30 cm length; CS616, Campbell Scientific, Logan, UT, USA) in two randomly selected pots (one each for *well-watered* and *drought*, respectively) for each species in each of the four C and T treatments. Measurements were logged every hour, and data were stored in a data logger (CR3000, Campbell Scientific). Using regular measurements of pot weight from seedlings with TDRs, we obtained a linear regression between changes in pot weight (i.e., pot weight during drought progression compared with pots that were *well-watered*) and soil VWC for each species. We used this relationship to estimate soil VWC for each pot using pot weight.

Gas exchange measurements

Leaf gas exchange measurements were taken on recent fascicles (*P. radiata*) or small branches (*C. rhomboidea*) of seedlings using a portable open path gas exchange system (Licor-6400, Li-Cor, Lincoln, NE, USA) supplying photosynthetic photon flux density (PPFD) by red-blue light source (6400-02B). Leaf photosynthesis under saturating light (A_{sat}, µmol m^{-2} s^{-1}) and stomatal conductance (g_s, mol m^{-2} s^{-1}) were measured between 09:30 and 14:00 h on a weekly basis throughout the experiment, at PPFD of 1500 µmol m^{-2} s^{-1}, growth C (400 or 640 µl l^{-1}) and midday growth T (26 or 30 °C) ($n = 4$). The daytime leaf-to-air VPD in Licor-6400 cuvettes was 1.90 ± 0.02 kPa in T_a treatments and 2.42 ± 0.04 kPa in T_e treatments, respectively ($n = 103$; across C and species treatments), representing the environmental conditions in the glasshouse during the day. Leaf dark respiration at night (R_n, µmol m^{-2} s^{-1}) was measured 2 h after sunset following the daytime measurement of A_{sat} on the same leaf at zero PPFD, growth C (400 or 640 µl l^{-1}) and night-time growth T (16 or 20 °C) ($n = 3$).

Water status and hydraulic measurements

Pre-dawn (Ψ_{pd}, MPa) and mid-day (Ψ_{md}, MPa) leaf water potentials were measured on Days 46, 81, 102 and 118 using a Scholander-type pressure chamber (PMS Instruments, Corvallis, OR, USA). On the evening prior to measurements, three seedlings from each treatment for each species were randomly selected ($n = 3$). Each seedling was covered overnight using a large black plastic bag to minimize nocturnal transpiration and to achieve water equilibrium between the soil and the seedling. Approximately 1 h before sunrise, Ψ_{pd} of one small fascicle (or branch) from each seedling was measured. At mid-day (12:00–14:00 h), Ψ_{md} was determined on the same seedling as the Ψ_{pd} measurements. Leaf water potential was recorded as −10 MPa when it was more negative than −10 MPa (i.e., beyond the operating range of the instrument).

Stem hydraulic measurements were conducted at each harvest (i.e., Days 1, 58, 120 and 206) ($n = 3$). On the night before each harvest, to maximize the equilibrium between leaf and xylem and to estimate xylem pressure by leaf water potential, two small fascicles (or branches) from each seedling were wrapped with cling tape and covered with aluminium foil. Meanwhile, seedlings were covered by black plastic bags and sealed at the bottom of the pots to prevent nocturnal transpiration. All harvests were conducted at pre-dawn because xylem water potential was highest (i.e., least negative) and this could minimize problems associated with air entry into the cut surface. Therefore, ~1 h before sunrise on the harvest day, aboveground sections of seedlings were cut at the bottom of the stem and the stem was recut under water immediately to exclude air entry into the xylem. The cut surface was wrapped with parafilm and the seedling was sealed in a black plastic bag, humidified with wet paper towelling, and returned immediately to the laboratory.

Xylem water potential (Ψ_{xylem}) was estimated using methods described in Duan et al. (2014). Stem segments of 5 cm in length were cut under perfusing solution (i.e., 2 mM KCl solution) for hydraulic measurements. Hydraulic conductivity measurements were conducted by the pressure-flow method described by Sperry et al. (1988). The initial hydraulic conductivity ($K_{initial}$) of the segment was measured with a pressure head of 3.4–4.4 kPa. High-pressure flushing causes pit aspiration and reduced flow in conifers, therefore the segment was vacuum infiltrated with water for 48–60 h to remove any embolism that may have been present before a hydraulic measurement was taken to establish K_{max}. The percentage loss of conductivity (PLC) of the stem segment was calculated as:

$$PLC = 100\% \times \frac{K_{max} - K_{initial}}{K_{max}} \qquad (1)$$

Seedling mortality

Seedlings were considered 'dead' in this study when all needles were brown, brittle and necrotic. We assessed death visually by estimating the percentage of brown needles compared with all plant needles. Although 100% of leaf browning represents the canopy mortality of trees, it has also been proved to be a reliable visual indicator of individual mortality (Anderegg and Anderegg 2013, Mitchell et al. 2013). Regular monitoring for mortality for *drought* seedlings ($n = 4$–7) commenced on Day 82.

Destructive harvests

Three seedlings within each species assigned to *well-watered* treatment ($n = 3$) in each of the four C and T treatments were randomly selected for destructive harvest to establish a pre-drought baseline at the onset of the experimental drought (Day 1). Harvests were subsequently conducted on both *well-watered* and *drought* plants on Days 58 and 120 ($n = 3$). At the end of the experiment, when all *drought* seedlings were considered 'dead' (Day 206), the remaining seedlings were harvested ($n = 4$). At each harvest, seedlings were separated into leaves, stem and roots. Roots were washed free of soil. All harvested fresh organs were immediately placed into an oven at 110 °C for 1 h to kill any biological activity. The plant organs were then dried at 70 °C for 72 h and dry mass was determined.

NSC analyses

Oven-dried plant organ samples from Days 1 to 206 were ground to fine powder in a ball mill. NSC concentration (mg g⁻¹) (as the sum of starch concentration and soluble sugar concentration) was assayed following procedures described in Mitchell et al. (2013). Organ samples (20 mg) were weighed and then extracted with 5 ml of 80% aqueous ethanol (v/v) in a polyethylene tube. The mixture was boiled in a water bath at 95 °C for 30 min, and then centrifuged at 3000 rpm for 5 min. The supernatant was collected and the pellet re-extracted once with 5 ml of 80% aqueous ethanol (v/v) and once with 5 ml of distilled water, then boiled and centrifuged as before. The supernatants were reserved and evaporated to the last 1–3 ml in a Rotational Vacuum Concentrator at 40 °C. Total starch was determined on the pellets remaining after the ethanol and water extractions, and assayed enzymatically using a total starch assay kit (Megazyme International Ireland Ltd, Wicklow, Ireland). Soluble sugars were determined on the supernatants by the anthrone method (Ebell 1969).

Statistics

Time-series measurements (i.e., water use, soil VWC, A_{sat}, g_s, R_n and leaf water potential) were analysed using three-way repeated-measures ANOVA to test temporal effects of the factors (species, C and T), after checking for the assumption of normality and homogeneity of variances. Thereafter, two-way ANOVAs were assessed to test the main and interactive effects of C and T within each species for each time step. Duncan post hoc tests were assessed to compare means among C and T treatments when interactions of C and T were significant. Dry

mass, PLC and NSC for each species within each water treatment were assessed with three-way ANOVAs (time, C and T). When the time effects on NSC were significant, the temporal changes of NSC were analysed by Duncan post hoc tests within each C and T treatment. The effects of C and T on the time-to-mortality were analysed using 95% confidence intervals. In all cases, results were considered significant if $P \leq 0.05$. All analyses were performed in SPSS 20.0 (SPSS, Chicago, IL, USA).

To generate the mechanistic understandings of how C_e and T_e would affect the drought sensitivity of gas exchange traits (i.e., A_{sat}, g_s and R_n), gas exchange as a function of soil VWC was fitted with three-parameter sigmoid regression within each C and T treatment:

$$y = \frac{y_{asym}}{1 + e^{(-(VWC-VWC_{mid})/k)}}$$ (2)

with y_{asym} being the estimated asymptote for each sigmoid regression, VWC_{mid} the inflection point of soil VWC (where $y = y_{asym}/2$) and k a scaling parameter. The effects of C and T on parameters for each estimate were analysed using 95% confidence intervals. However, the data for R_n of P. radiata could not be significantly fitted with sigmoid function, but was fitted with linear regression:

$$y = mVWC + y_0$$ (3)

with m and y_0 the two parameters. All fittings were conducted using regression function in Sigmaplot 12.0 (Systat Software Inc., San Jose, CA, USA).

Results

Plant water use and soil VWC during drought

Whole-plant water use in both species declined continuously during drought (Figure 1a and c). Water use of *drought* seedlings in P. radiata was not different among C and T treatments. By contrast, water use in C. rhomboidea was larger in T_e than T_a for the second and third weeks (two-way ANOVA: $P < 0.001$ for those cases), but it had more rapid declines in T_e treatments thereafter (two-way ANOVA: $P < 0.001$ for the fifth week), particularly in C_eT_e.

Across C and T treatments, soil VWC declined as drought progressed, with sharper declines in C. rhomboidea than P. radiata (three-way repeated-measures ANOVA: $P < 0.001$ for species) (Figure 1b and d). In P. radiata, soil VWC in the *drought* treatment did not differ among C and T treatments. By contrast, in C. rhomboidea, declines in soil VWC were steeper in T_e compared
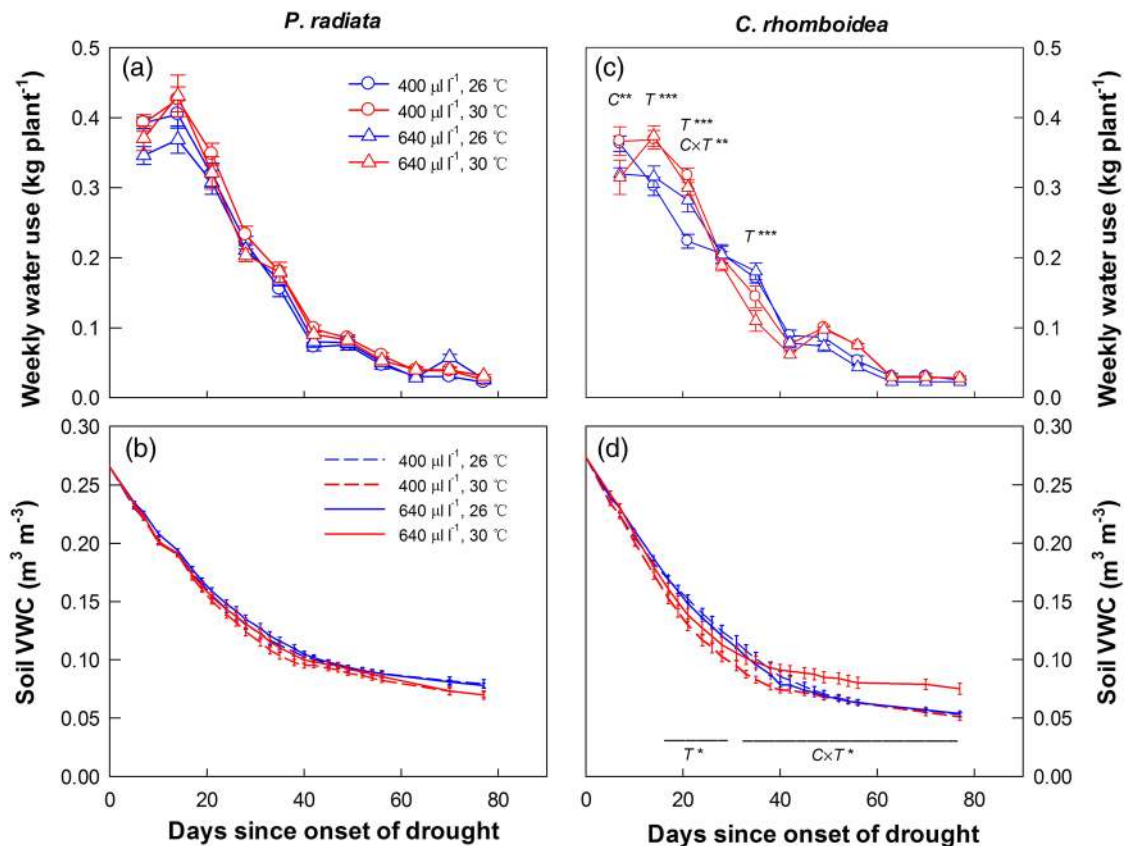

Figure 1. (a and c) Plant weekly water use and (b and d) estimated soil VWC in P. radiata and C. rhomboidea seedlings subjected to *drought* treatment under two C (C_a (400 µl l^{-1}) and C_e (640 µl l^{-1})) and two T (T_a (26 °C) and T_e (30 °C)) conditions over the course of the experiment. We estimated soil VWC by relationships of pot weight and monitored TDR values. Values are means ± SE ($n = 4–10$). Asterisks indicate significant differences in C or T treatments: *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$.

with T_a between Days 16 and 30 (two-way ANOVA: $P < 0.05$ for all cases) (Figure 1d). Thereafter, reductions in soil VWC slowed down in C_eT_e (two-way repeated-measures ANOVA: $P = 0.025$ for $C \times T$ interaction), possibly reflecting the treatment effects on initial cumulative plant water use.

Gas exchange as a function of soil VWC

The relationships between gas exchange and soil VWC were fitted with three-parameter sigmoid functions. Across C and T treatments, A_{sat} and g_s exhibited earlier and sharper declines in *P. radiata* than in *C. rhomboidea* as drought intensified, suggesting

that *P. radiata* had greater drought sensitivity of A_{sat} and g_s (Figure 2, Table 1). When soil water was not limiting, the estimated asymptote for A_{sat} of *P. radiata* was 56% higher in C_e than C_a, while it was 19% lower in T_e than T_a ($P < 0.05$ for all cases) (Figure 2a, Table 1). However, the estimated asymptote for g_s of *P. radiata* was 25% lower in T_e than T_a at the same stage ($P < 0.05$) (Figure 2b, Table 1). As drought progressed, A_{sat} and g_s of *P. radiata* converged among C and T treatments, thereby promoting 50% loss of A_{sat} and g_s at similar soil VWCs across C and T treatments (i.e., the inflection point VWC_{mid} did not differ among treatments; Figure 2a and b, Table 1). By contrast, in
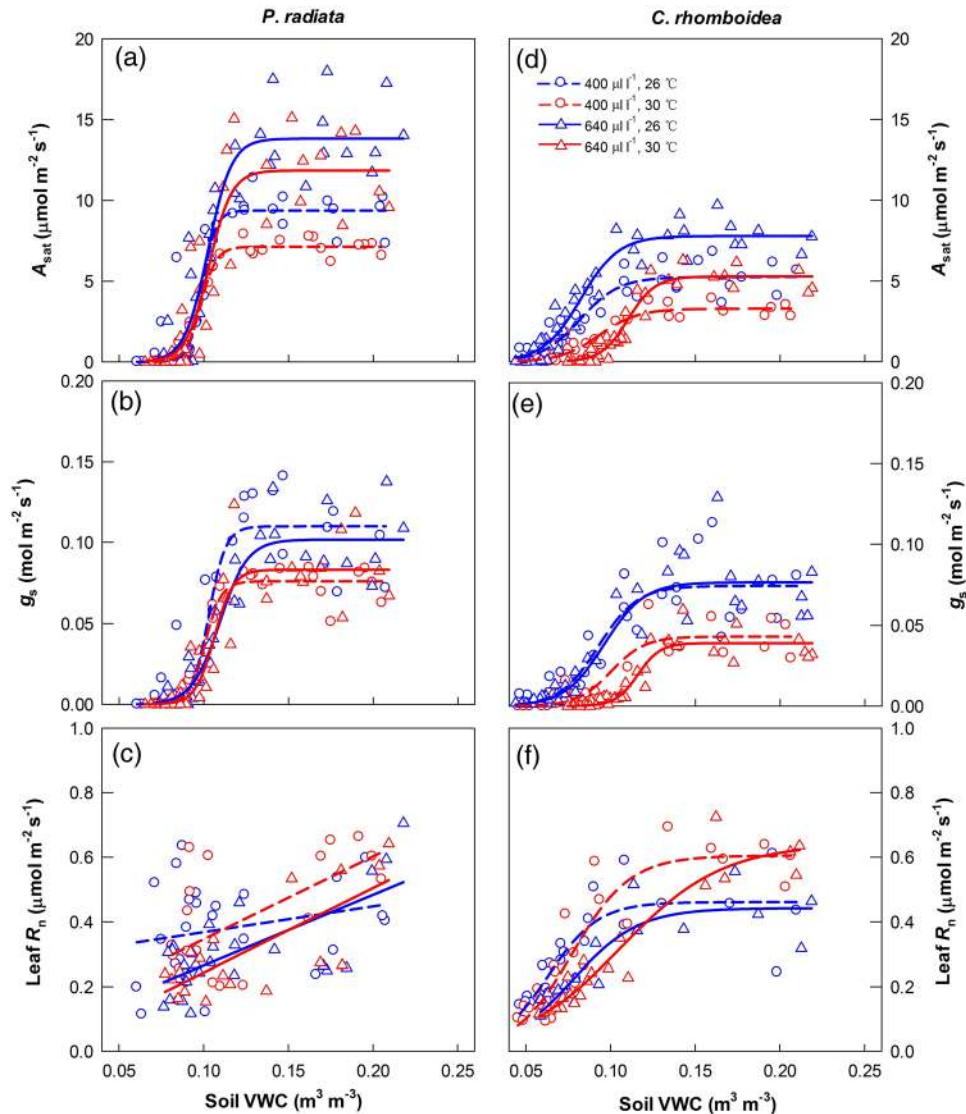

Figure 2. (a and d) Photosynthesis at saturating light (A_{sat}), (b and e) stomatal conductance (g_s) and (c and f) leaf dark respiration at night (R_n) of *P. radiata* and *C. rhomboidea* seedlings in the *drought* treatment as a function of soil VWC exposed to two C (C_a (400 µl l^{-1}) and C_e (640 µl l^{-1})) and two T (T_a (26 °C) and T_e (30 °C)) conditions. Data points for A_{sat} and g_s are raw data from the entire experiment, but those for R_n were just to Day 80 because SWC was not monitored after that date. Data are fitted with the three-parameter sigmoid relationships for each of the four C and T treatments: $y = y_{asym}/(1 + e^{(-(VWC-VWC_{mid})/k)})$ ($P < 0.001$, $R^2 = 0.663$–0.981, $n = 25$–48). 'y_{asym}' means the asymptote for each regression. 'VWC_{mid}' means the inflection point of soil VWC (where $y = y_{asym}/2$) and 'k' means a scaling parameter. Details of parameters can be found in Table 1. Data for R_n of *P. radiata* cannot be significantly fitted with sigmoid regressions, but are fitted with linear regressions, i.e., C_aT_a: $y = 0.798VWC + 0.288$ ($R^2 = 0.025$, $P = 0.211$); C_aT_e: $y = 2.561VWC + 0.090$ ($R^2 = 0.369$, $P = 0.002$); C_eT_a: $y = 2.182VWC + 0.047$ ($R^2 = 0.469$, $P < 0.001$); C_eT_e: $y = 2.610VWC - 0.018$ ($R^2 = 0.544$, $P < 0.001$).

Table 1. Summary of parameters in the fitted sigmoid regressions relating gas exchange traits of *P. radiata* and *C. rhomboidea* with soil VWC under two C (C_a (400 µl l^{-1}) and C_e (640 µl l^{-1})) and two T (T_a (26 °C) and T_e (30 °C)) treatments. The three-parameter sigmoid regressions were presented as $y = y_{asym}/(1 + e^{(-(VWC-VWC_{mid})/k)})$ ($P < 0.001$ for all parameters). 'y_{asym}' means the asymptote for each regression. 'VWC$_{mid}$' means the inflection point of soil VWC (where $y = y_{asym}/2$) and 'k' means a scaling parameter. '95% CI' means the 95% confidence interval. Different letters indicate a significant difference among C and T treatments for each parameter within each species based on 95% CI. '——' means no significant sigmoid relationship for R_n of *P. radiata*.

Species	Traits	Treatment	R^2	y_{asym} Estimate	95% CI	k Estimate	95% CI	VWC$_{mid}$ Estimate	95% CI
P. radiata	A_{sat}	C_aT_a	0.892	9.369b	8.605, 10.132	0.004	0.002, 0.006	0.099	0.097, 0.101
		C_aT_e	0.981	7.131b	6.890, 7.371	0.005	0.004, 0.005	0.098	0.097, 0.099
		C_eT_a	0.896	13.824a	12.751, 14.897	0.007	0.004, 0.010	0.102	0.099, 0.106
		C_eT_e	0.837	11.849a	10.536, 13.162	0.007	0.003, 0.010	0.103	0.098, 0.108
	g_s	C_aT_a	0.887	0.110a	0.100, 0.120	0.005	0.002, 0.008	0.103	0.099, 0.106
		C_aT_e	0.966	0.076b	0.073, 0.080	0.005	0.004, 0.006	0.101	0.097, 0.105
		C_eT_a	0.908	0.102a	0.094, 0.110	0.008	0.005, 0.011	0.109	0.103, 0.115
		C_eT_e	0.838	0.083b	0.074, 0.092	0.006	0.002, 0.009	0.106	0.102, 0.111
	R_n	C_aT_a	——	——	——	——	——	——	——
		C_aT_e	——	——	——	——	——	——	——
		C_eT_a	——	——	——	——	——	——	——
		C_eT_e	——	——	——	——	——	——	——
C. rhomboidea	A_{sat}	C_aT_a	0.857	5.253b	4.800, 5.705	0.012a	0.007, 0.016	0.082b	0.077, 0.087
		C_aT_e	0.902	3.285c	2.986, 3.583	0.012a	0.007, 0.016	0.093b	0.086, 0.100
		C_eT_a	0.920	7.791a	7.270, 8.313	0.011a	0.008, 0.015	0.082b	0.078, 0.087
		C_eT_e	0.916	5.282b	4.872, 5.691	0.008b	0.006, 0.010	0.110a	0.107, 0.113
	g_s	C_aT_a	0.819	0.074a	0.066, 0.083	0.012a	0.006, 0.017	0.093b	0.085, 0.101
		C_aT_e	0.862	0.043b	0.038, 0.048	0.010a	0.004, 0.016	0.100b	0.090, 0.110
		C_eT_a	0.857	0.076a	0.069, 0.084	0.010a	0.005, 0.016	0.093b	0.084, 0.101
		C_eT_e	0.870	0.039b	0.035, 0.043	0.007b	0.004, 0.010	0.116a	0.112, 0.120
	R_n	C_aT_a	0.663	0.461b	0.393, 0.529	0.015b	0.005, 0.024	0.063b	0.055, 0.071
		C_aT_e	0.851	0.605a	0.539, 0.670	0.017b	0.010, 0.024	0.078b	0.070, 0.085
		C_eT_a	0.759	0.442b	0.378, 0.507	0.020b	0.007, 0.032	0.078b	0.067, 0.088
		C_eT_e	0.920	0.641a	0.561, 0.720	0.030a	0.020, 0.041	0.106a	0.092, 0.120

C. rhomboidea, the estimated asymptote for A_{sat} was 55% higher in C_e than C_a when soil water was not limiting, but it was ~35% lower in T_e than T_a ($P < 0.05$ for all cases) (Figure 2d, Table 1). At the same stage, the estimated asymptote for g_s of *C. rhomboidea* was consistently lower in T_e than T_a ($P < 0.05$) (Figure 2e, Table 1). As drought intensified, *C. rhomboidea* exhibited 50% loss of A_{sat} and g_s at higher soil VWCs in C_eT_e than in other C and T treatments, indicating that C_eT_e induced earlier onset of stomatal closure at a given soil VWC (Figure 2d and e, Table 1). Leaf R_n of both species was less sensitive to soil drying than A_{sat} and g_s (Figure 2c and f). As drought progressed, R_n of *P. radiata* did not generally display significant differences among C and T treatments, while R_n of *C. rhomboidea* exhibited a 50% decline at higher soil VWC in C_eT_e than in other C and T treatments (Figure 2f, Table 1).

Leaf water potentials and xylem cavitation during drought

Pinus radiata and *C. rhomboidea* exhibited differing responses of water potential to drought (Figure 3). Across C and T treatments, declines in leaf Ψ_{pd} and Ψ_{md} of *P. radiata* were generally slower than those of *C. rhomboidea* (three-way repeated-measures

ANOVA: $P < 0.001$), as could be expected from tighter stomatal regulation in *P. radiata*. C_e did not have significant effects on Ψ_{pd} and Ψ_{md} in either species (Figure 3). However, T_e led to more rapid declines in Ψ_{pd} and Ψ_{md} of *P. radiata* compared with T_a (two-way repeated-measures ANOVA: $P < 0.001$ for both cases), but it did not significantly affect the descending trend in water potentials of *C. rhomboidea*.

Increases in PLC of both species were also observed as drought progressed (Figure 4). On Day 58, PLC of both species was lower than 40% and did not differ among C or T treatments. On Day 120, however, PLC of both species was ~100% and did not differ in C and T treatments, indicating that catastrophic xylem cavitation occurred in both species prior to mortality.

Plant mortality

On average, *P. radiata* seedlings were dead at 156 days following the onset of drought treatment across C and T treatments, while *C. rhomboidea* seedlings were dead after 191 days (Figure 5). The time-to-mortality of *P. radiata* seedlings was 40 days sooner in T_e compared with T_a (based on 95% confidence intervals), but it did not differ between C treatments.
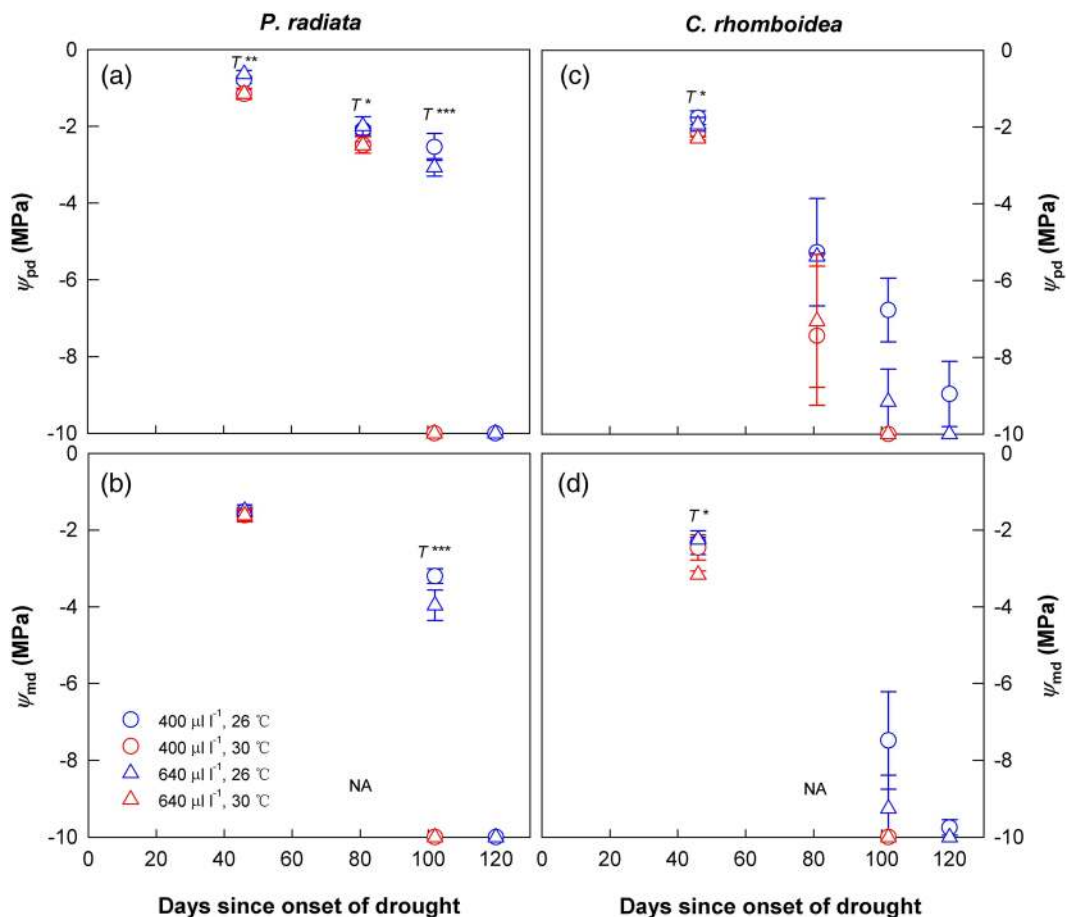
Figure 3. (a and c) Pre-dawn (Ψ_{pd}) and (b and d) mid-day (Ψ_{md}) leaf water potentials of *P. radiata* and *C. rhomboidea* seedlings subjected to *drought* treatment under two *C* (C_a (400 μl l^{-1}) and C_e (640 μl l^{-1})) and two *T* (T_a (26 °C) and T_e (30 °C)) treatments over the course of the experiment. Values are means ± SE ($n = 3$). In leaf water potential measurements, values were considered as −10 MPa when leaf water potential was more negative than −10 MPa. Ψ_{md} of both species on Day 81 was not reliable and thus was not used (i.e., NA). Asterisks indicate significant differences in *C* or *T* treatments: *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$.

In *C. rhomboidea*, however, the mortality pattern was not significantly different among *C* and *T* treatments.

Plant dry mass

In the *well-watered* treatment, the dry mass of *P. radiata* increased in a similar manner across all *C* and *T* treatments throughout the experiment (Figure 6), while temporal increases in dry mass of *C. rhomboidea* were highest in C_eT_e (Day 206; two-way ANOVA: $P = 0.005$). Dry mass accumulation of both species was limited by drought throughout the experiment (Figure 6), but did not differ among *C* and *T* treatments on any sampling date.

Organ NSC

Pre-drought, starch and soluble sugar concentrations of all organs in *P. radiata* and *C. rhomboidea* did not differ among *C* and *T* treatments (see Figures S2 and S3 available as Supplementary Data at *Tree Physiology* Online). Over the duration of the experiment, in *P. radiata*, leaf and stem starch concentrations of

well-watered seedlings were reduced by 49 and 70%, respectively in C_a treatments compared with pre-drought values ($P < 0.05$ for all cases) (Figure 7a and b), but leaf starch concentrations of *drought* seedlings were reduced by 53% (on average) in all *C* and *T* treatments ($P < 0.05$ for all cases except for C_eT_e) (Figure 7a). However, root soluble sugar concentrations of *well-watered* and *drought* seedlings were increased by ~150% (on average) across *C* and *T* treatments ($P < 0.05$ for all cases) (Figure 7i). By contrast, in *C. rhomboidea*, leaf starch concentrations of *well-watered* seedlings were significantly increased by 250% in C_eT_a treatment ($P < 0.05$) (Figure 7d), while those of *drought* seedlings were remarkably reduced (~79%) in all *C* and *T* treatments ($P < 0.05$ for all cases). Root starch concentrations of *well-watered* seedlings were increased in T_e treatments ($P < 0.05$ for all cases) (Figure 7f), while stem starch concentrations of *drought* seedlings were reduced in C_aT_a and C_eT_e treatments ($P < 0.05$ for all cases) (Figure 7e). Leaf and stem soluble sugar concentrations of *drought* seedlings were increased to a larger degree than those of *well-watered* seedlings, particularly
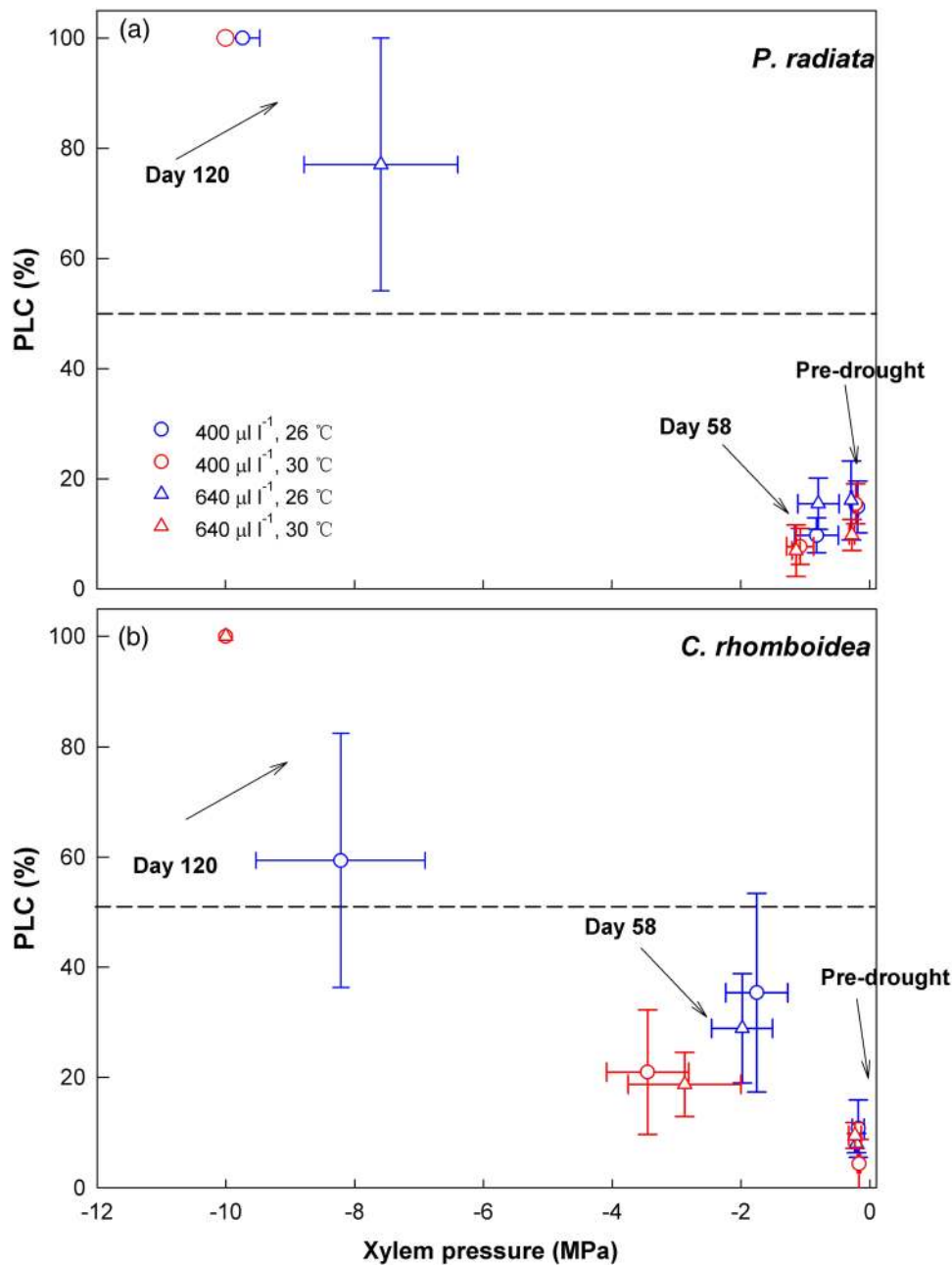
Figure 4. Percentage loss of stem hydraulic conductivity (PLC) as function of xylem pressure in (a) *P. radiata* and (b) *C. rhomboidea* seedlings exposed to two C (C_a (400 μl l^{-1}) and C_e (640 μl l^{-1})) and two T (T_a (26 °C) and T_e (30 °C)) treatments. Data are from pre-drought, Days 58 and 120. Data from the final harvest (i.e., Day 206) are not shown in this figure because PLC in all harvested seedlings among treatments were 100% on that day. Values are means ± SE ($n = 3$). The dash lines show 50% loss of hydraulic conductivity. Significant differences of PLC were not detected among C and T treatments on any sampling date.

under C_e conditions (Figure 7j and k). Overall, *P. radiata* exhibited similar trends in carbohydrate concentrations of *well-watered* and *drought* seedlings, indicating that drought did not lead to carbohydrate depletion in *P. radiata*. By contrast, depletion of leaf and stem starch in *C. rhomboidea* was offset by greater accumulation of leaf and stem soluble sugar; therefore, drought did not result in significant carbohydrate depletion in *C. rhomboidea*. Overall, C and T treatments did not have consistent effects on temporal changes of carbohydrates.

Discussion

Major findings

Although *P. radiata* and *C. rhomboidea* studied here exhibited differential gas exchange and leaf water potential responses to drought, our study showed that hydraulic failure preceded seedling mortality with little carbohydrate depletion observed in both species. However, the effects of T_e and C_e on physiological responses to drought varied in the two species. T_e led to larger
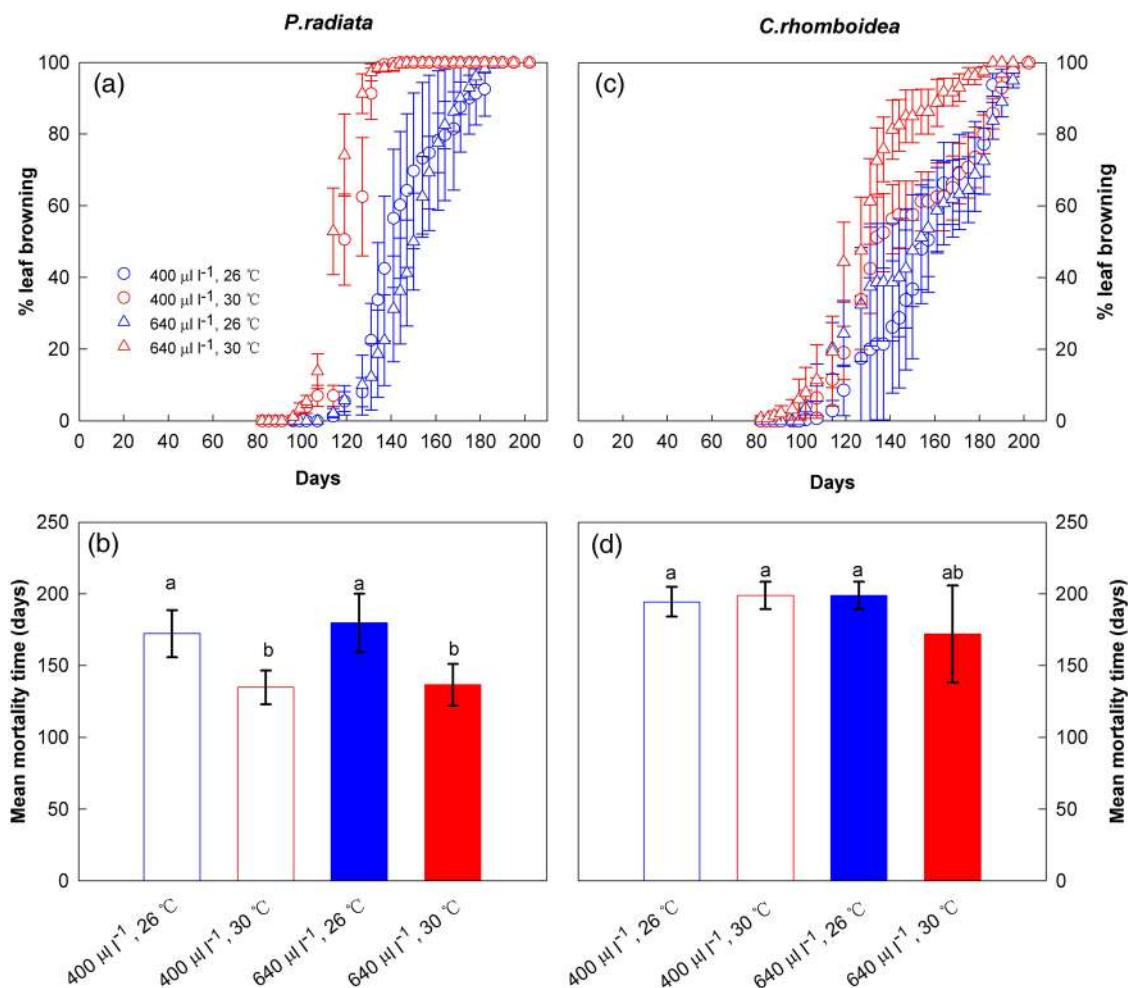
Figure 5. (a and c) Leaf browning and (b and d) mean time-to-mortality in *P. radiata* and *C. rhomboidea* seedlings in the *drought* treatment exposed to two C (C$_a$ (400 μl l^{-1}) and C$_e$ (640 μl l^{-1})) and two T (T$_a$ (26 °C) and T$_e$ (30 °C)) treatments. Seedlings with no brown leaves are 0% browning and seedlings with all brown leaves are 100% browning. Leaf browning tracking started on Day 82. In (a) and (c), values are means ± SE (n = 4–7). In (b) and (d), values are means ± 95% confidence intervals (n = 4). Different letters in the two figures indicate significant differences in C or T treatments.

declines in leaf water potential and faster mortality in *P. radiata*, but it did not affect physiological responses to drought in *C. rhomboidea*. By contrast, C$_e$ did not delay the effects of drought and T$_e$ on leaf water potential, whole-plant water use, NSC or time-to-mortality in either species. Our study highlights that the intensity and duration of drought may play an important role in carbohydrate utilization of plants. Elevated temperature (ambient +4 °C) rather than elevated [CO$_2$] (ambient +240 μl l^{-1}) appears to be the dominant environmental factor affecting gymnosperm seedling survival during drought in future climates.

Hydraulic failure occurred in both species but not carbohydrate depletion

Isohydric species are hypothesized to consume considerable carbohydrates during drought-induced mortality (McDowell et al. 2008). Significant carbohydrate depletion has been previously observed in the potted study of relatively isohydric

P. radiata seedlings (Mitchell et al. 2013). However, the present study shows that carbohydrate depletion in isohydric species was not observed, which is supported by two studies of relatively isohydric *P. edulis* saplings (see Anderegg and Anderegg 2013, Sevanto et al. 2014). We note that tree size and soil volume differed among these four studies, with bigger trees and pots in the *P. edulis* studies. Therefore, the inconsistency among those potted studies indicates that the consumption of carbohydrates may depend on some confounding factors such as the intensity and duration of drought (Woodruff et al. 2015). The drought stress imposed by Mitchell et al. (2013) was more controlled and gradual (i.e., replacing 80–90% of initial water loss) than our and the other two studies that generally withheld water completely. Rapid soil drying and dehydration in the present study could prevent the utilization of stored carbohydrates in plant organs (Hartmann et al. 2013), thus having minimal effects on NSC concentrations of the two studied species. Therefore, the present study shows that hydraulic failure during
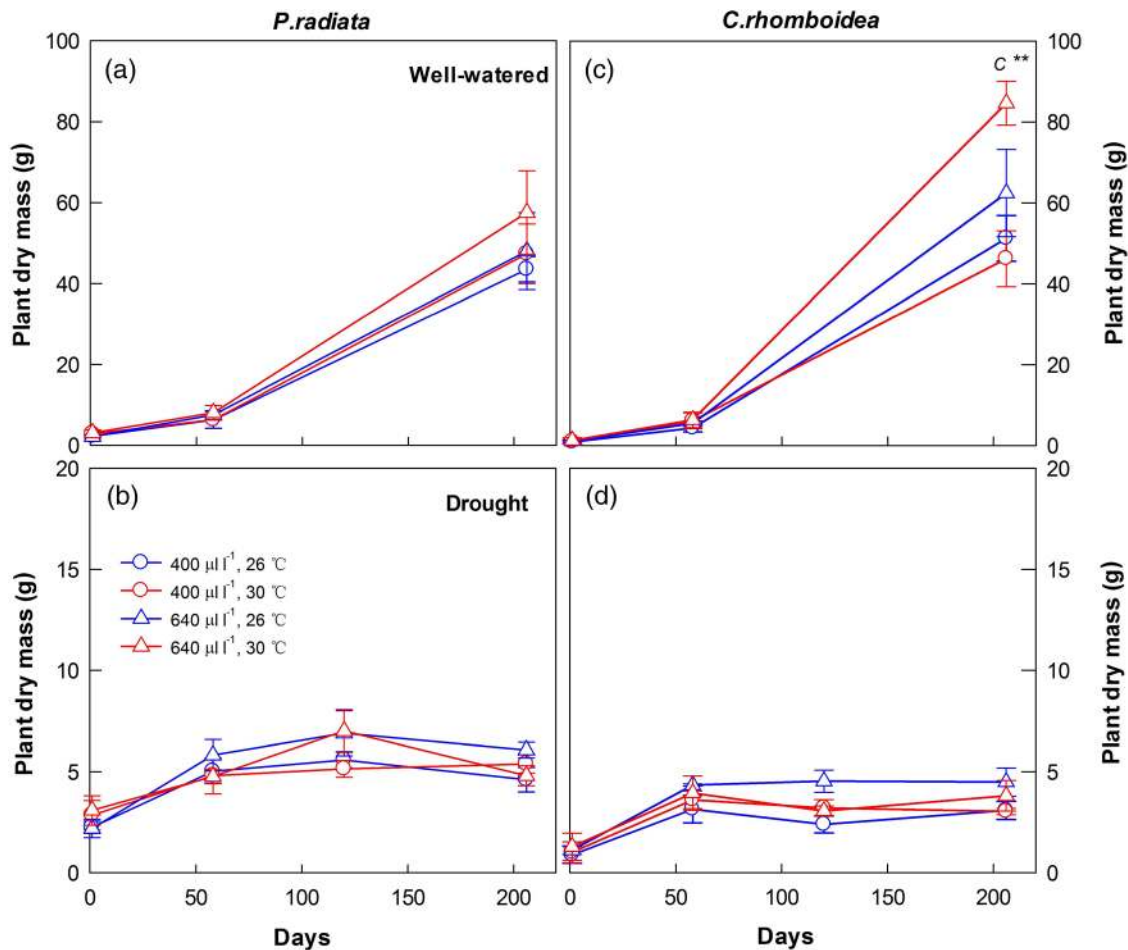
Figure 6. Plant dry mass of (a and b) *P. radiata* and (c and d) *C. rhomboidea* seedlings subjected to *well-watered* and *drought* under two C (C_a (400 µl l⁻¹) and C_e (640 µl l⁻¹)) and two T (T_a (26 °C) and T_e (30 °C)) treatments over the course of the experiment. Values are means ± SE. Pre-drought (Day 1), only *well-watered* seedlings were harvested as the baseline. Replicates: $n = 3$ for the first two/three harvests and $n = 4$ for the final harvest. Asterisks indicate significant differences in C or T treatments: *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$.

severe drought ultimately killed seedlings of both species without significant carbohydrate depletion irrespective of the contrasting stomatal regulation strategies. These results suggest convergence in the mechanisms of drought-induced mortality during severe drought along the iso-anisohydry continuum and support the conclusion of Hartmann et al. (2013) that 'thirst beats hunger'.

It is worth noting that the conclusions drawn from studies with potted seedlings/saplings need to be carefully considered when extrapolating to predict drought-induced mortality in mature trees in the field. Compared with seedlings/saplings, mature trees usually have larger root networks to explore soil available water in larger areas and bigger carbohydrate reserves to supply carbon to meet metabolic requirements (McDowell et al. 2013); thus, their response to drought may vary considerably from seedlings/saplings (McDowell et al. 2008). Nonetheless, studies on small potted trees can provide substantial insights into physiological processes during drought-induced mortality (McDowell et al. 2013). Overall, further studies are needed to examine water

relations and carbon dynamics in trees of varying sizes and ages under natural prolonged, but less intense droughts.

T_e treatments resulted in more rapid mortality in *P. radiata* but not in *C. rhomboidea*

Previous experimental studies have indicated that T_e accelerates drought-induced mortality of seedlings under environmentally controlled conditions (Adams et al. 2009, 2013, Will et al. 2013, Zhao et al. 2013, Duan et al. 2014), due mainly to two factors: increased evapotranspiration and increased rates of respiration. In this study, *P. radiata* died ~40 days sooner in T_e treatments than in T_a treatments, but *C. rhomboidea* died at approximately the same time across T treatments. Whole-plant water use in *P. radiata* did not significantly differ between T treatments, although VPD in T_e treatments was ~70% higher than in T_a treatments (note: plant size was similar between T treatments). However, with closed stomata, *P. radiata* in T_e treatments exhibited faster declines in leaf water potential than in T_a treatments. This finding suggests that T_e and associated higher VPD
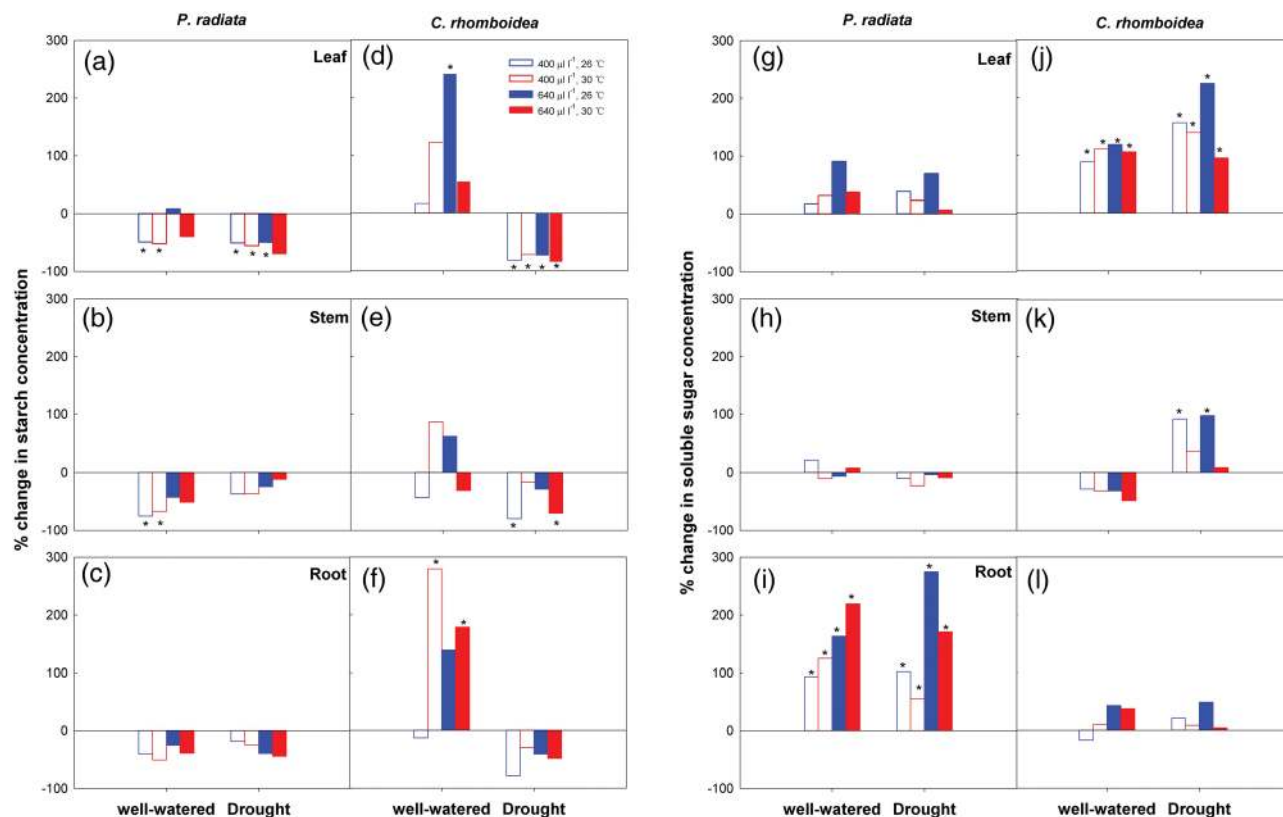
Figure 7. Percentage change in (a–f) starch and (g–l) soluble sugar concentrations in different organs (leaf, stem and root) of *P. radiata* and *C. rhomboidea* seedlings subjected to *well-watered* and *drought* treatment under two C (C_a (400 µl l⁻¹) and C_e (640 µl l⁻¹)) and two T (T_a (26 °C) and T_e (30 °C)) treatments. Changes in carbohydrate concentrations are calculated as follows: dividing concentration at mortality by pre-drought values and then minus 100%. Positive values indicate accumulation of carbohydrates and negative values indicate depletion of carbohydrates. Asterisks indicate significant differences between the pre-drought and final harvests within each C and T treatments ($P \leq 0.05$).

also have significant effects on plant dehydration after stomatal closure. The higher temperature treatments may have accelerated transpiration water loss from closed stomata and through the cuticle, thereby leading to faster desiccation. In contrast to *P. radiata*, leaf water potential in *C. rhomboidea* did not differ between T treatments, which could be related to the ecological characteristics of *C. rhomboidea* (Brodribb and McAdam 2013). *Callitris rhomboidea* is naturally distributed in areas of low rainfall concurrent with high temperatures, and the highly drought-resistant water transport system may have greater capacity to withstand higher temperature. Here, we observed that +4 °C warming did not significantly increase the impact of drought on water transport in the xylem or water loss from surfaces of *C. rhomboidea* seedlings relative to ambient temperature.

There was no evidence that carbohydrates at mortality were significantly different between T_a and T_e treatments in *P. radiata* or *C. rhomboidea*, which was consistent with the study on *Eucalyptus radiata* Sieber ex DC seedlings (Duan et al. 2014). Leaf respiration in *P. radiata* and *C. rhomboidea* seedlings under drought stress were not higher in T_e treatments than in T_a treatments, probably reflecting acclimation of respiration to rising T (Atkin and Tjoelker 2003, Wythers et al. 2005). Taken together,

mortality in our study was driven by hydraulic failure and was hastened by T_e in *P. radiata*, but not in *C. rhomboidea*.

Higher VPD due to T_e increases evaporative demand, potentially leading to more rapid stomatal closure with progressive drought (Eamus et al. 2013, Will et al. 2013, Zhao et al. 2013, Duan et al. 2014). For instance, the onset of stomatal closure occurred at higher soil VWC in T_e treatments (30 °C) compared with T_a treatments (26 °C) in *E. radiata* (Duan et al. 2014). In Zhao et al. (2013), *Thuja occidentalis* L. were treated under three temperature levels (i.e., 15, 25 and 35 °C) and the onset of stomatal closure occurred earlier in warmer temperatures (i.e., 10 °C temperature differences). However, in this study, *P. radiata* exhibited similar stomatal closure patterns among treatments, suggesting that stomatal sensitivity to drought stress was not modified by +4 °C warming treatments. Similar to *P. radiata*, *C. rhomboidea* did not exhibit greater stomatal sensitivity to drought in T_e alone treatments.

C_e did not delay time-to-mortality in either species under drought or T_e treatments

Contrary to our prediction, mortality of both species in the present study was not delayed by C_e. These results are consistent with

findings in an earlier study with *E. radiata* seedlings (Duan et al. 2014). In these two studies, C_e (i.e., 640 µl l^{-1}) was 240 µl l^{-1} higher than the ambient condition, which might not be high enough to reduce g_s and improve whole-plant water status. However, in most studies showing reductions in g_s, C_e usually ranged from 550 to 700 µl l^{-1} (see reviews Medlyn et al. 2001, Ainsworth and Rogers 2007), which encompasses the C_e in this study. Recently, a study of *Sequoia sempervirens* (D. Don.) Endl. reported that seedlings grown in very high *C* (i.e., 1500 µl l^{-1}) exhibited drought stress similar to seedlings grown in ambient *C* (Quirk et al. 2013). Therefore, the above findings suggest that C_e may have little capacity to affect plant responses during drought or alter survival time, at least during early stages of plant establishment. In fact, the effects of C_e on plant response to drought are complex (Franks et al. 2013) and could be confounded by other factors such as night-time g_s, plant morphology (e.g., leaf area and root characteristics), hydraulic traits, carbohydrate storage, drought intensity and CO_2 concentration (Ainsworth and Rogers 2007, Atwell et al. 2007, Niinemets 2010, Duursma et al. 2011, Warren et al. 2011, Zeppel et al. 2012). We argue that studies applied in a systematic manner are required to provide insight into the mechanisms underpinning plant response to drought and C_e.

In relatively isohydric *P. radiata*, C_e did not delay the onset of drought stress or increase survival time in T_e treatments (i.e., $C_eT_e = C_aT_e$); these data are consistent with findings from Duan et al. (2014). By contrast, in relatively anisohydric *C. rhomboidea*, the onset of stomatal closure was earlier in C_eT_e than other *C* and *T* treatments, suggesting C_e and T_e interactively increased stomatal sensitivity to drought stress. However, as drought progressed, leaf water potential and the time-to-mortality of *C. rhomboidea* seedlings did not differ among *C* or *T* treatments. Despite differing stomatal responses among *C* and *T* treatments at an early stage of drought stress, the xylem water transport and water status of highly cavitation-resistant *C. rhomboidea* were not significantly affected by *C* or *T* treatments under late severe drought stress.

Implications of this study

This research improves the understanding of the interactive effects of drought, and elevated [CO_2] and temperature on gymnosperm seedlings. The two investigated species are not naturally co-occurring, and thus our study does not allow conclusions to be developed regarding future trajectories of forest succession in a [CO_2]-enriched, warmer and drier world. Nonetheless, our study highlights that species-specific differences in drought and temperature responses may affect mortality processes of seedlings, and ultimately may improve our understanding of physiological dynamics in seedlings in natural ecosystems. Further research into mature trees coping with longer but less intense drought will provide more information for predicting forest responses in future climates.

Supplementary data

Supplementary data for this article are available at *Tree Physiology* Online.

Conflict of interest

None declared.

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