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Drought increases freezing tolerance of both leaves and xylem of *Larrea tridentata*

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ABSTRACT

Drought and freezing are both known to limit desert plant distributions, but the interaction of these stressors is poorly understood. Drought may increase freezing tolerance in leaves while decreasing it in the xylem, potentially creating a mismatch between water supply and demand. To test this hypothesis, we subjected *Larrea tridentata* juveniles grown in a greenhouse under well-watered or drought conditions to minimum temperatures ranging from –8 to –24 °C. We measured survival, leaf retention, gas exchange, cell death, freezing point depression and leaf-specific xylem hydraulic conductance (k_l). Drought-exposed plants exhibited smaller decreases in gas exchange after exposure to –8 °C compared to well-watered plants. Drought also conferred a significant positive effect on leaf, xylem and whole-plant function following exposure to –15 °C; drought-exposed plants exhibited less cell death, greater leaf retention, higher k_l and higher rates of gas exchange than well-watered plants. Both drought-exposed and well-watered plants experienced 100% mortality following exposure to –24 °C. By documenting the combined effects of drought and freezing stress, our data provide insight into the mechanisms determining plant survival and performance following freezing and the potential for shifts in *L. tridentata* abundance and range in the face of changing temperature and precipitation regimes.

Key-words: Chihuahuan Desert; freezing; xylem hydraulic conductance.

INTRODUCTION

The well-documented independent effects of drought and freezing demonstrate that physiological tolerance of both stresses can impact plant survival and productivity (e.g. Boorse, Ewers & Davis 1998b; Drake & Franks 2003). Across a variety of ecosystems, evergreens frequently experience drought and freezing simultaneously, raising the possibility of interactions between them (Pratt *et al*. 2005; Mayr *et al*. 2006). This interaction may be particularly important at the high latitude margins of arid lands, where low winter temperatures are often accompanied by low precipitation. Furthermore, climate models for Western North America predict that winter temperatures will increase by as much as 3 °C in the coming century, accompanied by increased winter precipitation (e.g. Christensen *et al*. 2007). Understanding the combined effects of drought and freezing stress on the survival and performance of desert evergreens is imperative in light of these predicted climate changes, which may potentially alter the relative abundance of arid shrublands and mesic grasslands in the temperate zones of North America (Epstein *et al*. 2002).

Freezing has been argued as an important limitation on the distribution of the warm desert evergreen *Larrea tridentata* [(Sessé & Moc. ex DC.) Coville; Pockman & Sperry 1997; Martínez-Vilalta & Pockman 2002]. The additional stress of drought may further constrain plant growth and survival in years when they occur simultaneously. Alternatively, warm desert species like *L. tridentata* which remain physiologically active during drought may exhibit higher tolerance for these combined stresses than might be expected because physiological changes in living cells of plants subjected to water deficit can also occur as part of cold acclimation (Xin & Browse 2000). Strong overlap in gene regulation during drought and cold exposure has been demonstrated in *Rhododendron* (Peng *et al*. 2008a), *Arabidopsis* (Puhakainen *et al*. 2004) and barley (Tommasini *et al*. 2008). In *Thelluginella*, however, limited overlap in gene expression was reported (Wong *et al*. 2005), suggesting that the relative benefits of drought in conferring cold tolerance varies.

In addition, leaf and xylem freezing responses may be differentially altered under drought conditions such that the freezing temperatures which damage living leaf cells may not be the same as those which reduce function of the dead xylem elements (Feild & Brodribb 2001; Cavender-Bares *et al*. 2005). In the xylem sap, dissolved gases are forced out of solution during ice formation, resulting in bubbles which may expand to form xylem embolism. Bubble formation is more likely to cause freeze-thaw embolism in the presence of drought because the critical bubble size necessary to cause cavitation is reduced at lower water potentials (Davis, Sperry & Hacke 1999). In the living leaf cells, mechanical damage to cell membranes during ice formation and dehydration may lead to the death of individual cells and/or damage to the photosynthetic

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apparatus (Pearce 2001). Plant cells that have acclimated to drought prior to freezing may be able to tolerate greater mechanical deformations because drought induces changes in membrane properties (Serrano *et al*. 2005). In addition, active osmotic adjustment during drought can reduce the freezing temperature of cell sap (López, Rodríguez-Calcerrada & Gil 2009), allowing super-cooling of living cells to at least –10 °C, while freezing of water contained in the apoplast and xylem conduits occurs at temperatures closer to 0 °C (Sakai & Larcher 1987).

The lack of correlation between leaf and xylem freezing tolerance in some species demonstrates that these can be independently evolving traits (Feild & Brodribb 2001; Cavender-Bares *et al*. 2005); therefore, the resulting whole-plant responses are difficult to predict from independent studies. If leaves are more freezing tolerant than xylem during drought, as was seen in *Ceanothus crassifolius* (Ewers *et al*. 2003), a disparity between transpiring leaf area and water transport capacity may result. When transpiration resumes following freezing, reduced xylem hydraulic conductance may limit water supply to the leaves, increasing the limits on stomatal opening and reducing carbon gain (Sperry *et al*. 2002). Conversely, frost-sensitive leaves and/or petioles could allow for leaf drop at temperatures too high to cause xylem embolism (Sakai & Larcher 1987).

Although the freezing tolerance of xylem in *L. tridentata* is apparently correlated with long-term minimum temperature at the high latitude edges of the North American warm deserts such as the Sonoran (Pockman & Sperry 1997) and Chihuahuan (Martínez-Vilalta & Pockman 2002), the interaction of drought and freezing stress has not been determined for this widespread species, nor have the potential differential responses of leaves and xylem been investigated. To understand how the interaction of drought and freezing may influence the distribution of *L. tridentata* at the high latitude edge of the Chihuahuan Desert, we addressed two questions: (1) what is the effect of drought on the freezing tolerance of leaves and xylem; and (2) how does the interaction of drought and freezing impact whole-plant function and survival following freezing? We compared leaf, xylem and whole-plant responses of drought-exposed and well-watered 1-year-old greenhouse-grown juveniles before and after exposure to –8, –15, –19 or –24 °C. To determine leaf responses, we monitored leaf retention and measured leaf cell damage. To determine xylem responses, we measured cell damage in green stems and leaf-specific xylem hydraulic conductance. To determine the effects on whole-plant function, we measured gas exchange and monitored survival and re-sprouting. We also measured plant water potential and freezing point depression of green stems and wood to determine the effects of imposed drought treatments. Because drought acclimation stimulates the accumulation of solutes in leaves, we expected to observe greater freezing tolerance of leaves under drought. In contrast, decreasing xylem water potential increases the potential for freeze-thaw embolism; therefore, we expected to observe a mismatch between water supply and demand, resulting in reduced whole-plant function when *L. tridentata* plants experienced the simultaneous stresses of drought and freezing.

MATERIALS AND METHODS

Seed collection site description

Seeds of *L. tridentata* were collected at the high latitude edge of the Chihuahuan desert, near the Five Points area of the Sevilleta LTER (34°20′ N 106°45′ W) at an elevation of 1610 m. Long-term minimum temperature at the Sevilleta LTER for the period from 1989 to 2009 was –20 °C. At Five Points, the mean yearly minimum temperature for the period from 1999 to 2009 was –14.4 ± 1.1 °C and the minimum temperature was –16 °C with an average of five nights per month during winter where the temperature fell to –8 °C or below. Mean annual precipitation was 248 mm [standard deviation (SD) = 105 mm; Moore 1989–2009].

Experimental design

Field-collected seeds were grown in the University of New Mexico research greenhouse for 1 year and then exposed to a single freezing treatment either in the presence or absence of drought. Two separate experiments were performed to test for the effects of drought: in 2007, we measured gas exchange before and after freezing, and in 2008, we measured leaf-specific xylem hydraulic conductance, cell death and freezing point depression. In both years, we measured plant water potential to assess the effectiveness of watering treatments and monitored survival, leaf retention and re-sprouting following freezing.

Watering treatments

In both years, the well-watered treatment consisted of spray irrigation for 5 min every day. In 2007, drought-exposed plants experienced a 50% reduction in the volume of water applied compared to well-watered plants. Because plant water potential (Ψ) differences generated by this method were small (see results for details), this regime was altered in 2008 when drought-exposed plants experienced 80% reduction in the volume of water applied. Plant water potential was measured using a pressure chamber (PMS, Corvalis, OR, USA). In 2007, measurements of Ψ were made on 12 plants at midday in the greenhouse and following 7 d of acclimation. In 2008, Ψ of 15 plants was measured at midday only, following 7 d of acclimation. Plants that were returned to the greenhouse following freezing (gas exchange and survival treatments) were watered in the same manner as before freezing.

Temperature treatments

Because cold acclimation is known to significantly impact freezing tolerance (Sakai & Larcher 1987), we preceded freezing treatments with 7 d of cold temperatures (1 °C night/12 °C day) in a growth chamber (Model #E8

Controlled Environments Limited, Winnipeg, Manitoba, Canada). Freezing took place with plants intact in 1 L pots in a double-chambered freezing apparatus consisting of two stacked coolers lined with coiled copper tubing and separated by an insulated barrier, creating a lower root chamber where temperatures were maintained between 1 and 6 °C by a temperature bath (Model RTE140, Neslab, ThermoFisher Scientific, Waltham, MA, USA) and an upper canopy chamber where freezing occurred, controlled by a separate temperature bath (Model 1197, VWR Scientific Products, West Chester, PA, USA). In the canopy chamber, freezing treatments started and ended at 10 °C, and cooling/warming proceeded at 1 °C min^{-1} for above-zero temperatures and 0.1 °C min^{-1} below 0 °C. Minimum temperature was maintained for 150 min. For each minimum temperature treatment (T_{min}), we randomly chose plants from drought-exposed and well-watered treatments. In 2007, measurements were made before freezing (T_{min} = 20 °C) and after exposure to –8, –15 and –19 °C. Experiments in 2007 revealed: (1) little to no effect of exposure to –8 °C in both drought-exposed and well-watered plants; and (2) survival of drought-exposed plants following exposure to –19 °C; therefore in 2008, the –8 °C treatment was replaced by a –24 °C treatment. In 2008, only post-freeze measurements were made because hydraulic conductance and electrical conductivity measurements are destructive; a control treatment (T_{min} = 20 °C) was added to allow determination of changes caused specifically by freezing.

Survival, leaf retention and re-sprouting

We assessed survival, leaf retention and re-sprouting of 10 plants in each watering treatment following five T_{min} treatments (20, –8, –15, –19 and –24 °C). Because plants that lost leaves following freezing lost the entire plant canopy, leaf retention data are reported as the proportion of plants retaining all leaves. Plants that lost all of their green tissue but sprouted new branches from the woody stems were considered re-sprouts.

Leaf gas exchange

To determine the effects of freezing on whole-plant performance, we measured gas exchange of 10 plants in each watering treatment before freezing (T_{min} = 20 °C) and subsequently for the same plants following one of three T_{min} treatments (–8, –15 or –19 °C). Measurements were made using a LiCor 6400 (LiCor Biosciences, Lincoln, Nebraska, USA). The same branch was measured before and after freezing. Each observation reported here is a mean of five observations recorded by the LiCor 6400 after steady state was attained.

Freezing point depression

Freezing point depression was measured to determine whether there were differences between drought-exposed and well-watered plants in the freezing temperature of plant tissues. We measured low temperature exotherms in green stems and wood of 12 plants in each watering treatment following three T_{min} treatments (20, –15 and –19 °C) using a copper–constantan thermocouple (Model # SMP, Omega Engineering, Inc., Stamford, CT, USA) installed in the middle of each stem, within the outer layer of xylem (method described in Pockman & Sperry 1997).

Cell death

To quantify electrolyte leakage from cells ruptured during freezing treatments, we measured relative electrical conductivity (*REC*; method described in Boorse *et al*. 1998a) of leaves and green stems for four plants in each watering treatment following three T_{min} treatments (20, –15 and –19 °C). Following freezing, tissue samples were incubated overnight at room temperature in water treated using a Millipore gradient to an electrical conductivity of <1 μS cm^{-1}, and initial electrical conductivity was determined using a temperature correcting multi-meter (Model pH/Cond 340i, WTW, Germany). Samples were autoclaved to completely lyse cells, and then maximum electrical conductivity was measured. *REC* was determined as initial electrical conductivity/maximum electrical conductivity.

Xylem hydraulic conductance

We quantified the effects of freezing on xylem by measuring absolute xylem hydraulic conductance (k_h) and leaf-specific xylem hydraulic conductance (k_l) for eight plants in each watering treatment immediately following one of four T_{min} treatments (20, –15, –19 or –24 °C). Using an Ultra Low Flow Meter (method described in Tyree *et al*. 2002), measurements were made on the entire above-ground portion of plants, between 10 and 20 cm above-ground height. Following removal of leaves just below the leaf base, the main stem of the plant was cut under water and attached to Tygon tubing (Saint-Gobain Performance Plastics, Paris, France) containing a solution of water and 20 mM KCl. The entire above-ground portion of the plant was then placed into a vacuum flask for measurement of flow through the plant at five levels of partial pressure. Absolute xylem hydraulic conductance was determined as the slope of the relationship between the flow of water through the plant and the partial pressure in the vacuum flask. For all measurements, R^2 of this relationship was ≥0.95. Leaf-specific hydraulic conductance (k_l) was determined by dividing k_h by the total leaf area of the sample prior to freezing, so that reported k_l values reflect reductions in leaf area caused by watering treatments, but not those that may have resulted from freezing treatments. Leaf area was determined using a regression relating dry weight to surface area (Medeiros & Pockman 2010). We also computed the proportional change in k_l compared to control for comparison with *REC* data. We first calculated a mean control k_l for drought-exposed and well-watered plants, then for each post-freeze observation of k_l, we calculated the proportional change from mean control k_l for drought-exposed or well-watered plants, respectively.

Table 1. Pairwise χ^2 tests for the effects of freezing on the proportion of drought-exposed and well-watered *Larrea tridentata* plants surviving, surviving with leaves and re-sprouting following the application of watering treatments (minimum temperature 20 °C) and following freezing to –15 °C or –19 °C

Minimum temperature	Dependant variable	d.f.	χ^2
20 °C	Survived	1	19.17****
	Survived with leaves	1	56.54****
	Re-sprouting	1	28.57****
–15 °C	Survived	1	1.15
	Survived with leaves	1	11.25***
	Re-sprouting	1	6.56*
–19 °C	Survived	1	1.17
	Survived with leaves	1	3.41
	Re-sprouting	1	0.05

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$.

Data analysis

Data analysis was performed in SAS (Ver. 9.2, SAS Institute Inc., Cary, NC, USA). For plant water potential data, differences between watering treatments were determined using a standard two-tailed *t*-test. All other physiological measurements were analysed using analysis of variance (ANOVA). Independent variables for each analysis were as follows: (1) freezing point depression, watering treatment and tissue type (green stems or wood); (2) absolute xylem hydraulic conductance and leaf-specific hydraulic conductance (data log transformed to conform to assumptions of normality), watering treatment and T_{min}; and (3) *REC*, watering treatment, T_{min} and tissue type (leaves or green stems). Pairwise comparisons were made using least squares means and only planned comparisons were used. Gas exchange measurements were analysed using repeated measures MANOVA. Only plants which survived freezing were included in this analysis and watering treatment and T_{min} were specified as the independent variables. Survival, leaf retention and re-sprouting data were analysed using pairwise χ^2 tests to compare drought-exposed and well-watered plants within each T_{min}. We used a sequential Bonferroni correction to determine significance for these pairwise comparisons (Rice 1989).

RESULTS

Plant water potential

A *t*-test revealed significant differences in mean midday plant water potential (Ψ) between well-watered and drought-exposed plants in 2007 (–2.7 ± 0.3 MPa, and –3.9 ± 0.7 MPa; $t = 4.46$, d.f. = 1, $P = 0.0002$), however, differences were smaller and not significant following 1 week in the acclimation chamber (–2.4 ± 0.8 MPa and –2.8 ± 1.4 MPa, respectively; $t = 0.6981$, d.f. = 1, $P = 0.4951$). In 2008, well-watered plants exhibited significantly higher mean Ψ than drought-exposed plants following 1 week in the acclimation chamber (–1.8 ± 0.3 MPa and –3.5 ± 1.3 MPa, respectively; $t = 4.78$, d.f. = 1, $P < 0.0001$).

Freezing point depression, exotherms

Mean exotherm temperature was lower for drought-exposed plants (–9.2 ± 2.2 °C) than for well-watered plants (–7.5 ± 1.3 °C), and this difference was significant in a two-way ANOVA ($F = 6.03$, d.f. = 1, $P = 0.0208$). There was a trend for exotherms to occur at lower temperatures in green stems than in woody tissues (–8.7 ± 2.1 °C and –7.6 ± 1.6 °C, respectively), but this difference was not significant ($F = 2.15$, d.f. = 1, $P = 0.1544$) and there was not a significant interaction between watering treatment and tissue type ($F = 0.05$, d.f. = 1, $P = 0.8250$).

Survival, leaf retention and re-sprouting

Drought-exposed plants experienced significantly lower survival rates than well-watered plants over the period during which watering treatments were applied prior to freezing (minimum temperature 20 °C, Table 1). Following exposure to –8 °C, there were no differences between drought-exposed and well-watered plants in proportion of plants surviving (Fig. 1a), proportion of plants retaining leaves (Fig. 1b) or proportion of plants re-sprouting (all

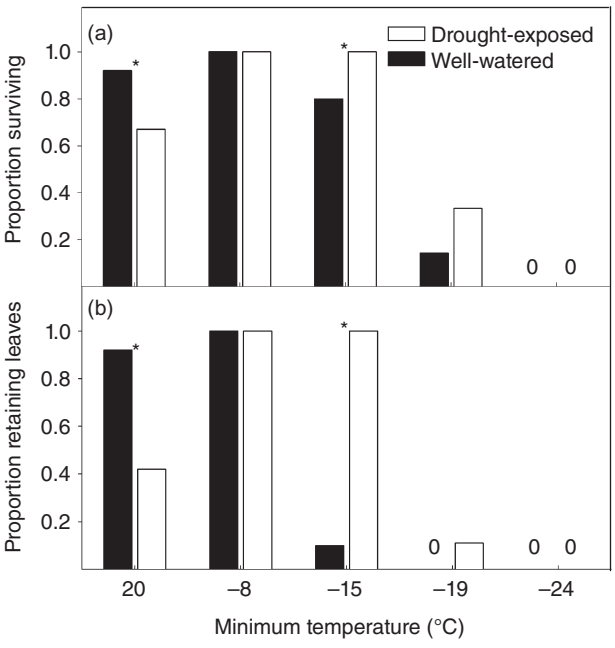

Figure 1. Performance and survival of drought-exposed and well-watered *Larrea tridentata* plants following five minimum temperature treatments, (a) proportion of plants surviving; (b) proportion of plants surviving with leaves. Error bars represent one standard error and * represents significant differences at $\alpha = 0.05$ in least-squares pairwise comparisons where only planned comparisons were used. A zero indicates cases where all plants died or lost all leaves. Data from are from 2007, except for data in the –24 °C treatment, which were collected in 2008.

surviving plants which lost leaves re-sprouted; therefore, re-sprouting data are not shown). All drought-exposed and well-watered plants died following exposure to –24 °C. There were differences between the response of the two watering treatments when plants were exposed to –15 and –19 °C. In this range, there was a trend for more drought-exposed plants to survive than well-watered plants (Fig. 1a), although a χ^2 test revealed no significant differences between the two watering treatments (Table 1). A significantly greater proportion of drought-exposed plants retained leaves compared to well-watered plants following exposure to –15 °C (Fig. 1b). In addition, more of the surviving plants in the well-watered treatment were re-sprouts.

Gas exchange

Because most plants in the –19 °C minimum temperature treatment (T_{min}) did not survive freezing, only gas exchange data from the –8 and –15 °C treatments were analysed. Table 2 presents the means and SD of gas exchange parameters for the two watering treatments before and after freezing. Before freezing, drought-exposed plants exhibited lower rates of net photosynthetic assimilation (A), transpiration (E) and stomatal conductance (g_s) than well-watered plants. Following freezing, the opposite was true, and well-watered plants exhibited greater reductions in gas exchange than drought-exposed plants. Immediately following exposure to –8 °C, we observed a positive effect of freezing on the gas exchange of drought-exposed plants. Two weeks later, drought-exposed plants exhibited significantly higher A, E and g_s than well-watered plants in both T_{min} treatments. By 4 weeks following freezing, the differences between drought-exposed and well-watered plants had diminished, caused both by a reduction in gas exchange rates among drought-exposed plants and an increase in rates among well-watered plants. Repeated measures MANOVA revealed a significant effect of time ($F = 62.48$, d.f. = 11, $P < 0.0001$), time × watering treatment ($F = 12.73$, d.f. = 11, $P < 0.0001$) and time × T_{min} ($F = 9.53$, d.f. = 11, $P = 0.0020$) on gas exchange; but there was not a significant three-way interaction (time × watering treatment × T_{min}; $F = 2.12$, d.f. = 11, $P = 0.1835$).

Cell death

Drought-exposed plants exhibited higher *REC* than well-watered plants in the control treatment ($T_{min} = 20$ °C) for both green stems (Fig. 2a) and leaves (Fig. 2b). Although water alone was not a significant predictor of *REC* in a two-way ANOVA ($F = 1.72$, d.f. = 1, $P = 0.1925$), freezing increased *REC* above control in well-watered plants more than in drought-exposed plants, resulting in a significant water × T_{min} interaction ($F = 13.62$, d.f. = 2, $P < 0.0001$). Leaves exhibited larger increases in *REC* than green stems following freezing, and there was a significant effect of tissue type ($F = 29.92$, d.f. = 1, $P < 0.0001$). There was also a significant effect of T_{min} ($F = 35.49$, d.f. = 2, $P < 0.0001$), although the differences between control and sub-zero T_{min} were

Table 2. Means and standard deviations for gas exchange characters, net photosynthetic assimilation (A; μmol m^{-2} s^{-1}), transpiration (E; mmol m^{-2} s^{-1}) and stomatal conductance (g_s; mmol m^{-2} s^{-1}) measured in drought-exposed and well-watered *Larrea tridentata* plants before, immediately, 2 and 4 weeks after freezing to two minimum temperatures, –8 and –15 °C

		Minimum temperature					
		–8 °C			–15 °C		
Time	Watering treatment	A	E	g_s	A	E	g_s
Before freezing	Drought-exposed	$6.60^a \pm 5.15$	$2.01^a \pm 1.82$	$0.08^a \pm 0.08$	$8.72^a \pm 6.08$	$2.77^a \pm 1.88$	$0.10^a \pm 0.07$
	Well-watered	$17.21^b \pm 10.32$	$5.41^b \pm 3.79$	$0.27^b \pm 0.25$	$10.87^a \pm 3.09$	$3.43^a \pm 1.27$	$0.13^a \pm 0.06$
Immediately after freezing	Drought-exposed	$2.02^a \pm 1.11$	$0.36^a \pm 0.16$	$0.02^a \pm 0.01$	$0.36^a \pm 0.60$	$0.21^a \pm 0.08$	$0.01^a \pm 0.01$
	Well-watered	$0.92^b \pm 0.54$	$0.23^a \pm 0.13$	$0.01^b \pm 0.01$	$0.12^a \pm 0.66$	$0.13^a \pm 0.06$	$0.01^a \pm 0.01$
Two weeks after freezing	Drought-exposed	$24.73^a \pm 3.68$	$6.04^a \pm 1.05$	$0.33^a \pm 0.09$	$20.70^a \pm 3.20$	$5.06^a \pm 1.07$	$0.29^a \pm 0.08$
	Well-watered	$14.12^b \pm 2.76$	$3.28^b \pm 1.23$	$0.20^b \pm 0.08$	0^b	0^b	0^b
Four weeks after freezing	Drought-exposed	$13.47^a \pm 6.02$	$3.18^a \pm 2.00$	$0.16^a \pm 0.12$	$16.96^a \pm 8.60$	$4.29^a \pm 2.37$	$0.22^a \pm 0.15$
	Well-watered	$15.75^a \pm 8.57$	$4.13^a \pm 2.39$	$0.24^a \pm 0.19$	$23.09^a \pm 7.54$	$6.07^a \pm 1.63$	$0.34^a \pm 0.10$

Only plants that survived freezing are included. Two weeks following freezing to –15 °C all surviving plants in the well-watered treatment had lost all leaves and could not be measured. They were therefore assigned a gas exchange rate of zero. Superscript letters indicate significant differences at $\alpha = 0.05$ across watering treatments within each temperature treatment and time period using only planned comparisons of least squares means.

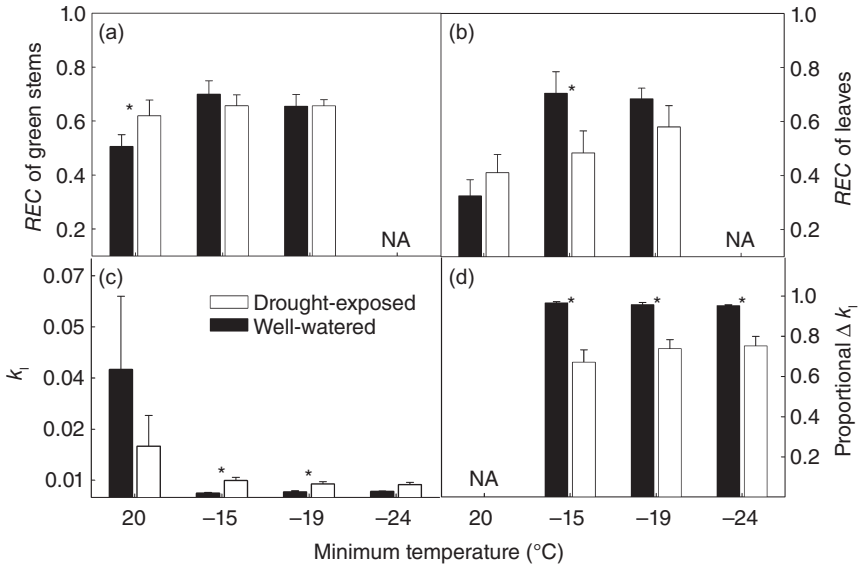

Figure 2. Physiological characteristics of leaves and xylem of drought-exposed and well-watered *Larrea tridentata* following four minimum temperature treatments; relative electrical conductivity (*REC*) in (a) green stems and (b) leaves, (c) leaf-specific hydraulic conductance (k_l; mmol kPa^{-1} s^{-1} m^{-2}), and (d) proportional change in leaf-specific hydraulic conductance compared to mean for control plants. Error bars represent one standard error and * represents significant differences at $\alpha = 0.05$ in least-squares pairwise comparisons where only planned comparisons were used. NA indicates that measurements of a particular type were not made at that temperature.

significantly larger in leaves than in green stems (T_{min} × tissue-type interaction; $F = 7.67$, d.f. = 2, $P = 0.0007$). Finally, there were differences between watering treatments in how the two tissue types responded to freezing. In drought-exposed plants, green stems exhibited negligible increases in *REC* following freezing, while a gradual increase in *REC* was observed for leaves as minimum temperature decreased. In contrast, both leaves and green stems of well-watered plants exhibited large increases in *REC* following exposure to –15 °C and no further increases following exposure to –19 °C, resulting in a significant watering treatment × tissue-type interaction ($F = 8.16$, d.f. = 1, $P = 0.0050$).

Xylem hydraulic conductance

Prior to freezing, mean absolute hydraulic conductance (k_h, units = 10^{-5} mmol s^{-1} kPa^{-1}) of well-watered plants tended to be higher than that of drought-exposed plants, while following freezing, this situation was reversed. Before freezing, k_h was 7.9 ± 14 for drought-exposed compared to 64 ± 96 for well-watered plants. Following exposure to –15 °C, drought-exposed plants exhibited higher k_h than well-watered plants (2.1 ± 0.49 and 1.9 ± 0.82, respectively). Compared to exposure to –15 °C, k_h was higher for both drought-exposed and well-watered plants following exposure to –19 °C (–2.4 ± 0.20 and 2.6 ± 1.6, respectively) and –24 °C (–2.3 ± 0.27 and 2.7 ± 0.88, respectively). The large variation in k_h within watering treatments resulted in no significant pairwise differences between them at any temperature.

Leaf-specific hydraulic conductance (k_l, Fig. 2c) responded in the same manner as k_h. Because these measurements are standardized for leaf area, this accounted for much of the variation observed in k_h. Both pre-freeze and post-freeze k_l were calculated using pre-freeze leaf area, with drought-exposed plants having on average 59% smaller canopy than well-watered plants across all treatments. This, along with the contrasting responses of drought-exposed and well-watered plants to freezing, indicates that observed differences in k_l between the two treatments were not simply due to differences in leaf area used in the calculation of this measure. Two-way ANOVA revealed a significant effect of watering treatment on k_l ($F = 7.40$, d.f. = 1, $P = 0.0092$) and a marginally significant effect on k_h ($F = 3.475$, d.f. = 1, $P = 0.0690$). There was a significant water × T_{min} interaction for k_h ($F = 3.701$, d.f. = 3, $P = 0.018$), but not for k_l ($F = 2.31$, d.f. = 3, $P = 0.0889$). As T_{min} decreased, we observed a trend of decreasing k_h and k_l in drought-exposed plants and increases in well-watered plants, and there was a significant effect of T_{min} on both measures ($F = 9.094$, d.f. = 3, $P < 0.0001$ and $F = 6.59$, d.f. = 3, $P = 0.0008$, respectively). This effect was driven by differences between control and T_{min} treatments, however not by differences between the freezing treatments, and pairwise comparisons revealed no significant differences in k_l or k_h between plants frozen to –15, –19 and –24 °C.

Relative balance between leaf and xylem vulnerability

Compared to well-watered plants, drought-exposed plants exhibited a smaller proportional change in k_l relative to control (Fig. 2d), and this difference was significant in a two-way ANOVA ($F = 45.07$, d.f. = 1, $P < 0.0001$). There was

not a significant effect of T_{min} ($F = 0.68$, d.f. = 2, $P = 0.5128$) or a significant watering treatment × T_{min} interaction ($F = 1.02$, d.f. = 2, $P = 0.3722$) on the proportional change in k_l. Following exposure to –15 °C, well-watered plants experienced nearly 100% loss of xylem function compared to control, accompanied by a 20% increase in cell death (*REC*) of green stems (Fig. 2a) and a 70–80% increase for leaves (Fig. 2b). In contrast, reductions in k_l and cell death of drought-exposed plants were more closely matched at 70–80% loss for xylem (Fig. 2d), a 5% increase in cell death for green stems and 50–60% for leaves.

DISCUSSION

Biophysical changes during drought may protect leaves from freezing damage, while at the same time, decreasing xylem water potential increases the likelihood of freeze-thaw embolism. Therefore, we predicted that the balance between water supply and demand following freezing would be disrupted when *L. tridentata* plants experienced freezing under drought conditions, resulting in reduced whole-plant performance and survival. We observed 100% mortality regardless of water status near the long-term minimum for the region (–24 °C). Contrary to our predictions, however, drought improved whole-plant function following exposure to –8 °C and –15 °C (Table 2; Fig. 2) and there was a trend for higher survival among drought-exposed plants following exposure to –15 and –19 °C (Fig. 1). Our results indicate that the interaction between drought and freezing may be an important consideration in understanding the effect of both year-to-year variability and directional change in thermal and water regimes on the establishment of *L. tridentata*.

Although the magnitude of the drought we imposed was larger in 2008 than in 2007, in both years, drought-exposed plants reduced growth and lost leaf area following the application of watering treatments, an indication that physiological changes were taking place. Water potentials of drought-exposed plants in our study were higher than those generally observed in *L. tridentata* in the field during the late spring/early summer and fall, but typical during the winters at our seed collection site (Martínez-Vilalta & Pockman 2002). While high water availability is still likely to favour seed germination, our results suggest that mild drought currently typical of winters in the Northern Chihuahuan Desert improves the performance and survival of juvenile *L. tridentata* experiencing freezing. Given that winter temperatures and precipitation are predicted to increase in Western North America (Christensen *et al*. 2007), our data indicate that *L. tridentata* will continue to experience low temperature limits on expansion into adjacent grasslands, as decreased performance and survival would be expected when temperatures below –15 °C co-occur with saturating precipitation events. Other modelling efforts have predicted increased drought frequency for Western North America (Sheffield & Wood 2008), in which case, our results predict increased abundance of *L. tridentata*, even given the most conservative estimates of increased winter minimum temperature, which are in the range of 1 °C (Christensen *et al*. 2007).

We found that leaves were more tolerant of freezing in the presence of drought, as has been observed in other woody evergreens (Ewers *et al*. 2003; Cavender-Bares *et al*. 2005). Furthermore, our observations of xylem embolism in intact plants experiencing a combination of drought and freezing are similar to those of Pockman & Sperry (1997) and Martínez-Vilalta & Pockman (2002) in which excised stems of *L. tridentata* became completely embolized between –16 and –20 °C after experiencing plant water potentials of –3 to –4 MPa in the field. Unexpectedly, we did not observe higher xylem vulnerability to freeze-thaw embolism in drought-exposed compared to well-watered plants, while in other experiments, drought significantly increased xylem vulnerability to freezing (Davis *et al*. 1999; Ewers *et al*. 2003). What differences could account for the opposite effect we observed? One difference may be that the plants in our study were intact throughout drought and freezing treatments, while previous studies have measured xylem embolism of branches that experienced freezing following their removal from the plant (e.g. Pockman & Sperry 1997). The preservation of whole-plant function by this method could have avoided cellular responses to cutting or maintained the ability of living cells to respond to freezing by further mobilizing a stress response which had already begun under drought.

A number of gene transcription factors induced in response to drought can confer enhanced cold tolerance. Dehydrin genes have been shown to be up-regulated during drought stress and subsequently contribute to cold tolerance, possibly through their activity in protecting enzyme (Peng *et al*. 2008b) and/or membrane function (Puhakainen *et al*. 2004). In barley, 34% overlap in transcriptomes of genes in the dehydrin family was observed when plants were exposed to either drought or freeze-thaw stress (Tommasini *et al*. 2008). In our experiment, drought-exposed plants may have benefitted from the up-regulation of drought-induced gene transcripts, allowing more rapid response and recovery following freezing. Greater whole-plant performance of drought-exposed compared to well-watered plants following exposure to –8 °C may also be due in part to osmotic adjustment during drought, resulting in greater super-cooling in both green stems and wood of drought-exposed plants. Supercooling only delayed the occurrence of freezing exotherms from –7 °C in well-watered plants to –9 °C in drought-exposed plants, however suggesting that the differences between the watering treatments in the –15 and –19 °C treatments were instead due to differences in other physical characteristics or responses.

Our results indicate that further investigation into the mechanism of drought-induced freezing tolerance is warranted in *L. tridentata*, particularly given our observation that freezing tolerance of leaves and xylem were more closely matched in the presence of drought compared to well-watered conditions. Although no relationship between leaf and xylem freezing tolerance has been found among

vessel-less angiosperms (Feild & Brodribb 2001) or conifers (Coopman *et al*. 2008), strong co-ordination has been observed in species of oaks (Cavender-Bares *et al*. 2005). In our study, greater vulnerability of leaf versus xylem tissue was accompanied by re-sprouting, which occurred in both drought-exposed and well-watered plants following exposure to a temperature near the long-term minimum recorded at our field site (–19 °C). Re-sprouting following freezing may be a cost-effective way of tolerating extreme, but infrequent, freezes and has been observed previously in *L. tridentata* (Cottam 1937) and in cold-tolerant species of *Ceanothus* (Ewers *et al*. 2003). Under severe drought, however, this balance may be disrupted and the range of temperatures where drought-exposed plants outperform well-watered plants may shift, as increased solute accumulation provides greater protection from freezing in living cells and the likelihood of xylem embolism increases.

We also observed a trend for embolism to increase with decreasing minimum temperature among drought-exposed plants, another pattern previously observed in *L. tridentata* (Martínez-Vilalta & Pockman 2002). One possible explanation is that mortality increased among the xylem parenchyma cells that may have supported refilling as minimum temperature decreased (Pockman & Sperry 1997; Martínez-Vilalta & Pockman 2002). At the current field site under study, large fluctuations in minimum temperatures occur over the winter months, and although plants experience an average of 14 ± 6 d per year on which temperatures fall to –8 °C or below, the average maximum daily temperature for the months of November–February from 1999 to 2008 was 19.5 ± 2.6 °C (Moore 1989–2009). Under such circumstances, embolism re-filling could increase the ability of plants to take immediate advantage of brief warm periods. A role for sugars in refilling of embolism has been suggested (Salleo *et al*. 2009), and sugar content increases not only in living cells during drought (Naser *et al*. 2010) and cold exposure (Walker, Romero & Correal 2010) but also in xylem sap (Wong, Baggett & Rye 2003). Furthermore, overlapping gene induction for compounds such as raffinose occurs under both drought and cold stress (Taji *et al*. 2002). The possibility and mechanism of vessel re-filling has yet to be investigated in the genus *Larrea*, however, and techniques that interrupt the phloem could further distinguish between physical and biological mechanism behind decreased xylem vulnerability to freezing in the presence of drought.

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