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Research paper

Root resistance to cavitation is accurately measured using a centrifuge technique

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Plants transport water under negative pressure and this makes their xylem vulnerable to cavitation. Among plant organs, root xylem is often highly vulnerable to cavitation due to water stress. The use of centrifuge methods to study organs, such as roots, that have long vessels are hypothesized to produce erroneous estimates of cavitation resistance due to the presence of open vessels through measured samples. The assumption that roots have long vessels may be premature since data for root vessel length are sparse; moreover, recent studies have not supported the existence of a long-vessel artifact for stems when a standard centrifuge technique was used. We examined resistance to cavitation estimated using a standard centrifuge technique and compared these values with native embolism measurements for roots of seven woody species grown in a common garden. For one species we also measured vulnerability using single-vessel air injection. We found excellent agreement between root native embolism and the levels of embolism measured using a centrifuge technique, and with air-seeding estimates from single-vessel injection. Estimates of cavitation resistance measured from centrifuge curves were biologically meaningful and were correlated with field minimum water potentials, vessel diameter (VD), maximum xylem-specific conductivity (K_{smax}) and vessel length. Roots did not have unusually long vessels compared with stems; moreover, root vessel length was not correlated to VD or to the vessel length of stems. These results suggest that root cavitation resistance can be accurately and efficiently measured using a standard centrifuge method and that roots are highly vulnerable to cavitation. The role of root cavitation resistance in determining drought tolerance of woody species deserves further study, particularly in the context of climate change.

Keywords: cavitation, centrifuge, drought, embolism, vessels, xylem.

Introduction

Plants transport water under negative pressure and this makes their vascular system prone to cavitation (the conversion of water from liquid to vapor). The end result of cavitation is a gas filled (embolized) vessel or tracheid that no longer transports water. Under water stress, if many conduits become embolized, a reduction in water transport efficiency can lead to greater negative pressures, furthering cavitation in a vicious cycle called runaway cavitation (Tyree and Sperry 1988). Coping with cavitation via avoidance, tolerance and repair is well established to be a dominant factor driving the evolution of xylem structure and function (Carlquist 2001, Tyree and Zimmermann 2002) and the ability to resist cavitation is a key trait that determines a woody plant's ability to survive water stress during drought (Rice et al. 2004, Pratt et al. 2008, Kursar et al. 2009). This is important in the context of climate change in order to predict the response of species, communities and ecosystems to a warming and drying climate (Hoffmann et al. 2011, McDowell et al. 2011, Anderegg et al. 2012*b*, Nardini et al. 2013, Pratt et al. 2014).

Cavitation can occur in the xylem of any plant organ, and a similar process can occur in the soil (Sperry et al. 1998). Some long-lived plants may restrict cavitation to distal organs (leaves or branches) as a means of reducing water loss during drought and protecting more costly structures such as main stems

(Zimmermann 1983, Rood et al. 2000). Roots are generally found to be more vulnerable to cavitation than stems when compared using losses of conductivity relative to a maximum value following emboli removal, i.e. percentage loss of conductivity (PLC) (however, see Tsuda and Tyree 1997, Hukin et al. 2005); however, when comparisons are made as xylem-specific conductivity (K_s) and compared on an absolute rather than a relative basis, roots may match or exceed conductivities of other organs in spite of being substantially more embolized. This is because roots tend to be much more hydraulically efficient than stems, i.e. they start at a much higher level of hydraulic conductivity (Martínez-Vilalta et al. 2002, Pratt et al. 2008). Nevertheless, roots often experience substantial losses of conductivity during drought, making them a weak point of the vascular system in both angiosperms and gymnosperms (Mencuccini and Comstock 1997, Matzner et al. 2001, Stout and Sala 2003, Domec et al. 2006, Maherali et al. 2006, Pratt et al. 2007*b*).

Methods to measure cavitation resistance of woody plants have been debated, and some have suggested that centrifuge methods to characterize cavitation resistance do not yield reliable results (Choat et al. 2010, Cochard et al. 2010). In contrast, others have found good agreement between vulnerability curves constructed with the standard centrifuge method using the Alder et al. (1997) rotor design, when data are compared with other non-centrifuge methods (Christman et al. 2012, Jacobsen and Pratt 2012, Sperry et al. 2012, Tobin et al. 2013, Jacobsen et al. 2014, Hacke et al. 2015). Some have suggested that roots are not amenable to study using centrifuge methods because they have long vessels (McElrone et al. 2004, Choat et al. 2010, McElrone et al. 2012). However, it may be premature to suggest that roots generally have long vessels, since there is a paucity of published data on root vessel lengths (Jacobsen et al. 2012). Nevertheless, a study of the accuracy of standard centrifuge-derived root vulnerability to cavitation estimates is lacking and would help resolve these issues.

It is a standard practice to validate one method by comparing it with different and independent methods. In the present study we examined estimates of root cavitation measured in the native state and compared them with estimates generated using centrifugal force. There are two standard methods that are generally agreed to yield robust estimates of the levels of embolism of intact plants. The first is to measure native embolism by carefully excising stems or roots from intact plants and then making measurements shortly thereafter. Wheeler et al. (2013) recently suggested that cutting xylem under tension nucleates cavitation, but a subsequent study has found that trimming at least 0.5 cm from sample ends prevented this potential effect from impacting measurements (Venturas et al. 2014). Thus, cutting samples slightly longer than what will be measured and removing the portion of affected xylem easily overcomes any artifact due to cutting under tension. A second method is to sample very large branches and to let them dry down under controlled conditions in a laboratory (Sperry et al. 1988*b*). It is generally not possible to harvest an intact root system and allow it to dry down on a benchtop, thus native embolism measurements are the chief standard by which to compare other methods for roots.

Recent studies have used imaging methods, MRI and HRCT, as a comparison with other methods (Choat et al. 2010, Torres-Ruiz et al. 2014). Imaging roots for the presence of embolism has not been done to our knowledge and will require removing them in much the same way they are removed for native embolism. It will be challenging to measure xylem water potential on a detached root for imaging (this will likely require psychrometers). Additionally, imaging methods are not generally available to researchers, are very expensive and have not been thoroughly tested (Hacke et al. 2015), thus more traditional and standard measurements such as native embolism measurements will continue to be an important benchmark to compare among methods, especially in the case of roots.

There is a third set of methods that push air across pit membranes to nucleate cavitation. In an intact vascular system, cavitation occurs when air is seeded into a vessel when it is pulled through a pit membrane. Positive pressure methods assume that the air-seed pressure is the same whether it is pulled into a vessel by negative pressures or pushed into a vessel by external positive pressures. There are different approaches in these methods, and one is to place most of an organ into a chamber and pressurize it (Cochard et al. 1992). Another approach is to insert micro-capillaries into individual vessels in small cut segments (typically ~5 cm long) and force air into the vessel (Zwieniecki et al. 2001, Melcher et al. 2003). The non-injected end of the cut organ is placed in a beaker of water and the air-seed pressure is taken as the point when gas streams from a vessel at the cut end under water.

In the present study we examined root resistance to cavitation using three methods: a standard centrifuge method (Alder et al. 1997), native embolism and single-vessel air injection. The chief goals were to evaluate the cavitation resistance of roots and to test the validity of root cavitation resistance as measured by the standard centrifuge method by comparing it with the other two methods. The standard centrifuge method is preferable over other methods because it is faster, provides an analysis across a full range of water potentials and yields information on hydraulic conductivity as well as resistance to cavitation. An additional goal of this study was to examine root vessel lengths. There are very few studies of this topic and some have assumed, possibly prematurely, that roots have long vessels. To put root vessel lengths into context, they were compared with stem vessel lengths measured on the same individuals and with a global data set (Jacobsen et al. 2012).

Materials and methods

Chaparral shrubs were propagated from cuttings or seedlings harvested from field sites in southern California and grown in the

ground in a common garden on campus at California State University, Bakersfield located in Bakersfield (35°20'46.79"N, 119°5'58.37"W), CA, USA (species listed in Table 1). All source sites contained a mix of chaparral species and all of them contained the common chaparral shrub *Adenostoma fasciculatum*. The field sites and Bakersfield have a Mediterranean-type rainfall pattern where rain falls in the winter and early spring months and does not fall in the summer and autumn months. Bakersfield is hotter and drier than the field sites where the plants naturally occur and, to compensate for this, the common garden was watered with spray-type sprinklers from October to July to supplement the natural precipitation so that the total for the year was ~500 mm per year (similar or slightly greater than what they typically experience in their native environment). For the months of July–October the common garden was not watered and rain did not fall. At the time of sampling, plants were established and several years old.

All of the sampled species are common members of the chaparral shrub vegetation type of southern California and they all co-occur at multiple points along their distribution. All of the species are relatively long-lived (30–100+ years). Two of the species are non-resprouters after fire (*Ceanothus vestitus* and *Trichostema lanatum*) and recruit abundant seedlings after fire from a seed bank. One of the species resprouts after fire and also recruits seedlings from a seedbank (*A. fasciculatum*), whereas the rest of the species resprout only after fire and do not typically recruit seedlings in a post-fire environment. Instead, their seeds will typically germinate in mature stands.

A series of measurements were made that relate to vascular functioning of the root systems of the shrubs. In all cases, the roots that were sampled were shallow (within the top 50 cm of the soil). We made efforts to standardize our sampled roots and the diameters to ~3–6 mm; however, this is more challenging than with stems because they have to be unearthed and the roots coming from the base of the plant tend to vary widely in diameter.

Cavitation resistance

In summer 2011, the vulnerability to cavitation of roots was measured for our target species using a centrifuge method (Alder et al. 1997). Roots were harvested from plants from the garden by cutting them off under water so as to avoid nucleating cavitation. Root lengths harvested in the field were mostly 20 cm or longer. Ten milliliter volume centrifuge tubes filled with filtered water were attached to the cut ends of root segments with Parafilm immediately after excising them, and roots were double bagged in opaque plastic bags with a moist paper towel. Roots were transported to a laboratory within 1 h of harvesting and were sampled the same day or within 3 days. Roots that were not sampled the same day were cold-stored at 4 °C in the plastic bags and wrapped in moist paper towels.

For measurements, roots were trimmed under water to remove the initially cut ends and to obtain segments slightly longer than 14 cm; a fresh razor blade was then used to shave root ends to achieve a clean measurement surface and a final length of 14 cm. Roots were flushed under high pressure with an ultradegassed (MiniModule membrane contactor, Membrana, Charlotte, NC, USA) and ultrafiltered (1 µm pore exclusion) 20 mM KCl solution for 1 h (this same solution was used to measure hydraulic conductivity). This was done to flush out any native emboli.

Table 1. Species mean (1 SE in parentheses) root maximum and mean vessel lengths (VL_{max} and VL_{mean}; $n = 3$), stem VL_{max}, the water potential at 50 and 90% loss of conductivity (P50, P90) from centrifuge vulnerability curves ($n = 6$), the predawn water potential (Ψ_{pd}), maximum xylem-specific conductivity (K_{smax}) and mean vessel diameter (VD).

Leaf habit and species	Family	VL_{max} (m) Roots	VL_{mean} (m) Roots	VL_{max} (m) Stems	P50 (MPa) Roots	P90 (MPa) Roots	Ψ_{pd} (MPa) Roots	K_{smax} (kg s^{-1} MPa^{-1} m^{-1})	VD (µm)
Evergreen									
A. fasciculatum Hook. & Arn.	Rosaceae	0.75 (0.09)	0.07 (0.01)	0.25 (0.04)	−1.46 (0.38)	−7.69 (1.62)	−1.75 (0.09)	5.25 (0.52)	30.51 (1.03)
C. vestitus Greene[1]	Rhamnaceae	0.46 (0.14)	0.04 (0.01)	0.18 (0.04)	−2.96 (0.30)	−9.46 (1.26)	−3.77 (0.07)	6.48 (0.76)	36.19 (1.95)
M. laurina (Nutt.) Nutt. ex Abrams	Anacardiaceae	0.86 (0.18)	0.07 (n/a)[3]	0.90 (0.03)	−0.24 (0.06)	−1.12 (0.28)	−0.76 (0.07)	14.25 (4.14)	65.96 (5.31)
R. ovata S. Watson	Anacardiaceae	1.05 (0.15)	0.08 (0.02)	0.49 (0.17)	−1.06 (0.18)	−2.55 (0.54)	−1.02 (0.06)	9.22 (2.73)	44.51 (3.77)
Deciduous									
Rhus aromatica Aiton[2]	Anacardiaceae	0.95 (0.11)	0.03 (0.01)	0.96 (0.31)	−1.03 (0.13)	−2.84 (0.33)	−1.75 (0.07)	6.09 (1.51)	40.86 (0.89)
S. nigra L. subsp. canadensis (L.) Bolli	Adoxaceae	0.75 (0.30)	0.02 (0.01)	1.37 (0.45)	−0.53 (0.10)	−1.83 (0.29)	−0.90 (0.07)	25.44 (4.00)	69.59 (2.04)
T. lanatum Benth.	Lamiaceae	0.84 (0.14)	0.09 (n/a)[3]	0.47 (0.05)	−0.66 (0.31)	−3.07 (0.40)	−1.79 (0.08)	6.69 (1.93)	41.42 (1.49)

[1]Formerly *C. greggii*.
[2]Formerly *R. trilobata*.
[3]An $n = 2$ precludes SE calculation.

Hydraulic conductivity was measured in the absence of emboli (K_{max}) using a gravity-induced pressure head in series with the root connected into a tubing system, with an outflow onto an analytical balance (CP124S, Sartorius, Goettingen, Germany) connected to a computer (Sperry et al. 1988*a*). After measuring K_{max}, roots were spun in a centrifuge for 5 min at a given rpm to achieve a negative xylem pressure, which caused cavitation and embolism in the roots (Sperry et al. 2012, Tobin et al. 2013). Then their hydraulic conductivity (K_h) was measured. Root segments were spun at increasing rpms until their K_h was near zero. Because roots typically have wide diameter vessels (Pratt et al. 2007*a*), and our samples contained some vessels that were open (did not contain a terminal vessel element), we kept the pressure head low (~2 kPa) to avoid refilling such vessels if they were embolized. We also took care when inserting roots into our tubing apparatus because we have previously observed that a pressure build-up upon insertion can lead to refilling. The loss of hydraulic conductivity was calculated as

$$\text{Loss in conductivity} = 1 - \frac{K_h}{K_{max}} \quad (1)$$

and multiplied by 100 to convert to a percentage. Vulnerability to cavitation was determined by fitting a Weibull curve to each vulnerability curve per root with the best curve fit being determined by minimizing the sum of the squared differences between the measured and fit values with Excel's solver function (Excel 2007, Microsoft, Bellingham, WA, USA). From each curve the water potential at 50 and 90% loss in K_h were calculated as estimates of cavitation resistance. Maximum specific conductivity of xylem (K_{smax}) was calculated dividing K_{max} by the xylem cross-sectional area. This takes into account root size and allows comparing conductivity across roots that differ in size.

Following the determination of the vulnerability curve, root segments were transversely thin-sectioned and analyzed microscopically. From each root sample, the cross-sectional area of ~100 vessels was measured freehand using ImageJ software (ImageJ 1.44p, National Institutes of Health, Bethesda, MD, USA). These areas were then used to calculate the vessel diameter (VD), assuming that vessels were circular, and the mean VD was then calculated.

Native embolism and centrifuge compared

Root native embolism was measured for roots of our target species on 3 days in the summer 2012: 31 August, 14 and 28 September. For each species, three to five different individuals were sampled. In some cases, multiple roots were sampled from the same individual, in which case the data were averaged prior to analysis to avoid pseudoreplication. The evening prior to a measurement day we went to the field and partially excavated roots, taking care not to damage them. Roots were tagged, reburied and a pin flag was put in the ground to facilitate finding them the following morning. At predawn the next morning the roots were unearthed and harvested by cutting them off underwater as previously described for the vulnerability curves. Roots were immediately transferred back to a laboratory located ~0.25 km from our study site. Roots were cut down under water to ~15 cm in length and then trimmed with a razor blade to 14 cm and their K_h was measured. Roots were then flushed and remeasured to obtain K_{max} (see methods for cavitation resistance above). Native state embolism was expressed as Eq. (1) multiplied by 100.

To estimate root water potential, the evening prior to harvesting of roots, four branchlets per plant were sealed in thick plastic bags. Branchlets or leaves were selected at the ground level from stems that were coming directly out of the soil and attached to a root (not the same root for K_h measures). At predawn the bagged leaves or small branchlets were excised, placed in a sealed bag in a cool box at 4 °C, carried to a laboratory and their water potential measured with a Scholander pressure chamber within 2 h (Model 2000, PMS Instruments, Albany, OR, USA).

After measuring K_{max} of roots, each root was individually spun in a centrifuge at an rpm that recreated the water potential measured at predawn. The K_h was measured following this spin and loss of conductivity was calculated [Eq. (1)]. Stems, even within the same plant, have been shown to vary in their levels of cavitation resistance, K_h, and native embolism, all of which are sources of error when making comparisons among different methods (see Hacke et al. 2015). It is likely that roots are also variable. Measuring the same root repeatedly is advantageous experimentally because it holds all sources of variation between roots constant and allows for a careful comparison between native state embolism and the level of embolism generated using centrifugal force. For example, flushing xylem to remove emboli may refill some xylem conduits that were not functional and may have been fatigued, i.e., they underwent cavitation and became more vulnerable to cavitation as a result (Hacke et al. 2001). Using matched samples as we have done controls for any fatigued vessels and makes native state levels of embolism directly comparable to centrifuge-generated embolism.

Single-vessel injection

Roots were collected in September 2014 from *Malosma laurina* in the same common garden as sampled for other measurements. Four different individuals were sampled and roots were harvested in a similar fashion to those for native embolism. Roots were flushed with 100 kPa of degassed and filtered KCl solution within 2 h of harvest in the manner described above. After flushing, roots were trimmed to 5 cm for measurements.

Micro-capillaries (Item 1B150-4, World Precision Instruments, Inc., Sarasota, FL, USA) were pulled so that the tips had very narrow diameters and then trimmed with a razor blade to achieve a hollow point 40–80 μm in diameter. The non-tapered end of a capillary was inserted into a brass fitting with the aid of Teflon

tape to get a tight fit between the metal and glass. The other end of the brass fitting was connected to PEEK tubing (green) that was inserted into a pressure chamber (Model 2000, PMS Instruments). Tapered capillary points were inserted into individual vessels with the aid of a dissecting microscope and a micromanipulator. Once inserted, a vessel was pressurized at low pressure (50 kPa) to determine whether it was open, i.e., if air flowed through the vessel at that low pressure it was assumed that it contained no terminal vessel element and another vessel was injected. If no air flowed through the vessel at this low pressure, the micro-capillary was glued into the vessel with fast drying cyanoacrylate glue (Loctite, Super Glue Gel Control, Henkel Corp., Rocky Hill, CT, USA). Vessels were slowly pressurized (increasing ~0.01 MPa every 10 s) with the control provided by the pressure chamber and the non-injected cut end of the roots were observed. When gas began to stream from a vessel, the pressure was recorded. We then carefully removed the capillary from the vessel and the glue in order to inject another vessel. We obtained one to five air-seeding pressures from every 5-cm-long root segment and evaluated 23 segments in total.

We report air-seed pressures for 117 vessels from our 5-cm-long segments. The approach that would be most directly comparable to embolism measurements sampled natively or in a centrifuge would be to only sample the single vessel being injected, which was not possible in the present study. Vessel lengths vary and it is likely that some vessels we measured were from two or more vessels in series with the injected vessel. We chose to sample 5-cm-long segments because it represented a good compromise in that it minimized the number of vessels sampled in our air-injection pathway while providing a reasonable number of vessels that contained at least one terminal vessel element that could be measured (~25% of the vessels we pushed air into were not open). Any error in our data due to sampling multiple vessels should lead to an overestimation of root cavitation resistance, which would not change the chief interpretations of the data.

The data were expressed as a cumulative loss of vessels (% of total sampled) plotted against the air-seed pressure recorded. The percentage of vessels that cavitate at a given pressure may not be the same as the PLC, since we do not know how much each vessel contributed to flow. However, it is possible that the data examined this way are closely correlated to the percentage loss of hydraulic conductivity if each injected vessel makes an equal contribution to the root conductivity. This is probably a realistic assumption for the present study, since we injected similar diameter vessels.

Maximum vessel length

Maximum vessel length (VL_{max}) of roots and stems were measured using the air-injection method (Greenidge 1952). Measurements were performed in late July and August 2014. Both stems and roots were sampled from the same individuals and all sampled plants were growing in the common garden at Bakersfield from which root vulnerability to cavitation and native embolism had been characterized. Sampled roots were similar to those collected for measuring native embolism but much longer (~0.75–2 m). Water-filled centrifuge tubes were connected to the ends of the roots to keep them hydrated prior to measurements.

After harvest, roots and stems were transported to a laboratory where they were measured the same day. For roots, a plastic tube was fitted to the cut end farthest from the plant base and a modest pressure of nitrogen gas was applied (50 kPa). The end nearest to the plant base of the root was submerged in water and was monitored for air bubbles. For stems, a branch tip was cut to expose a stem xylem segment similar in diameter to those injected for roots. Plastic tubing was connected to this stem segment and air was injected from this distal branch toward the proximal branch end and this end was monitored for air bubbles as the branch was shortened. Stems were measured using an applied pressure of 100 kPa. One-centimeter cuts were made from the non-injected end of roots or stems until a bubble was observed coming from a xylem vessel and in all cases it was clearly visible that the bubble was coming from a single vessel. The length of the branch or root was then measured and recorded as the VL_{max}.

Mean vessel lengths of roots were measured on samples harvested in 2011 at the same time and from the same plants from which the vulnerability curves were measured. Sample sizes varied from two to five individuals per species. Roots similar in diameter to those used for other measures were carefully excavated, wrapped in moist paper towels and transported to the laboratory where they were trimmed underwater to 25 cm using fresh razor blades. Roots were then flushed to remove emboli as described above. Flushed roots were fitted into a tubing apparatus and injected with silicon (Rhodorsil RTC-141, Bluestar Silicones, Rock Hill, SC, USA) containing a 1% fluorescent dye (Uvitex OB, Ciba Specialty Chemicals, Basel, Switzerland) dissolved in chloroform for 24 h at 50 kPa. Following injection, roots were cured at room temperature for several days. Roots were submerged underwater to soften tissue prior to their being sectioned for microscopic analysis. Roots were serially sectioned up to a distance of 24 cm from the injection end and the number of filled vessels recorded at increasing distance from the injection end. These values were then used to determine the mean vessel length. Because of the large influence of a few very long vessels on these calculations, we used the log-transformed length distribution for our mean vessel length calculations because the mean from the untransformed data is biased toward the longer vessels (Wheeler et al. 2005, Lens et al. 2011).

Statistics

Comparisons between native embolism and centrifuge-induced embolism were analyzed by comparing the slope between the two

variables. No difference between the two would be indicated by a slope of 1. Standardized major axis regression was used to estimate the slope because error in both the *y* and *x* variables requires a model that addresses this (Warton et al. 2006). In cases where we were examining relationships between two continuous variables, but not interested in the slope, Pearson correlation analyses were used. Because of the small sample size for these analyses ($n = 7$), we set $\alpha = 0.10$ to reduce the type II error rate.

Results

Species differed broadly in their root and stem vessel lengths, root cavitation resistance, predawn water potentials, K_{smax} and VD (Table 1). Maximum vessel length measured on roots and stems of the same individuals growing in a common garden was not correlated (Figure 1a). Four of the seven species had longer maximum vessels in their roots, but three did not and one had longer vessels in its stems (Figure 1a, Table 1). The mean and maximum root vessel lengths were not correlated ($r = 0.392$, $P = 0.385$). Two estimates of root cavitation resistance, the water potential at 50% (P50) and 90% (P90) loss in hydraulic conductivity, were strongly correlated (Figure 1c). Species with greater cavitation resistance (more negative P50) experienced more negative predawn and midday water potentials (Figure 1e), and tended to have shorter maximum vessels (Figure 1b). More negative predawn water potentials were associated with shorter

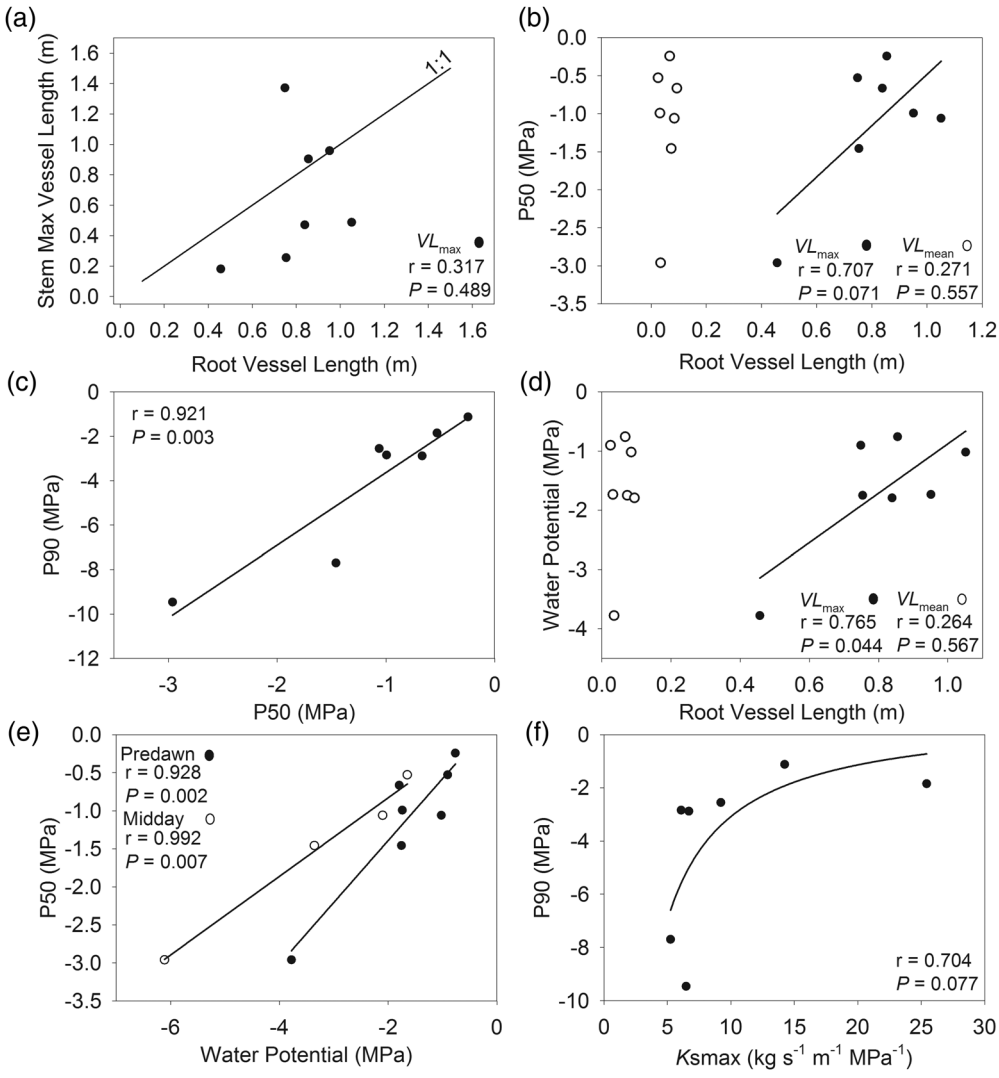

Figure 1. Scatterplots showing relationships between root and stem VL_{max} (a), the water potential at 50% (P50) and 90% (P90) loss in hydraulic conductivity [estimates of cavitation resistance (c)], cavitation resistance and predawn and midday water potential (e), the relationship between cavitation resistance and vessel length (b), water potential and vessel length (d) and cavitation resistance and hydraulic efficiency (K_{smax}) (f). Each point represents a mean for a species ($n = 3$–8; $n = 2$ for two VL_{mean} values as noted in Table 1) and statistics are from Pearson correlation analyses (the *r* and *P* values for panel f are for log/log data). Note, not all species were sampled for midday water potentials (e). In (a), the 1 : 1 line is shown to assist in the comparison of stem and root VL_{max} and all other lines are linear regression fits. The descriptive statistics, including errors, are listed by species in Table 1.

maximum vessels (Figure 1d). There was a tradeoff between safety and hydraulic efficiency (Figure 1f). Vessel diameter was not correlated with either VL_{max} ($r = 0.164$, $P = 0.726$) or mean vessel length ($r = -0.270$, $P = 0.557$). Xylem-specific conductivity was strongly correlated with VD ($r = 0.908$, $P = 0.005$), but was not correlated with maximum ($r = -0.303$, $P = 0.995$) or mean vessel length ($r = -0.414$, $P = 0.356$).

Native embolism and embolism generated using centrifugal force were not significantly different. This was compared by measuring root native embolism and predawn water potential for plants growing in a common garden. After flushing emboli out of the root xylem following native embolism measurements, the same roots were spun at their predawn water potential in a centrifuge. Native and centrifuged embolism showed good agreement when compared for all individual samples measured (Figure 2a), and also when analyzed as mean values for each species (Figure 2b).

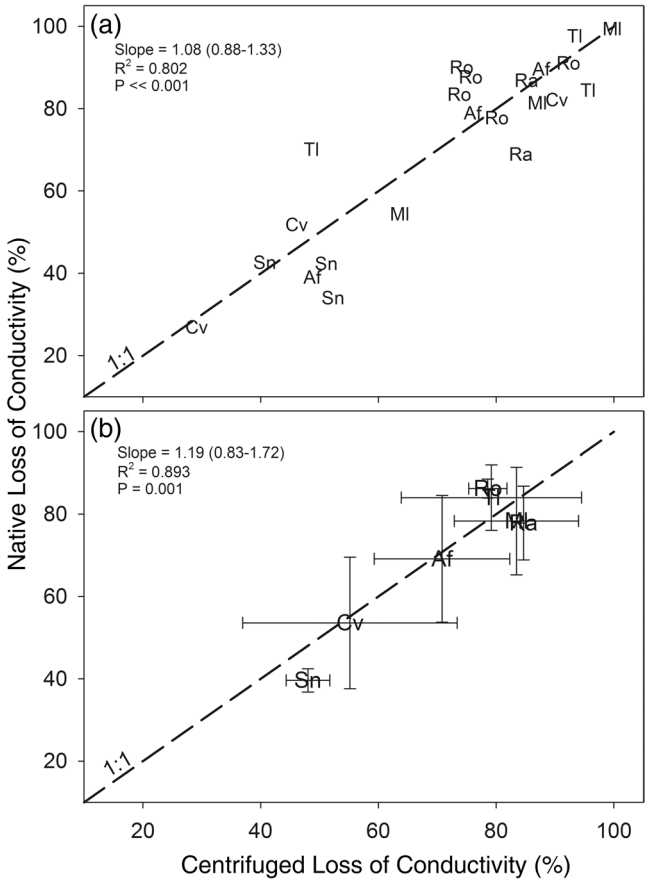

Figure 2. Percentage loss of conductivity (native embolism) was measured on roots (*y*-axis) and those same roots were spun in a centrifuge to a xylem tension that equaled the predawn water potential measured on plants in the field. Panel (a) shows data for each individual root measured and (b) shows the mean per species. Statistics are shown for standardized major axis regression, and the interval shown for the slopes are the upper and lower 95% confidence limits; neither slope was different from 1. Abbreviations are the genus and species initials (Table 1).

To assess if species with longer vessels were more prone to deviations when comparing native embolism with centrifuge-generated embolism, we calculated the % deviation from native embolism and plotted it against VL_{max} and VL_{mean}. In this analysis, values above zero are cases where the centrifuge tended to underestimate the level of native embolism, whereas negative values are cases where the centrifuge produced greater levels of embolism than what was measured in the native state. The long-vessel artifact predicts that species with longer vessels should have the most negative values. We found no relationship between VL_{max} and VL_{mean} and the degree of disagreement between native and centrifuge levels of embolism (Figure 3, $P > 0.10$), and some of the species with the longest vessels had positive deviation values, i.e., they were in the wrong direction predicted by the long-vessel artifact hypothesis. In all cases, deviations between centrifuge and native estimates were quite small and these values showed strong agreement, as already shown in Figure 2.

When native embolism values were compared with estimates of cavitation resistance from vulnerability curves generated using centrifugal force, there was generally good agreement (Figure 4). In one species, *Rhus ovata*, the levels of native embolism were greater than those predicted from the centrifuge vulnerability curve (Figure 4g), and for another species, *Sambucus nigra*, the native embolism values were less than those predicted by the centrifuge curve (Figure 4d). This discrepancy is likely due to changes in cavitation resistance over time: the vulnerability curves were measured in summer 2011 while the natives were measured in summer 2012. In the experiment shown in Figure 2, the same roots were sampled at the same time, thus minimizing error variation due to time and different individuals.

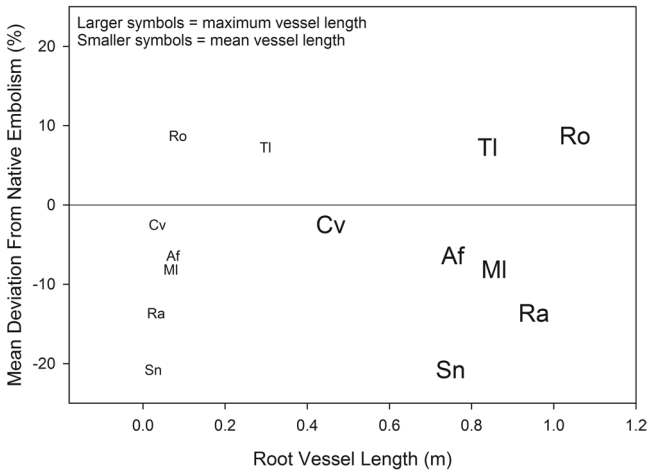

Figure 3. The deviation between the measured native and centrifuged levels of embolism plotted against root maximum and mean vessel lengths. Positive values indicate cases where the centrifuge slightly underestimated the amount of native embolism and negative values indicate cases where the centrifuge slightly overestimated levels of embolism. The long-vessel artifact predicts that mean deviations from native embolism should decrease as maximum vessel length increases.

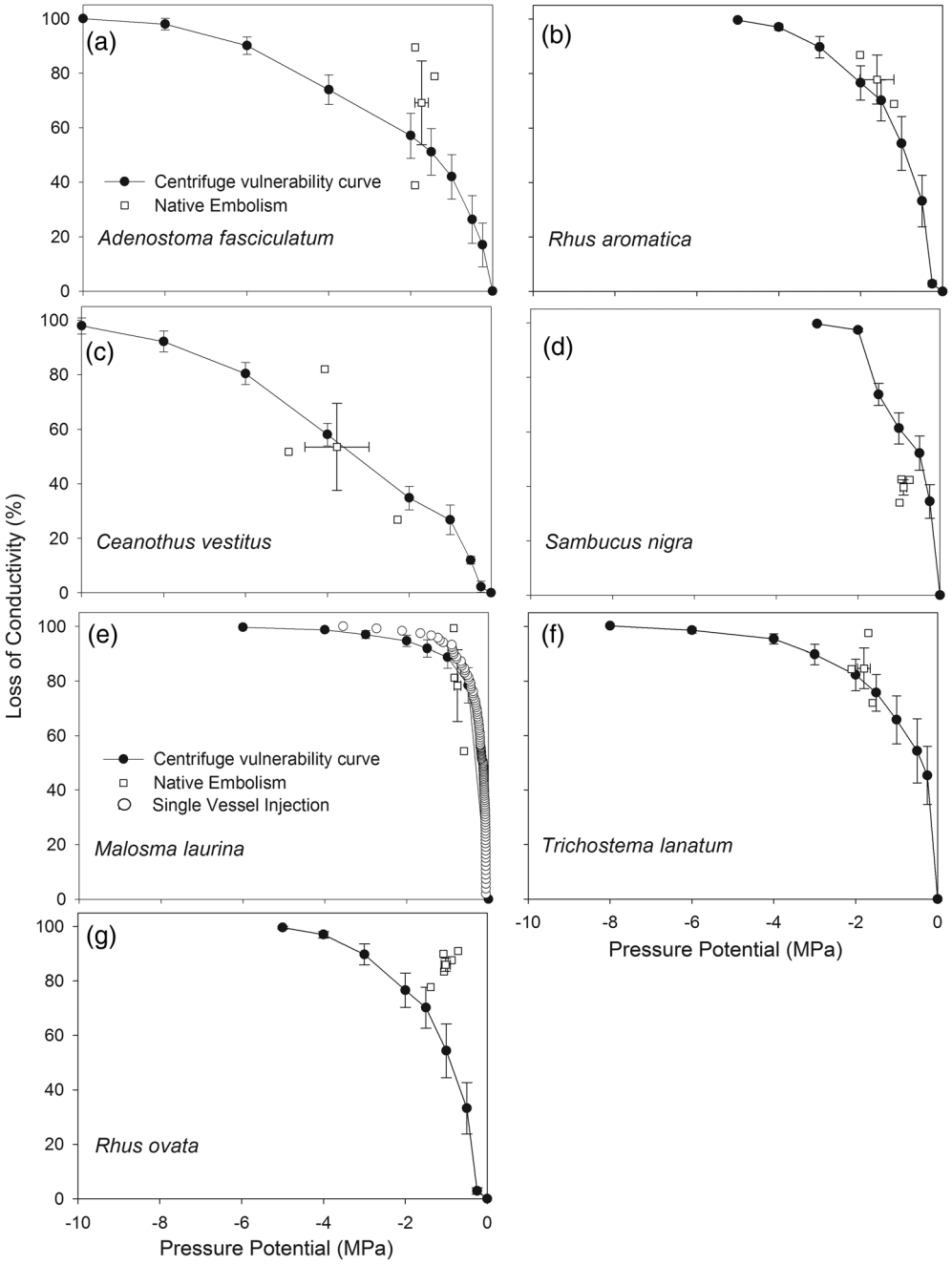

Figure 4. Percentage loss of conductivity measured for a range of xylem water potentials generated using centrifugal force (closed symbols) and native embolism values for roots sampled from plants growing in a common garden at their predawn water potentials. The *y*-axis in (e) also corresponds to the percentage of vessels that air-seeded at a particular positive pressure (MPa of *x*-axis) using the single-vessel air-injection method. Note that in this case, the *x*-axis refers to negative or positive pressure for the data in (e). Data with error bars are means ± 1 SE ($n = 6$ for centrifuge data).

In this more controlled experiment, the mean (±1 SE) native embolism values compared very well with the centrifuge values for these two species: 39.6 ± 2.8 to 48.0 ± 3.7%, respectively for *S. nigra*, and 86.2 ± 2.3 to 78.6 ± 3.2% for *R. ovata*.

Single-vessel air injection was measured for one species, *M. laurina*, and compared with native and centrifuge embolism estimates (Figure 4e). In this case, the centrifuge vulnerability curve was *r*-shaped and had a PLC between 0 and –0.25 MPa of ~80% (Figure 4e). For single-vessel injection, we observed the same *r*-shape and that ~80% of the vessels sampled air-seeded between 0.05 (the pressure used to determine if a vessel was open or not) and 0.25 MPa (Figure 4e). This indicates that a large proportion of *M. laurina* vessels cavitate at very moderate tensions, i.e., at high water potentials. The pressure potential at 50% loss in vessels was –0.14 MPa and the mean air-seed pressure was –0.35 MPa, both of which are similar to

the P50 of the centrifuge vulnerability curves that was –0.24 MPa and both of these values fell within the 95% confidence limits of centrifuge curve P50 (LCL = –0.37, UCL = –0.10). The pressure potential at 90% loss in injected vessels was –0.86 MPa, which was also not significantly different from the centrifuge estimated P90 of –1.12 MPa (Table 1; LCL = –1.73, UCL = –0.50).

Discussion

Root native and centrifuge-based embolism and 'r-shaped' vulnerability curves

Native embolism measures are an important benchmark for assessing the accuracy of different methods to estimate cavitation resistance. A recent study suggested that cutting xylem under tension for measurements of native embolism can introduce emboli in xylem vessels even if the cut is made under water (Wheeler et al. 2013). We analyzed this possibility on stems of five species, including four that had long vessels, and found that cutting under tension had no effect on native embolism measures (Venturas et al. 2014). Our study differed from that of Wheeler et al. (2013) in that they cut their samples under tension to their precise measurement length, whereas we found that if the sample is cut even 1 cm longer than the target length and the extra length is subsequently removed under water (at least 0.5 cm from each cut end) then artifacts are avoided (Venturas et al. 2014). Sampling for native embolism in the present study was done by cutting roots from plants underwater that were 5–20 cm longer than the target length, and subsequently cutting them to their final length under water thus avoiding cutting-under-tension artifacts.

Most of our sampled species displayed rapid losses in conductivity at mild water potentials (i.e., they had *r*-shaped curves) and some were extreme *r*-shapes, such as *M. laurina*. Recent studies have suggested that '*r*-shaped' curves are indicative of an artifact due to the presence of long vessels (Choat et al. 2010, Cochard et al. 2010, 2013); however, this interpretation is not supported by the present study. The veracity of these *r*-shaped curves for our measured roots is supported in the present study by the agreement between native embolism measurements and levels of embolism measured with the centrifuge. Our vulnerability curves and native embolism measures are further supported by single-vessel injection vulnerability analysis for *M. laurina*. Our results suggest that the standard centrifuge method produces accurate estimates of the vulnerability to cavitation of roots.

We are aware of only one other study that compared root native embolism with centrifuge-based measurements and they too found excellent agreement between native and centrifuge embolism measures (Sperry and Hacke 2002). Other studies have examined both conifer and angiosperm roots, but these have differed by using an air-injection method to construct vulnerability curves (Domec et al. 2004, 2006). These other studies also found agreement between root native embolism and the vulnerability curves, and many of their root vulnerability curves were *r*-shaped even for conifer species. Other studies that have examined conifer roots have found strongly *r*-shaped curves (Stout and Sala 2003, Pittermann et al. 2006, Domec et al. 2010). Conifers, with imperforate tracheids for water conducting cells, cannot be vulnerable to an artifact that results from the presence of long vessels. The agreement between native embolism values, single-vessel injection measurements and *r*-shaped vulnerability curves, as well as *r*-shaped vulnerability curves in conifers, suggests that *r*-shaped curves produced with a centrifuge are not generally due to an artifact as has been proposed by Cochard et al. (2013).

Our results support other recent studies on stems that have found good agreement between the centrifuge method described by Alder et al. (1997) and native embolism and benchtop dehydration (Jacobsen and Pratt 2012, Sperry et al. 2012, Tobin et al. 2013, Hacke et al. 2015). However, these results contrast with studies using the Cavitron centrifuge method described by Cochard et al. (2005) that has been shown to be affected by a long-vessel artifact in at least two independent labs (Cochard et al. 2010, Wang et al. 2014). It is important to note that the main difference between these centrifuge methods is that with the Alder et al. (1997) rotor design there is no flow going through the stem or root segment during centrifugation, whereas there is flow going through the segment during spinning with the Cavitron. This fundamental difference has been linked to the contrasting results obtained with both centrifuge methods (Sperry et al. 2012, Wang et al. 2014, Hacke et al. 2015).

Two of our seven sampled species showed poor agreement between native embolism and vulnerability curves. For one species, the level of native embolism was less than that predicted by the vulnerability curve (*S. nigra*), whereas the other had native embolism levels greater than that predicted by the curve (*R. ovata*). Based on a hypothesized long-vessel artifact, longer vesseled species should have centrifuge-based vulnerability curves that have artificially high levels of embolism compared with native levels. For *S. nigra*, this was the direction of the observed disagreement; however, *S. nigra* had the shortest vessels in our data set. For *R. ovata*, the species with the longest maximum vessels in our study, the direction of disagreement was in the opposite direction predicted by the long-vessel artifact.

There are a number of likely reasons why the native embolism between these two species diverged from the vulnerability curves. First of all, the vulnerability curves were constructed during the summer months in 2011, whereas the native embolism measurements were conducted in summer 2012. There is ample evidence that resistance to cavitation can change seasonally (Kolb and Sperry 1999, Jacobsen et al. 2007, 2014). In one study, the shape of the vulnerability curve shifted seasonally from being sigmoid to *r*-shaped (Tsuda and Tyree 1997). Thus,

when trying to compare data using different methods, it is vital to use a sampling approach that holds important sources of error as constant as possible so as to focus any variation on the methods comparison of interest (Jacobsen and Pratt 2012, Tobin et al. 2013, Hacke et al. 2015).

We overcame the possibility of temporally driven sampling errors by measuring native embolism and water potential on roots and then treating these same roots to their field water potential in a centrifuge. This experiment showed strong agreement between these measures. This paired experimental design has the advantage of eliminating errors associated with changes in cavitation resistance over time and by using the same roots for both treatments it minimizes errors due to variability among roots. This includes possible errors that may arise because sampled roots differ in the level of cavitation fatigue (Hacke et al. 2001) or age. A lack of standardization of plant material may have confounded some of the studies that have documented different results among different methods to measure cavitation resistance (Choat et al. 2010, Torres-Ruiz et al. 2014). Recommendations to avoid such errors have been discussed by Hacke et al. (2015).

Root structure and function

Some have suggested that roots generally have long vessels (Evert 2006, Choat et al. 2010, McElrone et al. 2012); however, we found that VL_{max} of roots were not longer than stem vessel lengths compared between the same individuals and across species (reviewed in Jacobsen et al. 2012). Moreover, the mean vessel lengths were not unusually long either. The interspecific VL_{mean} in the present study is 0.06 m, which is not different from stems of shrubs in a global data set where VL_{mean} = 0.06 m (Jacobsen et al. 2012). Roots typically have wider diameter vessels and some have suggested that vessel length and diameter are positively correlated across species, which may be related to the assumption that roots have long vessels (McElrone et al. 2004); however, a recent meta-analysis of published vessel length data for stems found that VD and length were not strongly correlated when compared interspecifically (Jacobsen et al. 2012). Likewise, in the present study VDs and lengths were not correlated across species for roots.

Root xylem cavitation resistance was correlated with a number of important parameters. There was a negative relationship between cavitation resistance and both K_{smax} and VD suggesting a tradeoff between safety and efficiency. Cavitation resistance was positively correlated with the predawn and midday water potentials measured during the dry season suggesting that the level of root cavitation resistance is adjusted to the water potentials that the plants experience (Hacke et al. 2000, Pratt et al. 2007*a*). This suggests that cavitation resistance as measured in roots in this study is informative and predictive regarding xylem function and plant water relations. Other studies reporting *r*-shaped vulnerability curves have also found that root cavitation resistance is correlated with important traits such as stomatal conductance (Domec et al. 2004, 2006) and drought survival (Sperry and Hacke 2002, Pratt et al. 2008). We conclude that *r*-shaped curves yield biologically relevant information that is applicable to a range of studies, including how woody plants succumb to mortality in response to climate change (Anderegg et al. 2012*a*).

We further hypothesize that the extreme vulnerability of some roots may represent adaptive traits. *Malosma laurina*, the species showing the highest vulnerability to cavitation, has some of the deepest roots (>12 m) among chaparral shrub species and experiences less negative water potentials than co-occurring species during the summer dry season and during drought (Thomas and Davis 1989). This species also has some shallow roots that are necessary for nutrient acquisition and it was these shallow roots that we sampled. Lifting water from deep soil layers may require the roots to have high levels of efficiency to overcome the drag of transporting water such long distances and this comes at the cost of cavitation resistance. Another possibility is that the extreme vulnerability of shallow roots might be a mechanism that leads to runaway embolism that would minimize hydraulic redistribution to dry shallow soil layers during drought, which is consistent with the higher water potentials maintained by this species compared with others that co-occur (Thomas and Davis 1989).

Some have suggested that *r*-shaped vulnerability curves imply diurnal cycles of cavitation and refilling (Cochard et al. 2013) and there is evidence that this can happen (Domec et al. 2006, Taneda and Sperry 2008). However, it is not axiomatic that an *r*-shaped cavitation response will lead to refilling and the ecological context is important to consider when analyzing this possibility. There is good evidence that there is hysteresis in the novel refilling response such that it will not occur at highly negative water potentials and that it only happens when the xylem reaches a threshold water potential (Hacke and Sperry 2003). In climates where rainfall is predictable and seasonal, like the Mediterranean-type climate where our study was conducted, refilling is likely not possible during the dry season because water potentials are too negative. Even if it were possible, we hypothesize that it is futile and maladaptive to spend resources to refill xylem if it will simply embolize again in a matter of hours. Instead of refilling, in seasonal environments the species that have *r*-shaped vulnerability curves accumulate increasing levels of embolism as the dry season progresses (Kolb and Davis 1994).

In conclusion, we found that roots did not have unusually long vessels when compared with stems. Cavitation resistance of roots was accurately measured using the standard centrifuge technique. Roots often have *r*-shaped curves and these curves provide useful estimates of cavitation resistance, in that they correlate with a range of plant functional and performance traits.

Roots represent a high proportion of transport resistance in the xylem pathway of plants (Pratt et al. 2010) and this resistance

increases when emboli develop in root xylem, thus roots represent a weak point in the transport system of many plants and are important to examine in many contexts such as understanding the drought tolerance of a species (Sperry et al. 1998, Anderegg et al. 2012*a*). Using a centrifuge method that is robust against long-vessel artifacts, such as the one used in this study, allows researchers to rapidly and efficiently determine how roots respond to water stress for a range of research questions. Expanded research on root structure and function will likely continue to illuminate how roots contribute to whole plant hydraulic function.

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Conflict of interest

None declared.

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