

Evidence for xylem embolism as a primary factor in dehydration-induced declines in leaf hydraulic conductance

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ABSTRACT

Hydraulic conductance of leaves (K_{leaf}) typically decreases with increasing water stress and recent studies have proposed different mechanisms responsible for decreasing K_{leaf} . We measured K_{leaf} concurrently with ultrasonic acoustic emissions (UAEs) in dehydrating leaves of several species to determine whether declining K_{leaf} was associated with xylem embolism. In addition, we performed experiments in which the surface tension of water in the leaf xylem was reduced by using a surfactant solution. Finally, we compared the hydraulic vulnerability of entire leaves with the leaf lamina in three species.

Leaf hydraulic vulnerability based on rehydration kinetics and UAE was very similar, except in *Quercus garryana*. However, water potentials corresponding to the initial decline in K_{leaf} and the onset of UAE in *Q. garryana* were similar. In all species tested, reducing the surface tension of water caused K_{leaf} to decline at less negative water potentials compared with leaves supplied with water. Microscopy revealed that as the fraction of embolized xylem increased, K_{leaf} declined sharply in *Q. garryana*. Measurements on leaf discs revealed that reductions in lamina hydraulic conductance with dehydration were not as great as those observed in intact leaves, suggesting that embolism was the primary mechanism for reductions in K_{leaf} during dehydration.

Key-words: cavitation; drought stress; transpiration; water potential.

INTRODUCTION

The water transport pathway through leaves must remain functional in order for them to acquire adequate carbon via photosynthesis. However, many studies have observed reductions in the hydraulic conductance of intact and excised leaves during dehydration. There has been much debate in the literature as to the mechanism of declining leaf hydraulic conductance (K_{leaf}) as leaf water potentials

decrease. Embolism, conduit deformation or implosion, and changes in the extra-xylary pathway (e.g. membrane porosity effects, aquaporin expression) have all been suggested as playing a role in this decline.

Recent studies have provided support for leaf xylem embolism as the primary cause of decreases in K_{leaf} (Nardini, Tyree & Salleo 2001; Nardini, Salleo & Raimondo 2003; Woodruff *et al.* 2007; Johnson *et al.* 2009a). Decreases in K_{leaf} have also been attributed to implosion or deformation of vein xylem (Cochard *et al.* 2004) and accessory transfusion tissue (Brodrribb & Holbrook 2005). In another recent study, leaf minor vein hydraulic vulnerability was closely associated with structural dimensions that dictate vulnerability to conduit collapse (Blackman, Brodrribb & Jordan 2010). Thus, the degree to which we can generalize about events inside the leaf xylem during dehydration is still unclear. Our understanding of the extraxylary pathway of water flow and its impact on K_{leaf} is even less clear.

It has been estimated that the extraxylary portion of the leaf hydraulic pathway may account for over 70% of the total leaf hydraulic resistance in some species (although this varies dramatically between species; see Sack & Holbrook 2006 for review). This is not surprising given that after crossing the vein bundle sheaths, water must travel either through symplastic or apoplastic pathways that present higher resistance than flow through xylem elements. As the extraxylary portion of the pathway often accounts for such a large fraction of the resistance, even small changes could make a large difference in overall K_{leaf} . In fact, this component of the water transport pathway has been shown to respond to both temperature and light, resulting in large changes in overall K_{leaf} (Sack, Streeter & Holbrook 2004; Cochard *et al.* 2007a; Voicu, Zwiazek & Tyree 2008).

The overall objective of this study was to determine the extent to which K_{leaf} decline during dehydration was associated with xylem embolism in four angiosperms and one coniferous species. To accomplish this goal, three experiments were conducted. Firstly, ultrasonic acoustic emission was used to detect xylem embolism in dehydrating leaves. Secondly, leaves were rehydrated in a solution containing a surfactant to reduce the surface tension of water by ~55% and were then dehydrated and K_{leaf} decline was compared

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with leaves that had been rehydrated in water. Finally, in three angiosperm species, the extraxylary versus xylary contribution to K_{leaf} decline was assessed by using leaf disc rehydration kinetics to estimate the decline in leaf lamina hydraulic conductance (K_{disc}). Midvein staining was also performed on one species to determine if the amount of embolized xylem was related to losses in hydraulic conductance.

MATERIALS AND METHODS

Plant material

Four woody angiosperm species (three deciduous, one evergreen) and one coniferous species were chosen for the study based on ease of access and as being representative of several different plant functional groups. Shoots of three to five individuals of *Corylus cornuta* Marsh., *Pinus ponderosa* Dougl., *Quercus garryana* Dougl., *Quercus rubra* L. and *Rhododendron macrophyllum* D. Don ex G. Don were collected between June and December 2009 (deciduous species collection ended in September 2009), sealed in plastic bags and returned to the lab. All samples were collected from fully sun-exposed branches. All leaves used in this study were fully expanded and from the previous year in the case of the evergreen species. Samples of *Q. rubra* were collected from a common garden plot near (non-irrigated) State College, PA, USA, and all other samples were collected from Oregon State University Campus and McDonald-Dunn University Forest located in Corvallis, OR, USA.

Leaf hydraulic conductance and vulnerability

Leaf hydraulic conductance was determined using a timed rehydration method (Brodribb & Holbrook 2003), which involved the use of the following equation based on an analogy between rehydrating a leaf and recharging a capacitor:

$$K_{\text{leaf}} = C \ln(\Psi_o/\Psi_t)/t \quad (1)$$

where C = capacitance, Ψ_o = leaf water potential prior to partial rehydration, Ψ_t = leaf water potential after partial rehydration and t = duration of rehydration. Branches approximately 30–50 cm long were collected from trees early in the morning prior to significant transpirational water loss and were transported back to the lab, recut under water and allowed to rehydrate for at least 4 h, at which point leaf water potentials were near -0.1 MPa. Shoots were dried on the benchtop for varying lengths of time, placed in a plastic bag, and sealed and then kept in the dark for at least 1 h to equilibrate. Measurements of leaf water potential were made using a Scholander-type pressure chamber (PMS Instruments, Albany, OR, USA) and were conducted over the next 3 d (shoots kept in the dark at 4°C , unless measured on the same day as they were dehydrated) on excised leaves/fascicles (three needles per fascicle for

the *Pinus*) for initial values (Ψ_o), and for final values (Ψ_t) after a period of rehydration of t seconds, which was between 90 and 120 s. Distilled water was used for rehydration of K_{leaf} samples and water temperature was maintained between 21 and 23°C . To determine the effects of the surface tension of water on leaf hydraulic vulnerability, stems were rehydrated in a surfactant solution [0.5% (v/v) Triton X-100 dissolved in deionized water] instead of water. Distilled water at room temperature has a surface tension of approximately 72 mN m^{-1} , whereas a 0.5% Triton X-100 solution has a surface tension of approximately 31 mN m^{-1} (Wu, Dai & Micale 1999; see also Cochard *et al.* 2009).

The inclusion of surfactant should not result in any bubbles forming in the surfactant solution at sub-atmospheric pressure, as the spontaneous cavitation pressure of water should be directly proportional to the surface tension (cohesive tension) of water. As the negative pressure required to cavitate pure water in an ideal tube is approximately 20–25 MPa (Briggs 1950; Caupin & Herbert 2006; Cochard *et al.* 2007b), the pressure required to cavitate the surfactant solution should be approximately 9–12 MPa. So the losses in K_{leaf} measured using the surfactant solution should be xylem embolism and not solution cavitation.

Rehydration of *C. cornuta*, *Q. garryana* and *R. macrophyllum* leaf discs was performed to assess the coordination between overall reductions in K_{leaf} and leaf lamina hydraulic conductance during dehydration. Leaf discs were cut from leaves (attached to stems) that had been rehydrated with distilled water and then dehydrated to various water potentials on the benchtop. Leaves were allowed to equilibrate overnight in sealed bags and several leaves were removed and their water potentials were measured (typically within 0.1 MPa of each other) to estimate the water potential of the leaves used for sampling. Two leaf discs were cut using a standard hole punch (leaf disc area approximately 28 mm^2) from portions of each leaf with minimal venation (no tertiary or lower-order veins). One disc was cut out under water (distilled) and allowed to rehydrate for a specified length of time, blotted dry, punctured 150 to 200 times with a 22 ga syringe needle to reduce cuticular resistance (Kikuta & Richter 1992; Nardini *et al.* 2008), and its water potential was measured and the other disc was cut out while the leaf was not under water, punctured 150 to 200 times with a syringe needle and its water potential was measured. Leaf disc water potentials were measured with a vapour pressure osmometer (VAPRO, Wescor Inc., Logan, UT, USA) and leaf disc hydraulic conductance (K_{disc}) was calculated as above (Eqn 1) for whole-leaf hydraulic conductance. Due to the difficulty of measuring leaf disc capacitance, and because previous research has shown similar pressure–volume relationships for whole leaves and leaf discs (Nardini *et al.* 2008), the whole-leaf capacitance from pressure–volume curves was used for calculations of leaf disc hydraulic conductance. Values of K_{leaf} and K_{disc} were converted to relative values, for ease of comparison, by dividing each measurement by the mean of the five highest values.

Values of C needed in Eqn 1 were estimated from pressure–volume curves (Scholander *et al.* 1965; Tyree & Hammel 1972) using the methods described by Brodribb & Holbrook (2003) for three to six leaves of each species. Briefly, the Ψ_L corresponding to turgor loss was estimated as the inflection point (the transition from the initial curvilinear, steeper portion of the curve to the more linear less steep portion) of the graph of Ψ_L versus relative water content (RWC). The slope of the curve prior to, and following turgor loss provided C in terms of RWC (C_{rwc}) for pre-turgor loss and post-turgor loss, respectively.

For leaves on which pressure–volume curves were performed, branch samples of approximately 30–50 cm were excised early in the morning and recut under water in the lab. Branches were allowed to rehydrate for at least 4 h before pressure–volume analyses were performed. Pressure–volume curves were created by plotting the inverse of Ψ_L against RWC with alternate determinations of fresh mass, and Ψ_L were repeated during slow dehydration of the twig on the laboratory bench. Leaf water potential was measured using a pressure chamber (PMS Instruments). For normalizing C on a leaf area basis, leaf areas for the broadleaf species were obtained with a scanner and ImageJ version 1.27 image analysis software (Abramoff, Magelhaes & Ram 2004; National Institute of Mental Health, Bethesda, MD, USA), and needle areas for *Pinus* were determined by multiplying mean needle length by circumference ($n = 8$ needles).

Ultrasonic acoustic emissions (UAEs)

Shoots were allowed to equilibrate overnight in plastic bags and five or six leaves were removed. Two leaves were used for acoustic emission measurements and three to four leaves were used for water potential measurements. Two sensors (R15 α , Physical Acoustics Corporation, Princeton Junction, NJ, USA) were connected to a UAE-specific datalogger (Pocket AE, Physical Acoustics Corporation) and emissions were amplified by 26 dB. Sensors were placed on the abaxial surface of each leaf midvein, 2–5 cm from the base of the petiole (on the proximal end of the leaf), and leaves were allowed to dry on the benchtop for 6–24 h. A small amount of silicone-based grease was placed at the leaf-transducer interface and was also applied to the same area of leaves used for water potential measurements (to minimize variation in treatment conditions). The entire UAE measurement apparatus was acoustically isolated by enclosing it in insulation wrap and 3 cm of foam-packing material. Leaves used for UAE and those used for water potential measurements were kept in the dark, inside the acoustic isolation material, for the duration of the measurements (except when water potential measurements were performed). Water potentials of leaves adjacent to the leaves being measured for UAE were measured every 30–60 min concomitantly while acoustic emission measurements were being made. Experiments were conducted prior to the study to ensure that, during dehydration, water potentials of UAE leaves were similar to those used for

water potential measurements. Mean water potentials for leaves with UAE sensors attached were typically within 3–7% of mean water potential values for leaves without sensors (using two to six leaves for each treatment).

Staining and imaging of midveins

Petioles were severed with a razor blade under water and a Tygon tube containing a 2% Safranin solution was attached to a leaf base. A pressure head of 10 cm (10 kPa, a low-enough pressure to avoid refilling) was used to deliver the stain solution to the midvein. After 10 min, the leaf was removed and the midvein was cut with a razor blade approximately 3 cm from the lamina attachment to the petiole. Hand sections were prepared and the sections were imaged immediately, in order to prevent diffusion of the stain. The images were captured using a digital camera attached to a Nikon Eclipse E400 compound microscope (Brighton, MI, USA). Image analysis was performed using ImageJ software (NIH, Bethesda, MD, USA).

Data analysis/statistics

For each species, K_{leaf} and UAE data were grouped (binned) over water potential ranges of approximately 0.3 to 0.5 MPa. Each bin contained 4 to 15 leaves, and a total of 39 to 105 leaves were used for K_{leaf} and 28 to 71 time points for UAE measurements in each species. However, curves were fitted through non-binned data, which reduced the correlation coefficient but reflected the truer fit of curves through the data. Least squares regression was performed using GraphPad Prism 5.0 (Graphpad Software, San Diego, CA, USA) and sigmoid models of the equation $y = y_0 + (a / (1 + e^{-[x-x_0] / b}))$, were fit through the data. To determine whether amplitudes of UAE decreased during dehydration, an analysis of variance (ANOVA) was performed with amplitude as the dependent variable and percent cumulative UAE as the independent variable.

RESULTS

Overall, K_{leaf} declined and accumulated UAE increased with decreasing (more negative) water potential (Figs 1 & 2) in all species measured. Patterns of K_{leaf} decline and UAE accumulation were generally similar across and within species except in *Q. garryana*. The difference in leaf water potential corresponding to 50% loss of K_{leaf} (P_{50}) and 50% accumulated UAE was 1.5 MPa in *Q. garryana*, but the two values of P_{50} were much more similar in the other three species (Table 1). UAE events in *Q. garryana* occurred at water potentials less negative than indicated by the sigmoidal curve fit through the data (Fig. 2b, dashed line). In fact, UAE events began to occur at about -2.6 MPa, a water potential that did not appear to result in substantial reductions in K_{leaf} in the same species.

Emissions with greater amplitudes tended to occur at lower % cumulative UAE [i.e. less negative leaf water potentials (Fig. 3)]. However, UAE amplitude increased in

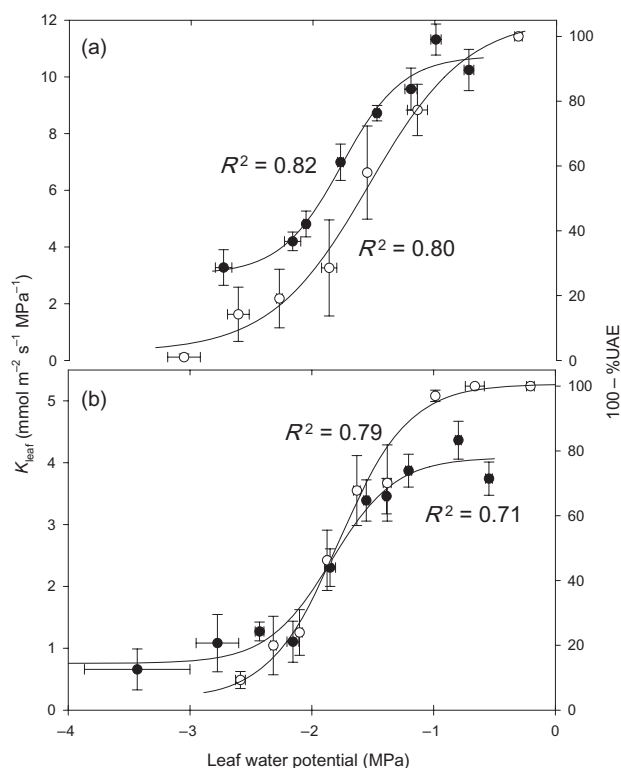


Figure 1. Leaf hydraulic conductance (K_{leaf} , closed symbols) and accumulated ultrasonic acoustic emissions (UAEs, open symbols; expressed as 100 minus the percentage of total accumulated emissions) at different leaf water potentials for the deciduous species (a) *Quercus rubra* and the evergreen species (b) *Rhododendron macrophyllum*. Error bars represent standard error.

C. cornuta until 40% accumulated UAE and then began to decline. Additionally, *Q. rubra* exhibited two peaks in amplitude, one at 20–30% cumulative UAE and one at 60% UAE. Overall amplitudes were greater in *Q. rubra* (32.8 db) than for any of the other species measured. ANOVA results indicated that there was a significant decrease in UAE amplitude with increasing % cumulative UAE for *Q. garryana* ($P < 0.001$) and *Q. rubra* ($P < 0.001$), but not for *C. cornuta* ($P = 0.36$) or *R. macrophyllum* ($P = 0.09$).

Leaves that were rehydrated in the surfactant solution were substantially more vulnerable to hydraulic dysfunction than those that had been rehydrated in water (Fig. 4, Table 1). Rehydration in surfactant solution increased P_{50} (less negative) by 0.5 to 1.6 MPa. The greatest increase in P_{50} (–3.6 to –2.0 MPa) was in *Q. garryana* while the smallest increase was in *R. macrophyllum* (–1.95 to –1.4 MPa).

Relative leaf hydraulic conductance in *Q. garryana* declined approximately 96% (slope = 0.347, $P < 0.0001$) over the range of –2.2 to –5.2 MPa. Estimated laminar hydraulic conductance declined 60% (slope = 0.197, $P < 0.0001$) over the same range of water potentials (Fig. 5), indicating that a large portion of the decline in hydraulic conductance occurred in the lamina. However, little loss of laminar hydraulic conductance was observed in either

C. cornuta or *R. macrophyllum*, suggesting that most of the loss in hydraulic conductance was in the leaf xylem in these species. Imaging of safranin-stained *Q. garryana* midveins revealed that fewer conduits were functional at more negative leaf water potentials (Fig. 6). The percentage of the vascular bundle area stained in *Q. garryana* midveins declined linearly (Fig. 7a, slope = 10.1% MPa^{-1} , $P < 0.001$) with decreasing water potential, and the percentage of the maximum K_{leaf} remaining declined exponentially (Fig. 7b, $P < 0.0001$) with decreasing functional conducting area of the midvein vascular bundle.

DISCUSSION

Leaves, the terminal portion of the plant hydraulic continuum, have the task of controlling water loss while maintaining adequate carbon gain. The maintenance of hydraulic conductance inside the leaf allows for carbon gain, transpirational cooling and even nutrient transport via transpiration. However, recent studies have observed that K_{leaf} declines in many species during dehydration (e.g. see Sack & Holbrook 2006, and references therein). In the current study, there was close correspondence between K_{leaf} decline and cumulative UAE during dehydration. This is consistent

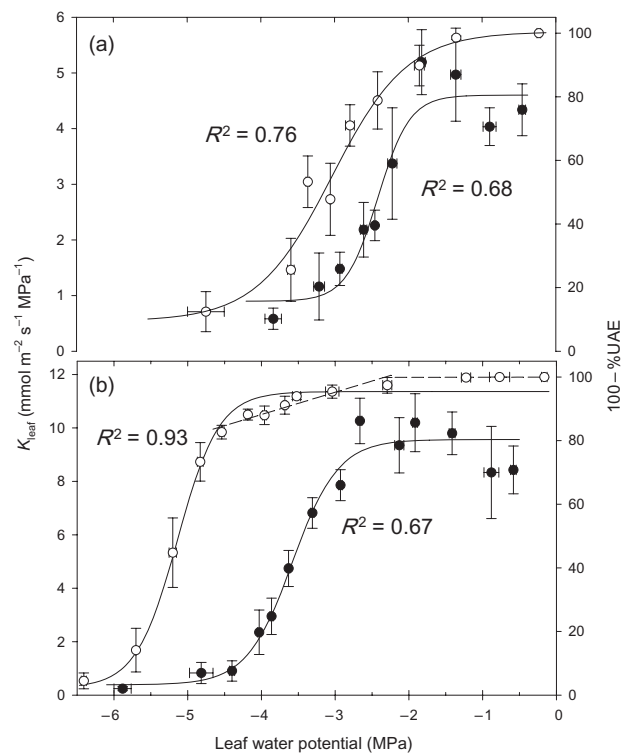


Figure 2. Leaf hydraulic conductance (K_{leaf} , closed symbols) and accumulated ultrasonic acoustic emissions (UAEs, open symbols; expressed as 100 minus the percentage of total accumulated emissions) at different leaf water potentials for the deciduous species (a) *Corylus cornuta* and (b) *Quercus garryana*. Dashed line represents linear fit through the data to indicate the initial accumulation of UAE. Error bars represent standard error.

Species	$K_{\text{leaf max}}$ (mmol m ⁻² s ⁻¹ MPa ⁻¹)	P_{50} (MPa)	P_{50} (MPa) with Triton	UAE ₅₀ (MPa)
<i>Corylus cornuta</i>	4.60	-2.51 (-2.37, -2.71)		-3.13 (-2.95, -3.35)
<i>Pinus ponderosa</i>	4.50	-1.62 (-1.54, -1.70)	-0.80 (-0.74, -0.86)	
<i>Quercus garryana</i>	9.57	-3.61 (-3.49, -3.74)	-1.98 (-1.85, -2.14)	-5.11 (-5.05, -5.20)
<i>Quercus rubra</i>	10.60	-1.98 (-1.87, -2.14)		-1.61 (-1.38, -1.81)
<i>Rhododendron macrophyllum</i>	4.10	-1.95 (-1.82, -2.12)	-1.42 (-1.19, -1.68)	-1.80 (-1.68, -1.89)

Table 1. Maximum leaf hydraulic conductance ($K_{\text{leaf max}}$) and water potentials corresponding to 50% loss of $K_{\text{leaf max}}$ (P_{50}) in water and surfactant (Triton) and 50% of total accumulated ultrasonic acoustic emission events (UAE₅₀). Numbers in parentheses correspond to 95% confidence intervals

with an earlier study where K_{leaf} decline and UAE were closely related in several evergreen species (Johnson *et al.* 2009a), and supports the hypothesis of embolism-induced decreases in K_{leaf} during desiccation. Furthermore, samples rehydrated in a solution with reduced surface tension were much more vulnerable to reductions in K_{leaf} , also supporting

this hypothesis. In *Q. garryana*, it appeared that a large portion of the decline in K_{leaf} coincided with reduced conductance in the lamina, but that the majority of K_{leaf} decline in *C. cornuta* and *R. macrophyllum* was due to hydraulic dysfunction in the xylem, based on a leaf disc rehydration experiment. Midvein staining in *Q. garryana* revealed that

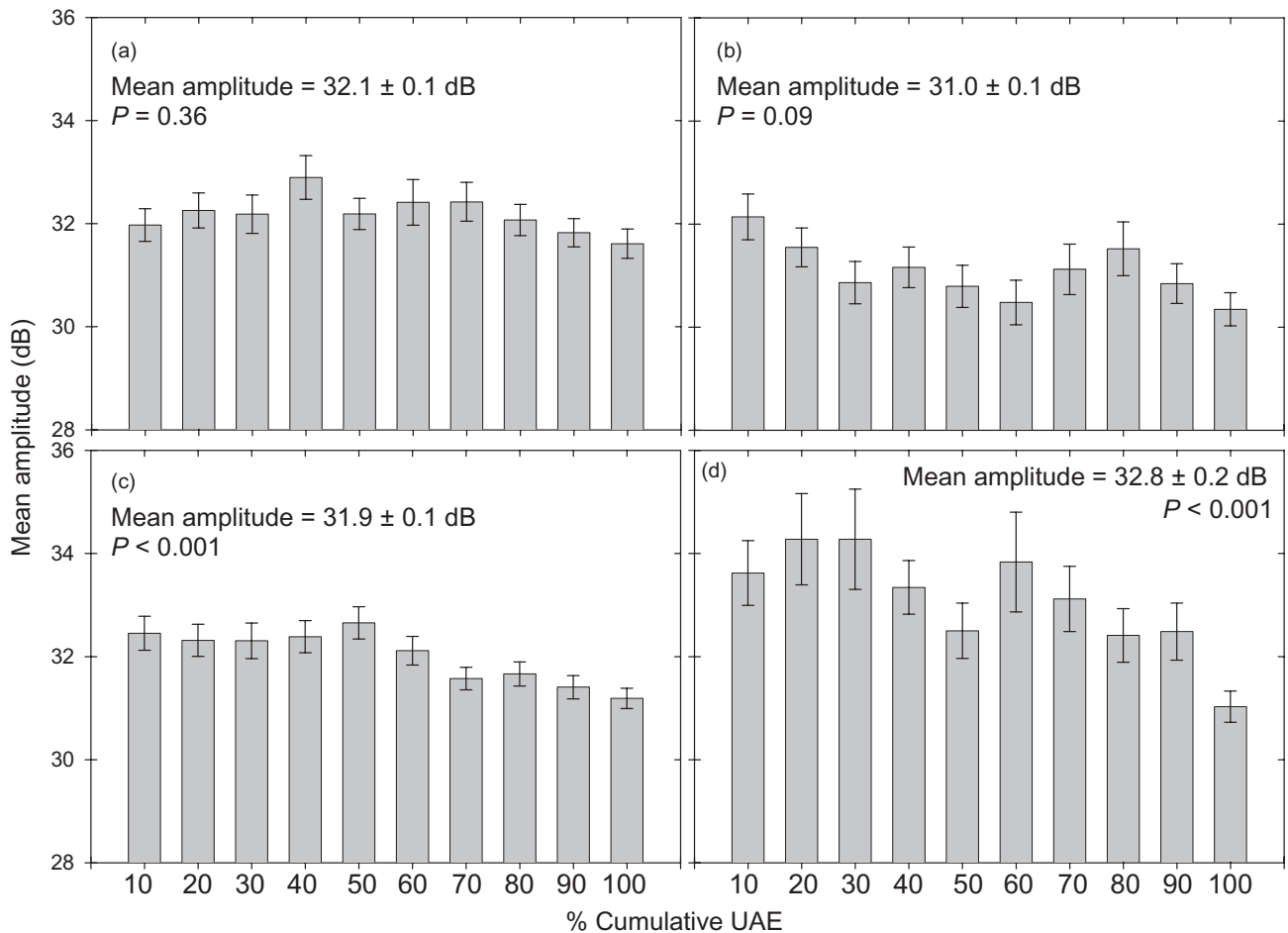


Figure 3. Mean amplitude of ultrasonic acoustic emission (UAE) versus the percentage of total acoustic emissions in (a) *Corylus cornuta*, (b) *Rhododendron macrophyllum*, (c) *Quercus garryana* and (d) *Quercus rubra*. Vertical error bars represent standard error. P values represent results of ANOVA testing reduction in amplitude with a greater percentage of accumulated acoustic emissions.

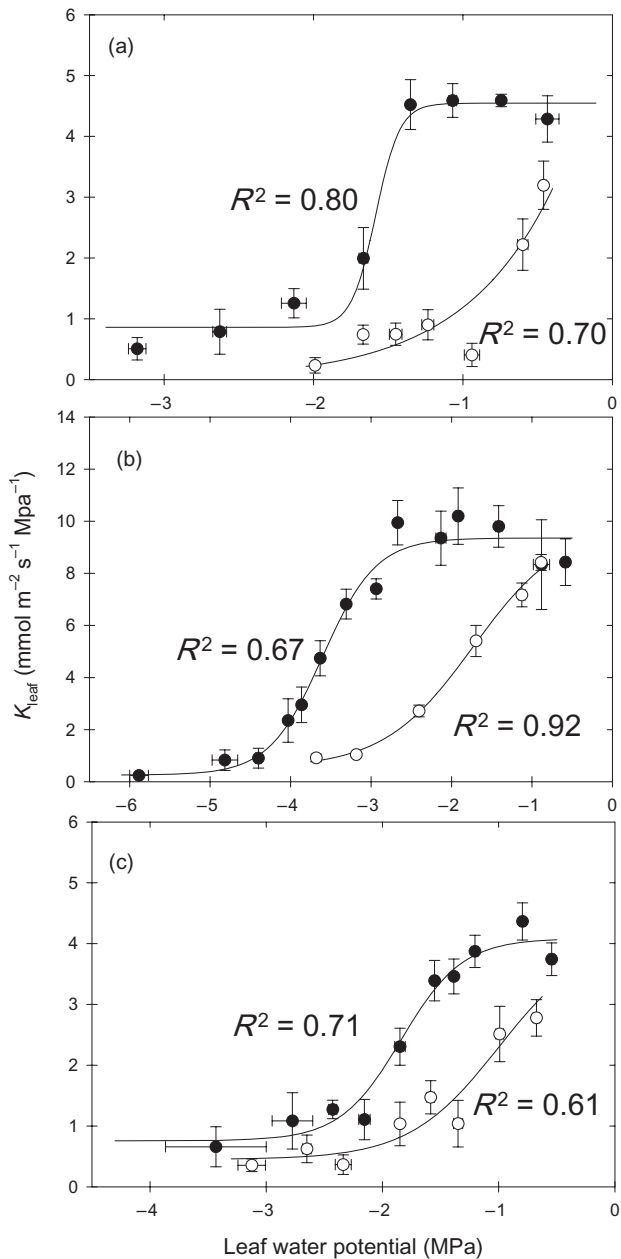


Figure 4. Leaf hydraulic conductance for leaves rehydrated with water (closed symbols) or a solution of 0.05% Triton X-100 (open symbols) in (a) *Pinus ponderosa*, (b) *Quercus garryana* and (c) *Rhododendron macrophyllum*. Error bars represent standard error.

xylem embolism was also occurring as K_{leaf} declined. Therefore, reductions in xylem conductance and reductions in laminar conductance may have occurred concomitantly in *Q. garryana*.

There has been much debate in the literature concerning the potential mechanisms underlying K_{leaf} decline during dehydration. Leaf xylem embolism, xylem implosion/deformation and extraxylary changes (e.g. changes in membrane permeability) have all been cited as impacting K_{leaf} during desiccation. Recently, Blackman *et al.* (2010)

observed that leaf P_{50} was correlated with xylem vulnerability to collapse across a number of species, and suggested that leaves may experience xylem collapse near their P_{50} values. This would agree with an earlier study that reported xylem collapse that corresponded to hydraulic dysfunction (Cochard *et al.* 2004).

However, other studies have shown that the decline in K_{leaf} is associated with hydraulic dysfunction and not with structural collapse. For example, multiple studies on a wide

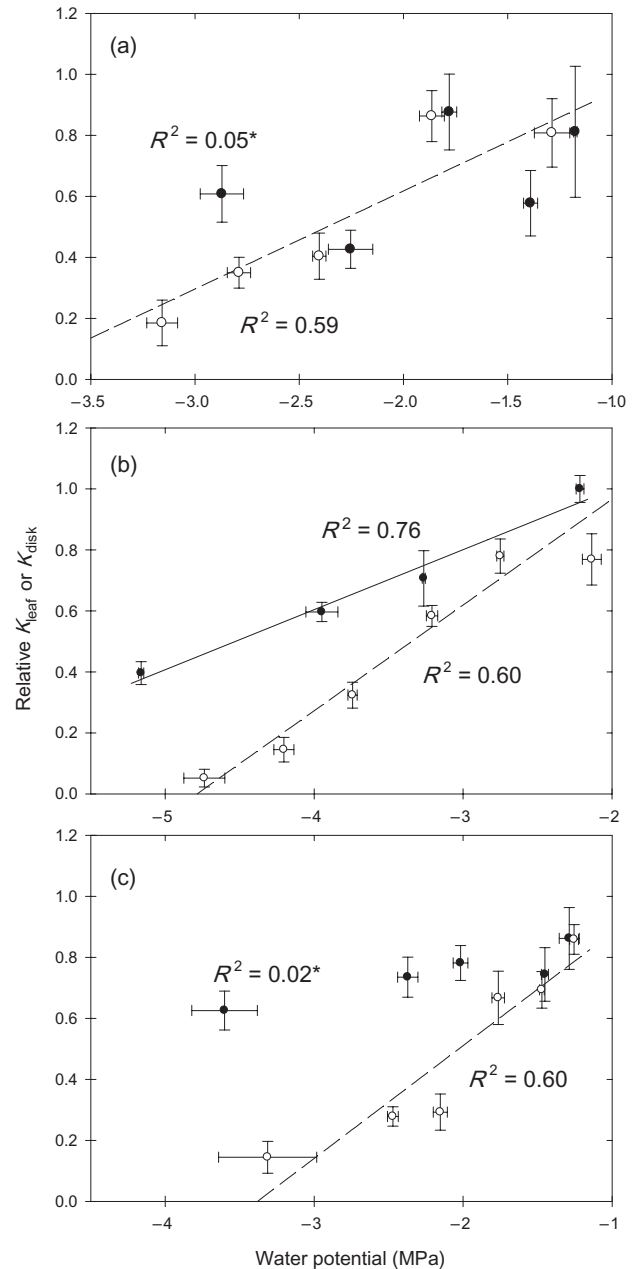


Figure 5. Relative leaf disc hydraulic conductance (closed circles, solid line) as compared with relative whole-leaf hydraulic conductance (open symbols, dashed line, linear fit through data) in (a) *Corylus cornuta*, (b) *Quercus garryana* and (c) *Rhododendron macrophyllum*. Error bars represent standard error and asterisks indicate non-significant linear fits.

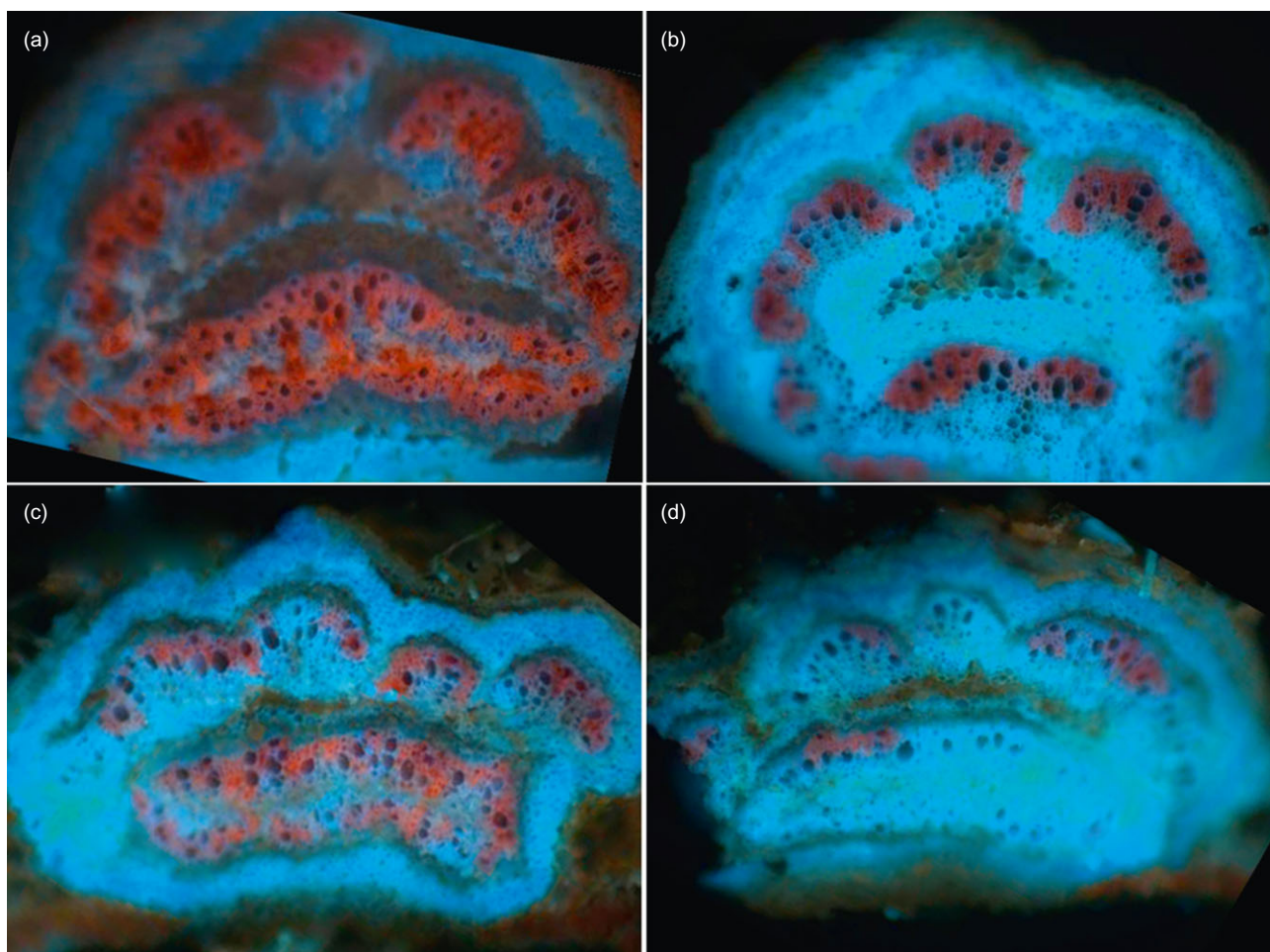


Figure 6. Leaf midveins of *Quercus garryana* stained with safranin at water potentials of (a) -2.1 , (b) -4.2 , (c) -5.2 and (d) -5.7 MPa.

range of species have found strong evidence that leaf xylem embolism is tightly correlated with reductions in K_{leaf} (Nardini *et al.* 2001; Bucci *et al.* 2003; Woodruff *et al.* 2007; Johnson *et al.* 2009a). These studies have documented a close association with leaf midvein embolism and hydraulic dysfunction. Portions of the hydraulic pathway outside the leaf xylem have also been shown to play a role in the decline of the overall leaf hydraulic pathway as leaves dehydrate. Changes in aquaporin expression or regulation and the resulting changes in membrane permeability can have large impacts on overall K_{leaf} (Cochard *et al.* 2007a; Kaldenhoff *et al.* 2008; Voicu *et al.* 2008), especially because the extraxylary pathway can represent as much as 70% of the overall leaf hydraulic resistance (e.g. Sack *et al.* 2004).

Acoustic emission has been used to measure leaf vein embolism and is typically closely correlated with K_{leaf} decline (Crombie, Milburn & Hipkins 1985; Kikuta *et al.* 1997; Nardini *et al.* 2001; Johnson *et al.* 2009a). In the current study, cumulative UAE curves and K_{leaf} curves were similar in three of the four species tested. Other useful parameters can be extracted from UAE data including amplitude and energy (Rosner *et al.* 2006, 2009; Mayr & Rosner 2011). However, as embolism of smaller conduits in leaves results

in much lower amplitudes (as compared with many wood samples), UAE energy is typically zero. This makes amplitude a more useful parameter when studying leaf UAE, because amplitude can provide clues as to why or why not K_{leaf} and UAE curves are similar. For example, in *Pinus ponderosa*, embolisms with greater amplitude (likely reflecting larger tracheids) occurred at relatively high water potentials and this appeared to explain the offset in K_{leaf} and UAE curves (Johnson *et al.* 2009a). In the current study, there was a strong relationship between K_{leaf} decline and UAE amplitude in *Q. garryana* and *Q. rubra*, but not in *C. cornuta* or *R. macrophyllum*. This may explain the discrepancy between K_{leaf} decline and UAE in *Q. garryana*. It is noteworthy, however, that there was little offset in K_{leaf} and UAE curves for *Q. rubra*, therefore there are likely multiple factors leading to reductions in K_{leaf} , but that xylem embolism has a particularly large effect on K_{leaf} decline in *Q. garryana*.

Previous work has shown that reducing the surface tension of the xylem water in stems results in embolism at less negative water potentials than when using water (Cochard *et al.* 2009). If K_{leaf} in leaves rehydrated in surfactant solution were also to decline at less negative water

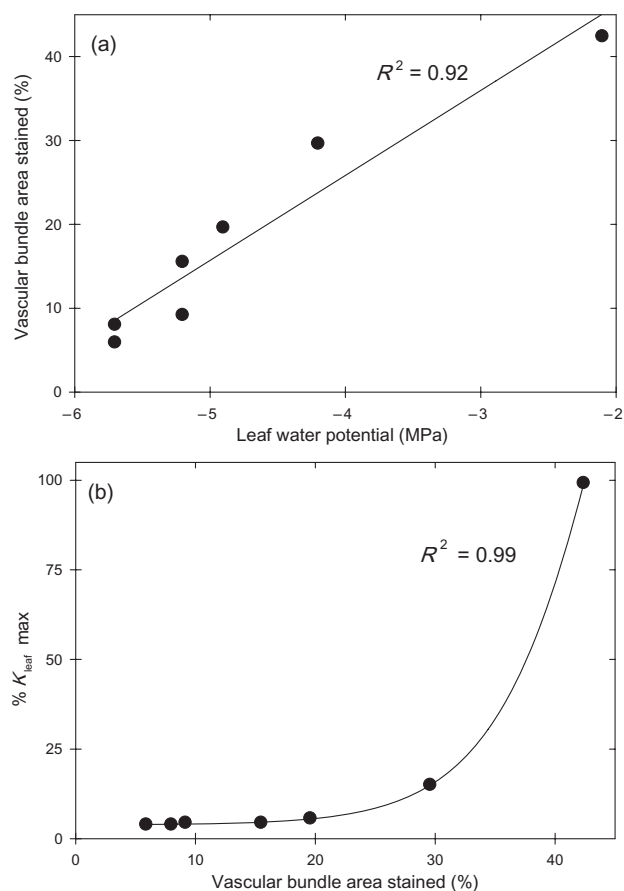


Figure 7. Results of image analysis of *Quercus garryana* midveins stained with safranin: (a) the percentage of the total vascular bundle area stained decreased linearly with declining leaf water potential and (b) the percentage of maximum leaf hydraulic conductance remaining decreased in a sigmoidal fashion with decreasing stained vascular bundle area.

potentials than in leaves rehydrated in water, this would support the hypothesis that leaf conduit embolism is at least partially responsible for the decline in K_{leaf} observed during leaf desiccation. Leaves of all three species tested in the current study showed marked increases in their P_{50} when rehydrated in solution containing surfactant, thus supporting the idea that embolism was responsible for K_{leaf} decline. In fact, Cochard *et al.* (2009) found a linear relationship between surface tension and stem P_{50} and a strong correlation between measured P_{50} and predicted P_{50} based on the Young–Laplace equation. Although the leaves are more hydraulically complex than stems, we had similar results to Cochard *et al.* (2009). Based on the Young–Laplace equation, the predicted increase in P_{50} in leaves from the surfactant treatment as compared with water alone would be approximately 53%. Both *Q. garryana* and *P. ponderosa* were very near this value with a 47 and 51% increase, respectively. Although *R. macrophyllum* only displayed a 27% increase in P_{50} , this may have been due to a poor curve fit for the leaves hydrated in water as the curve may actually be much steeper between -1.0 and -1.25 MPa than indicated by the sigmoid fit (see Fig. 4c).

Several studies have attempted to partition the hydraulic resistance of leaves into their component parts (e.g. petiole, veins, lamina). However, to our knowledge, little research has been performed on examining the differences in vulnerability to hydraulic dysfunction in different leaf parts. In *Q. garryana*, whole leaf hydraulic conductance declined much more rapidly with a given reduction in water potential than did the conductance of the leaf lamina. This would suggest that in *Q. garryana*, much of the decline in whole leaf hydraulic conductance was due to embolism in the midvein of the leaf. In fact, staining of midveins and image analysis supported this (Fig. 6). Either functional or partially collapsed xylem conduits should have been stained because they are still connected to the transpiration stream (i.e. not embolized). The pattern of more relative decline in total K_{leaf} than K_{disc} was even more distinct in *C. cornuta* and *R. macrophyllum*, both of which showed no measurable decrease in the laminar hydraulic conductance with more negative water potentials (Fig. 5).

Several studies have observed daily declines and recovery in K_{leaf} *in situ* (Bucci *et al.* 2003; Brodrigg & Holbrook 2004; Johnson *et al.* 2009b, 2011). The mechanism involved in refilling of embolism under negative pressure remains one of the most important unresolved questions in plant hydraulics (Zwieniecki & Holbrook 2009; Brodersen *et al.* 2010; Nardini, Lo Gullo & Salleo 2011; Secchi & Zwieniecki 2011). Additionally, the question of why some species appear to lose the majority of their K_{leaf} during a day while others lose none or very little, remains unanswered. The two questions may be related as it may be easier or more energetically economical to refill leaf xylem of some species than others. One prediction might be that species that are easier or cheaper to refill might experience a more significant loss of K_{leaf} on a daily basis. The trade-off would be keeping stomata open longer to generate more fixed carbon at the price of leaf xylem embolism. Examples of species with leaf xylem that might be more easily refilled, and that lose the majority of K_{leaf} daily, are several members of the *Pinaceae* (Johnson *et al.* 2011). Several of these species have been observed to lose the majority of K_{leaf} daily, several have slow stomatal responses to low leaf water potential and they all possess transfusion tissue adjacent to the leaf xylem. Transfusion tissue has been shown to store solutes (Canny 1993; Liesche, Martens & Schulz 2011) which could be released into adjacent, embolized tracheids to initiate the refilling process (Secchi & Zwieniecki 2011).

The current study provides evidence that reductions in K_{leaf} are directly related to leaf vein embolism. However, there are many unresolved facets to changes in leaf hydraulic conductance in general. The degree to which xylem collapses or implosion impacts K_{leaf} (e.g. Cochard *et al.* 2004) is still unknown for most species. Additionally, the effects of light (Lo Gullo *et al.* 2005; Scoffoni *et al.* 2008; Voicu *et al.* 2008), temperature (Sellin & Kopper 2007) and innate circadian rhythms (Nardini, Salleo & Andri 2005), all of which may be modulated through aquaporins (Cochard *et al.* 2007a; Kim & Steudle 2007; Voicu, Cooke & Zwiazek 2009), on K_{leaf} is not well understood. The underlying reason(s)

why some species lose K_{leaf} over the course of a day and others do not as well, as the mechanism(s) for refilling embolized conduits are also fascinating and poorly understood aspects of plant biology.

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