

leaf xylem embolism, detected acoustically and by cryo-SEM, corresponds to decreases in leaf hydraulic conductance in four evergreen species

DANIEL M. JOHNSON¹, FREDERICK C. MEINZER¹, DAVID R. WOODRUFF¹ & KATHERINE A. MCCULLOH²

¹USDA Forest Service, Pacific Northwest Research Station, OR 97331, USA and ²Department of Wood Science and Engineering, Oregon State University, Corvallis, OR 97331, USA

ABSTRACT

Hydraulic conductance of leaves (K_{leaf}) typically decreases with increasing water stress. However, the extent to which the decrease in K_{leaf} is due to xylem cavitation, conduit deformation or changes in the extra-xylary pathway is unclear. We measured K_{leaf} concurrently with ultrasonic acoustic emission (UAE) in dehydrating leaves of two vessel-bearing and two tracheid-bearing species to determine whether declining K_{leaf} was associated with an accumulation of cavitation events. In addition, images of leaf internal structure were captured using cryo-scanning electron microscopy, which allowed detection of empty versus full and also deformed conduits. Overall, K_{leaf} decreased as leaf water potentials (Ψ_l) became more negative. Values of K_{leaf} corresponding to bulk leaf turgor loss points ranged from 13 to 45% of their maximum. Additionally, Ψ_l corresponding to a 50% loss in conductivity and 50% accumulated UAE ranged from -1.5 to -2.4 MPa and from -1.1 to -2.8 MPa, respectively, across species. Decreases in K_{leaf} were closely associated with accumulated UAE and the percentage of empty conduits. The mean amplitude of VAEs was tightly correlated with mean conduit diameter ($R^2 = 0.94$, $P = 0.018$). These results suggest that water stress-induced decreases in K_{leaf} in these species are directly related to xylem embolism.

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

INTRODUCTION

When the transport of water to leaves is insufficient to resupply water during periods of rapid transpiration or drought, leaves tend to dehydrate. Hydraulic conductance in leaves (K_{leaf}) decreases as leaves desiccate, resulting in reduced water transport capacity and eventually stomatal closure and, thus, negligible photosynthesis (see Sack & Holbrook 2006 and references therein). However, the extent to which the observed fluctuations in K_{leaf} are related to reversible xylem cavitation, reversible partial conduit

collapse or changes in the extra-xylary portion of the hydraulic pathway is uncertain (Sack & Holbrook 2006). Moreover, the relative importance of mechanisms contributing to variation in K_{leaf} may differ among species.

Recent work has provided evidence that water stress-induced reductions in K_{leaf} resulted from leaf xylem cavitation (e.g. Bucci *et al.* 2003; Nardini, Salleo & Raimondo 2003; Woodruff *et al.* 2007). However, decreases in K_{leaf} in dehydrating pine needles appeared to be due to xylem element collapse, which occurred at less negative water potentials than cavitation (Cochard *et al.* 2004). Models have predicted that embolism should occur at less negative water potentials than implosion in stems and roots (Hacke *et al.* 2001; Hacke, Sperry & Pittermann 2004), although this may not be true for leaves. It is possible that walls of xylem conduits in leaves are less resistant to collapse, as compared to walls of secondary xylem conduits in stems and roots. Xylem collapse in leaves could be related to reduced mechanical support inside leaves as compared to wood. In addition, deformed conduits may be easier to refill under negative pressure than embolized conduits (Tyree & Yang 1992; Cochard *et al.* 2004).

Changes to extra-xylary pathways of water transport inside the leaf could also have a strong influence on K_{leaf} because the extra-xylary portion of the pathway may make up as much as 70% of the overall hydraulic resistance of leaves (Sack, Streeter & Holbrook 2004; Nardini & Salleo 2005; Brodribb, Field & Jordan 2007; Mott 2007). Therefore, changes in cell membrane permeability (e.g. via aquaporins) could have large impacts on K_{leaf} (Cochard *et al.* 2007; Kaldenhoff *et al.* 2008; Voicu, Zwiazek & Tyree 2008). Another example of extra-xylary impacts on K_{leaf} is the collapse of accessory transfusion tracheids in *Podocarpus grayi*, which coincided with depressions of K_{leaf} during dehydration (Brodribb & Holbrook 2005).

Ultrasonic acoustic emission (UAE) has been used for decades to detect cavitation events in tree stems (Millburn & Johnson 1966; Tyree & Dixon 1983; Nardini & Salleo 2000) and, more recently, in leaves (Kikuta *et al.* 1997; Nardini, Tyree & Salleo 2001; Salleo *et al.* 2001). During cavitation events, waves of energy likely result from sudden relaxation of tension inside the conduit lumen as air replaces water (Tyree & Sperry 1989). In wood, cavitation

Correspondence: D. M. Johnson Fax: +1541 7507250; e-mail: danieljohnson@fs.fed.us

events can occur in both conductive (tracheids and vessels) and non-conductive elements (ray tracheids and fibres). Furthermore, a single acoustic emission can cause a decline in the total hydraulic conductance by varying amounts depending on the diameter of the conduit. This can result in a relationship between hydraulic conductance and accumulated acoustic emissions that is not proportional (Rosner *et al.* 2006). However, because the vascular bundles of leaves are composed nearly entirely of xylem and phloem, we would expect close correspondence between accumulated acoustic emissions and K_{leaf} . Additionally, it has been proposed that the frequency range and/or amplitude of UAEs may be related to the dimensions of the cavitating conduits (Ritman & Milburn 1988, 1991; Rosner *et al.* 2006).

In the present study, we measured hydraulic conductance and UAEs in drying leaves of four evergreen species: two conifers and two angiosperms. Our goals were to assess the coordination between accumulated emissions and depressions in K_{leaf} and to assess the extent to which water stress-induced loss of K_{leaf} was associated with xylem cavitation. Additionally, cryo-scanning electron microscopy (cryo-SEM) was performed on leaf samples flash-frozen at different water potentials as an independent measure of xylem embolism and to detect xylem deformation or changes in the distribution of water within leaves as they dried.

MATERIALS AND METHODS

Plant material

Four evergreen, woody species were chosen as representative of tracheid-bearing and vessel-bearing species as well as based on local occurrence. Shoots of three to five individuals of *Pinus ponderosa* C. Lawson, *Pinus nigra* Arnold, *Castanopsis chrysophylla* (Dougl.) A. DC., and *Pieris japonica* (Thunb.) D. Don ex G. Don were collected between October 2007 and March 2008, sealed in plastic bags and returned to the lab. All samples were collected from branches in full sunlight from individuals >2 m in height. All leaves used in this study were from the previous year and were fully expanded. Samples were collected from Oregon State University Campus, McDonald-Dunn University Forest and Dan Farmer Tree Farm, all located near Corvallis, OR, USA.

K_{leaf} and vulnerability

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

$$K_{\text{leaf}} = C \ln(\Psi_0 / \Psi_t) / t$$

where C = capacitance, Ψ_0 = leaf water potential before 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 before significant transpirational water loss and were transported back to the lab, recut under water and allowed to rehydrate for at least 4 h. Shoots were dried on the bench top 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 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 for initial values (Ψ_0), and for final values after a period of rehydration of t seconds (Ψ_t), which was between 30 and 120 s. Distilled water was used for rehydration of K_{leaf} samples and water temperature was maintained between 21 and 23 °C. The photosynthetic photon flux density at the foliage was maintained at approximately 1000 $\mu\text{mol m}^{-2} \text{s}^{-1}$ during K_{leaf} measurements.

Values of C 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_t 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_t versus relative water content (RWC). The slope of the curve before and after turgor loss provided C in terms of RWC (C_{RWC}) for pre-turgor loss and post-turgor loss, respectively.

Pressure-volume curves were conducted on individual leaves for the broadleaf species and on fascicles of three needles for the two *Pinus* species. 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_t against RWC, and alternate determinations of fresh mass and Ψ_t were repeated during slow dehydration of the twig on the laboratory bench until values of Ψ_t exceeded the measuring range of the pressure chamber (–4.0 MPa). Leaf water potential was measured using a pressure chamber (PMS Instrument Company, Corvallis, OR, USA). For normalizing C on a leaf area basis, leaf areas for the broad leaf 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 the *Pinus* species were determined by multiplying mean needle lengths and circumferences (n = six needles per species).

UAE

Shoots were allowed to equilibrate overnight in plastic bags, and six to eight leaves were removed. Two leaves were used for acoustic emission measurements, and three to four leaves were used for water potential measurements. Two sensors (R 15 α , Physical Acoustics Corporation, Princeton Junction, NJ, USA) were connected to a UAE-specific data-logger (Pocket AE, Physical Acoustics Corporation) and emissions were amplified by 26 dB. Sensors were placed on

the abaxial surface of each leaf mid vein, 2–5 cm from the base of the petiole or fascicle sheath (on the proximal end of the leaf), and leaves were allowed to dry on the bench top 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). We detected little variation in adaxial versus abaxial UAE, in contrast to earlier work by Kikuta *et al.* (1997). The entire UAE measurement apparatus was acoustically isolated by enclosing it in insulation wrap and 3 cm 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). Measurement sensitivity was set to 29 dB, below which the instrument detected acoustic signals that were not from leaves [e.g. Radio Frequency Interference (RFI) from fluorescent lights - communication from Physical Acoustics Corp.]. Water potentials of leaves adjacent to the leaves being measured for UAE were measured every 30–60 min while acoustic emission measurements were being made. Experiments were conducted before 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).

Additionally, to test for introduction of embolism due to removing leaves from stems for UAE measurements, petioles were excised from both *Pieris* and *Castanopsis* leaves, including the proximal third of the midvein (the same approximate location used for both cryo-SEM and UAE) under water, attached to a pressurized air source and pressure was applied to the section (under water) to see if there were air bubbles. Then sections were shortened by approximately 0.5 cm and pressure was applied again. At no point were there bubbles coming from the end of the section, indicating that the vessels were shorter than the section used. This process was repeated until we could not discriminate between air bubbles originating from the coupling of the petiole/midvein to the tubing (approximately 1 cm petiole/midvein left inside tubing).

Cryo-SEM

Shoots were dried on the bench top for varying lengths of time, sealed in a plastic bag and then kept at 4 °C for 1 h to equilibrate. One leaf/fascicle was removed, the shoot was returned to the plastic bag, and Ψ_t was determined. Leaves were selected for cryo-SEM based on Ψ_t that corresponded to different points on Ψ_t versus K_{leaf} and UAE curves. Then one leaf was removed from the shoot; if a broad leaf, the midvein was removed (see next paragraph); and a portion of the midvein/needle was inserted into a fracture rivet (Electron Microscopy Sciences, Hatfield, PA, USA) containing Tissue-Tek Embedding Compound (Sakura Finetek

USA, Torrance, CA, USA). Portions of needles/midveins used for cryo-SEM corresponded to the same approximate location as UAE sensors were placed (approximately 3 cm from the petiole or fascicle sheath).

To minimize the potential for introducing embolism due to severing veins, angiosperm leaves were flash-frozen in liquid nitrogen (LN_2). Subsequently, midribs were excised with scissors that were also cold, and then midveins were dropped back into LN_2 . Midveins were then placed into fracture rivets (room temperature) containing Tissue-Tek and then immediately dropped back into LN_2 (approximately 2 s was required to transfer tissue into rivets and then return it to LN_2). Pine needles were inserted into Tissue-Tek/rivet, and the rivet/needle was dropped into a vial containing LN_2 . The needle was then trimmed, while frozen, around the fracture rivet to leave approximately 0.5 cm of protruding tissue, then immediately placed back into LN_2 . Vials containing samples were then placed into a cryo-shipper (CX500; Taylor-Wharton Cryogenics, Theodore, AL, USA) that had been pre-charged with LN_2 . Samples were shipped to the microscopy facility at University of British Columbia, where they were fractured and imaged. Midveins/needles embedded in fracture rivets were mounted in a cryo-prep apparatus (K1270; Emitech USA, Houston, TX, USA) and were fractured by touching them with a metal prong. They were then sublimed for approximately 10 min and imaged in the cryo-SEM unit (S4700 FESEM; Hitachi High-Technologies Corp., Berkshire, UK).

Seventeen images, each corresponding to a different sample, were quantitatively analysed using ImageJ. The percentage of conduits that were full/empty was determined and all the diameters of all empty conduits were measured. The reported values of mean conduit diameter were determined from images of leaf tissue containing empty conduits.

Statistics/data analysis

For each species, K_{leaf} and UAE data were grouped (binned) over water potential ranges of approximately 0.3 MPa (i.e. -0.61 to -0.90 MPa, -0.91 to -1.20 MPa, etc.) with the exception of the first bin for each species, which corresponded to 0–0.6 MPa. Each bin contained 2–10 leaves, and a total of 42–55 leaves were used for K_{leaf} and 42–65 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 were fit through the data. Akaike's Information Criterion was used to decide whether to fit linear or sigmoid models through the data.

RESULTS

Overall, K_{leaf} and cumulative UAEs followed similar trends as Ψ_t declined (Figs 1,2). Differences between Ψ_t at 50% loss of K_{leaf} and 50% cumulative UAE (Table 1) were small

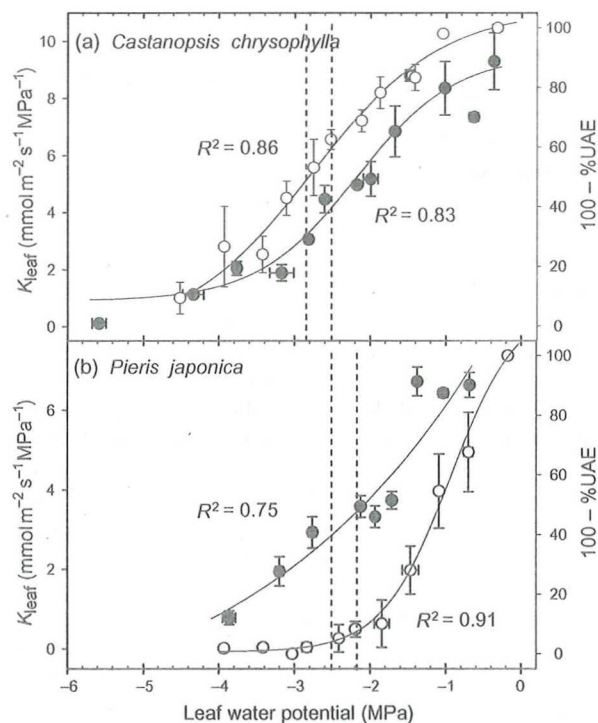


Figure 1. Leaf hydraulic conductance (K_{leaf} , closed circles) and accumulated ultrasonic acoustic emission (UAE, open circles; expressed as 100 - the percentage of total accumulated emissions) at different leaf water potentials for the evergreen broad leaf species (a) *Castanopsis chrysophylla* and (b) *Pieris japonica*. Dashed lines represent mean turgor loss point ± 1 standard error and vertical error bars represent standard error.

in the *Pinus* species (0.13 and 0.29 MPa for *P. nigra* and *P. ponderosa*, respectively), but greater in the two broad leaf species (0.42 and 1.04 MPa in *Castanopsis* and *Pieris*, respectively). At the bulk leaf turgor loss point, K_{leaf} had fallen to approximately 39 and 45% of its maximum value in *Castanopsis* and *Pieris*, respectively, and only 13 and 21% of its maximum in *P. nigra* and *P. ponderosa* (Figs 1, 2, Table 2). Additionally, turgor loss in *Pieris*, *P. nigra* and *P. ponderosa* corresponded to 95, 88 and 84% cumulative UAE, respectively, but only 46% cumulative UAE in *Castanopsis*.

Mean amplitude of UAE was greater in *P. ponderosa* (38.8 dB) than in the other species in the study, and *Castanopsis* had the lowest overall amplitude (32.8 dB, Fig. 3). In

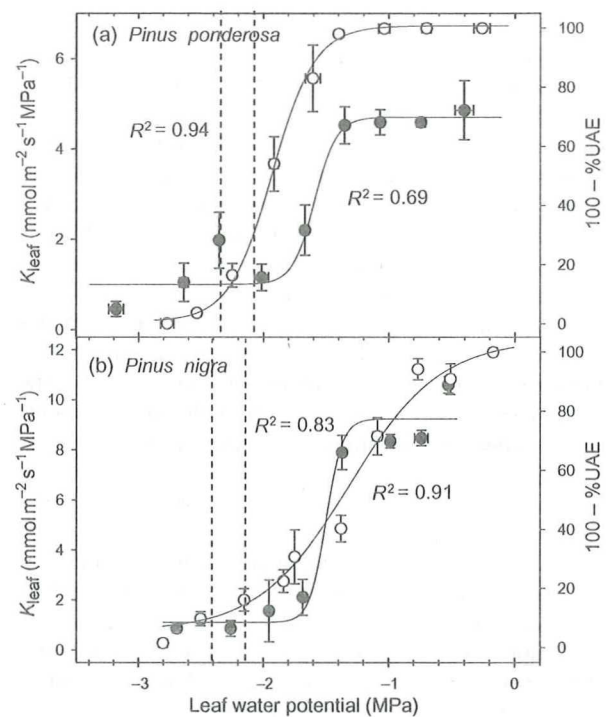


Figure 2. Leaf hydraulic conductance (K_{leaf} , closed circles) and accumulated ultrasonic acoustic emission (UAE, open circles; expressed as 100 - the percentage of total accumulated emissions) at different leaf water potentials for the evergreen needle-leaved species (a) *Pinus ponderosa* and (b) *Pinus nigra*. Dashed lines represent mean turgor loss point ± 1 standard error and vertical error bars represent standard error.

addition, mean amplitude of UAE was strongly correlated to mean conduit size ($R^2 = 0.94$, $P = 0.018$, Figs 3, 4), and the lower detection limit of UAE corresponded to a conduit size of approximately 4.5 μm .

At full hydration (Ψ_l approximately -0.1 MPa), no xylem conduits were empty in any of the four species, but, as leaves desiccated, an increasing fraction of conduits appeared to be embolized in cryo-SEM images (Figs 5, 6). In addition, the majority of transfusion tissue surrounding the vascular bundles of *P. ponderosa* was empty at -1.4 MPa (Fig. 5c), a water potential that corresponded to only 4% of accumulated UAE and approximately 92% of maximum K_{leaf} . At a given Ψ_l the percentage of conduits that remained water-filled in cryo-SEM images was strongly

Table 1. Leaf water potential values (Ψ_l), from best-fit regressions, corresponding to 10 and 50% of total accumulated emissions and 10 and 50% loss in K_{leaf} . Numbers in parentheses represent 95% confidence intervals

Species	Ψ_l at 10% UAE	Ψ_l at PLC10	Ψ_l at 50% UAE	Ψ_l at PLC50
<i>Castanopsis chrysophylla</i>	-1.29 (-0.98/-1.56)	-1.11 (-0.40/-1.44)	-2.82 (-2.68/-2.97)	-2.40 (-2.20/-2.60)
<i>Pieris japonica</i>	-0.43 (-0.19/-0.58)	-0.87 (-0.50/-1.12)	-1.08 (-0.94/-1.22)	-2.12 (-1.90/-2.31)
<i>Pinus nigra</i>	-0.67 (-0.50/-0.85)	-1.37 (-1.25/-1.46)	-1.39 (-1.28/-1.52)	-1.52 (-1.47/-1.57)
<i>Pinus ponderosa</i>	-1.58 (-1.49/-1.65)	-1.43 (-1.08/-1.55)	-1.94 (-1.90/-1.97)	-1.65 (-1.54/-1.75)

PLC, percent loss of conductance.