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

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ABSTRACT

Hydraulic conductance of leaves (Kleaf) typically decreases with increasing water stress. However, the extent to which the decrease in Kleaf is due to xylem cavitation, conduit deformation or changes in the extra-xylary pathway is unclear. We measured Kleaf concurrently with ultrasonic acoustic emission (UAE) in dehydrating leaves of two vessel-bearing and two tracheid-bearing species to determine whether declining Kleaf 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, Kleaf decreased as leaf water potentials (Ψleaf) became more negative. Values of Kleaf corresponding to bulk leaf turgor loss points ranged from 13 to 45% of their maximum. Additionally, Ψleaf 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 Kleaf were closely associated with accumulated UAE and the percentage of empty conduits. The mean amplitude of VAEs was tightly correlated with mean conduit diameter (P = 0.94, P = 0.018). These results suggest that water-stress-induced decreases in Kleaf 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 (Kleaf) 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 Kleaf are related to reversible xylem cavitation, reversible partial conduit collapse or changes in the extra-xylary portion of the pathway is uncertain (Sack & Holbrook 2006). Moreover, the relative importance of mechanisms contributing to variation in Kleaf may differ among species.

Recent work has provided evidence that water stress-induced reductions in Kleaf resulted from leaf xylem cavitation (e.g. Bucci et al. 2003; Nardini, Salleo & Raimondo 2003; Woodruff et al. 2007). However, decreases in Kleaf 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 Kleaf 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 Kleaf (Cochard et al. 2007; Kaldenhoff et al. 2008; Voicu, Zwiazek & Tyree 2008).

Another example of extra-xylary impacts on Kleaf is the collapse of accessory transfusion tracheids in Podocarpus gracilis, which coincided with depressions of Kleaf 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...
Acoustic detection of leaf embolism

Japonica 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 KL,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 KL,leaf and to assess the extent to which water stress-induced loss of KL,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.

KL,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:

\[ KL,leaf = \frac{C \ln(\Psi_f/\Psi_i)}{t} \]

where \( C \) = capacitance, \( \Psi_f \) = leaf water potential before partial rehydration, \( \Psi_i \) = 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 transpiration 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 (\( \Psi_i \)), and for final values after a period of rehydration of \( t \) seconds (\( \Psi_f \)), which was between 30 and 120 s. Distilled water was used for rehydration of KL,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 KL,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 \( \Psi_f \) 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 \( \Psi_i \) versus relative water content (RWC). The slope of the curve before and after turgor loss provided \( C \) in terms of RWC (Cw) 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 \( \Psi_i \) against RWC, and alternate determinations of fresh mass and \( \Psi_i \) were repeated during slow dehydration of the twig on the laboratory bench until values of \( \Psi_i \) 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 (\( n = 6 \) 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 156, 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 ΨI was determined. Leaves were selected for cryo-SEM based on ΨI that corresponded to different points on ΨI versus KIab 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 (LN2). Subsequently, midribs were excised with scissors that were also cold, and then midveins were dropped back into LN2. Midveins were then placed into fracture rivets (room temperature) containing Tissue-Tek and then immediately dropped back into LN2 (approximately 2 s was required to transfer tissue into rivets and then return it to LN2). Pine needles were inserted into Tissue-Tek/rivet, and the rivet/needle was dropped into a vial containing LN2. 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 LN2. Vials containing samples were then placed into a cryo-shipper (CX500; Taylor-Wharton Cryogenics, Theodore, AL, USA) that had been pre-charged with LN2. 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; Emetech 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 for the abaxial surface of each leaf midvein, 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).

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Statistics/data analysis

For each species, KIab 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 KIab 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, KIab and cumulative UAEs followed similar trends as ΨI declined (Figs 1,2). Differences between ΨI at 50% loss of KIab 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 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 $\mu$m.

At full hydration ($\Psi_{f}$, 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 $\Psi_{f}$ the percentage of conduits that remained water-filled in cryo-SEM images was strongly

Table 1. Leaf water potential values ($\Psi_{f}$), 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

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

PLC, percent loss of conductance.