

Aquaporin-mediated changes in hydraulic conductivity of deep tree roots accessed via caves

ANDREW J. MCELRONE^{1,2}, JUSTIN BICHLER², WILLIAM T. POCKMAN³, ROBERT N. ADDINGTON⁴, C. RANDAL LINDER⁵ & ROBERT B. JACKSON⁴

¹USDA-ARS, Crops Pathology and Genetics Research Unit, Department of Viticulture and Enology, University of California, Davis, CA 95616, ²Department of Biology, Saint Joseph's University, Philadelphia, PA 19131, ³Department of Biology, University of New Mexico, Albuquerque, NM 87131, ⁴Department of Biology and Nicholas School of the Environment, Duke University, Durham, NC 27708 and ⁵Section of Integrative Biology, University of Texas, Austin, TX 78712, USA

ABSTRACT

Although deep roots can contribute substantially to whole-tree water use, little is known about deep root functioning because of limited access for *in situ* measurements. We used a cave system on the Edwards Plateau of central Texas to investigate the physiology of water transport in roots at 18–20 m depth for two common tree species, *Quercus fusiformis* and *Bumelia lanuginosa*. Using sap flow and water potential measurements on deep roots, we found that calculated root hydraulic conductivity (RHC) fluctuated diurnally for both species and decreased under shading for *B. lanuginosa*. To assess whether these dynamic changes in RHC were regulated during initial water absorption by fine roots, we used an ultra-low flowmeter and hydroxyl radical inhibition to measure *in situ* fine root hydraulic conductivity (FRHC) and aquaporin contribution to FRHC (AQPC), respectively. During the summer, FRHC and AQPC were found to cycle diurnally in both species, with peaks corresponding to the period of highest transpirational demand at midday. During whole-tree shade treatments, *B. lanuginosa* FRHC ceased diurnal cycling and decreased by 75 and 35% at midday and midnight, respectively, while AQPC decreased by 41 and 30% during both time periods. A controlled growth-chamber study using hydroponically grown saplings confirmed daily cycling and shade-induced reductions in FRHC and AQPC. Winter measurements showed that the evergreen *Q. fusiformis* maintained high FRHC and AQPC throughout the year, while the deciduous *B. lanuginosa* ceased diurnal cycling and exhibited its lowest annual values for both parameters in winter. Adjustments in FRHC and AQPC to changing canopy water demands may help the trees maintain the use of reliable water resources from depth and contribute to the success of these species in this semi-arid environment.

Key-words: aquaporins; deep roots; hydraulic signals; long distance water transport; root hydraulic conductivity; water channel gating; whole-tree water use.

Correspondence: A. J. McElrone. Fax: 530 752 1819; e-mail: ajmcelrone@ucdavis.edu

INTRODUCTION

On a global basis, the vast majority of root biomass is found in the top 2 m of soil (Weaver 1919; Schenk & Jackson 2002a,b), yet numerous woody species have been shown to grow roots below 5 m depth, with a documented maximum exceeding 60 m (Canadell *et al.* 1996; Jackson *et al.* 1996; Schenk & Jackson 2002b). Although they account for only a small proportion of total root biomass, deep roots can contribute substantially to whole-tree water use, meeting as much as ~75% of daily transpirational demands (e.g. Nepstad *et al.* 1994). Deep water resources acquired by woody plants can also increase water cycling at the ecosystem scale and modify regional temperature regimes by increasing latent heat exchange (Lee *et al.* 2005). Such deep water utilization may increase in the future as woody plant encroachment and afforestation increase the proportion of deeply rooted plants across the globe (Scholes & Archer 1997; Jackson, Sperry & Dawson 2000; Engel *et al.* 2005). Therefore, a more thorough understanding of deep root form and function is needed to predict water-use patterns now and in the future.

Despite their importance for water uptake, deep roots have rarely been studied *in situ* without extensive excavation and rhizosphere disturbance. To overcome this limitation, Jackson *et al.* (1999) utilized limestone caves in central Texas, USA to study belowground community composition and maximum rooting depth. They linked deep roots to aboveground shoots using DNA sequence variation (Jackson *et al.* 1999; Linder, Moore & Jackson 2000) and found that six of the dominant tree species on the Edwards Plateau grew roots below 5 m, and at least four of the six reached 18 m. Deep roots from several of these species had extremely high hydraulic efficiencies resulting from increased xylem conduit diameters (McElrone *et al.* 2004). A similar pattern has been found for roots of *Prunus* spp. accessed at ~6 m depth using caves in mesic habitats of Pennsylvania, USA (McElrone *et al.* unpublished data). Physiological adjustments of fine roots at depth may also help to maximize the hydraulic efficiency of deep roots.

Water moving across fine roots to the xylem encounters substantial radial resistance from living cells (Steudle 2001).

The radial resistance of fine roots is typically much higher than that of the axial flow path in the xylem because water must cross plasma and vacuolar membranes (transcellular pathway), cell walls (apoplastic pathway) or plasmodesmata connections between adjacent cells (symplastic pathway) before entering xylem conduits in the stele (Steudle & Peterson 1998). Plants regulate the resistance of the transcellular pathway to facilitate water uptake during midday periods of high transpiration. For example, root hydraulic conductivity (RHC) of *Lotus japonicus* has increased fivefold during the day compared to measurements at night (Henzler *et al.* 1999).

Diurnal fluctuations in root resistance have been linked to regulation of water-specific membrane protein channels called aquaporins (AQPs) (Javot & Maurel 2002). AQPs form narrow, hydrophilic pores in plasma and vacuolar membranes, and are found in most types of root cells (Agre, Bonhivers & Borgnia 1998; Javot & Maurel 2002). Functional regulation of AQPs has been attributed to changes in protein abundance and/or channel gating (Luu & Maurel 2005). Despite a recent flurry of research on the localization and regulation of root AQPs, little work has addressed their importance to plants in field conditions. During periods of high canopy water demand, elevated AQP conductivity may be especially advantageous in deep roots, where axial conductivity is particularly high (McElrone *et al.* 2004) but where water uptake could be limited by the high radial resistance of fine roots.

In the current study, we used cave systems in central Texas to study the physiology of deep roots *in situ* for two of the dominant tree species in this region, *Quercus fusiformis* and *Bumelia lanuginosa*. In 2002, we initiated measurements of deep root water potential and sap flow on woody tap roots at this site, and calculations using this data revealed that the hydraulic conductivity of the roots changed dramatically diurnally and seasonally. Here we present the calculated RHC data for deep woody roots coupled with detailed physiological measurements from both field and growth chamber experiments to assess the contribution of fine root AQPs to changes in RHC. We tested the following three hypotheses: (1) AQPs contribute to the temporal changes in RHC that we observed in woody tap roots; (2) fine root hydraulic conductivity (FRHC) and aquaporin contribution to FRHC (AQPC) are lower under periods of lower transpirational demand (e.g. at night, while shaded, and in winter); (3) FRHC and AQPC differ seasonally for the evergreen species *Q. fusiformis* compared to the deciduous species *B. lanuginosa* because of changes in canopy transpirational demands.

MATERIALS AND METHODS

Field site, study species and root–shoot connections

The Edwards Plateau is a 100 000 km² region in central Texas characterized by shallow soils overlaying fractured limestone (Elliot & Veni 1994). The vegetation is primarily

savanna and woodland dominated by trees in the genera *Quercus*, *Bumelia*, *Juniperus*, *Ulmus*, *Celtis* and *Prosopis* (van Auken *et al.* 1980). The current work was conducted at Powell's Cave in Menard, TX, USA, which contains a perennial underground stream at ~18–20 m depth and is part of the second largest cave network in the state. The two principal tree species investigated were the evergreen *Q. fusiformis* (live oak) and the deciduous *Bumelia lanuginosa* syn. *Sideroxylon lanuginosum* (Gum Bumelia).

Woody tap roots and fine roots in the cave were identified either to species or individual levels using DNA sequence variation. Species level identification was achieved by comparing DNA sequences of the internal transcribed spacer (ITS) region of the 18S–26S nuclear ribosomal DNA repeat, derived from root samples, with a reference ITS region database for species at the site. Details of this method can be found in Linder *et al.* (2000). Individual trees were linked to their corresponding roots using inter-simple sequence repeats (ISSR), a DNA fingerprinting technique. Leaf material from stems above the cave and root material within the cave were collected and placed in ziplock bags on ice while in transit to the lab. Samples collected from trees located at distant field sites were used as negative controls. Sample DNA was extracted using a 1.0 M borate buffer (see protocols in Jackson *et al.* 1999; Linder *et al.* 2000). Leaf DNA was purified by either Elu-Quik (Schleicher & Schuell Bioscience, Inc., Keene, NH, USA) or the DNeasy Plant Mini Kit (QIAGEN, Inc., Valencia, CA, USA), while root DNA was gel purified from 1.5% agarose buffered with 0.5X TRIS, boric acid, EDTA (TBE) using the QIAEX II Gel Extraction Kit (QIAGEN, Inc.). Twelve ISSR primers (#843, 844, 807–812, 814, 815, 818, 820) were purchased from the University of British Columbia's Nucleic Acid Protein Services Unit, consistently produced polymorphic banding patterns, and were used for PCR amplification. Reaction cocktails, amplification cycles and gel runs were similar to those of Linder *et al.* (2000), and all runs included a negative control consisting of all reaction components except genomic DNA. Gels were stained with ethidium bromide; bands were visualized with UV light, and digital images of the gels were captured with an Alpha-Imager and the accompanying AlphaEase software (AlphaInnotech, San Leandro, CA, USA). The images were imported into Gel-Pro Analyzer (Media Cybernetics, Bethesda, MD, USA) and band sizes were analyzed using the 1 KB ladder as a standard. Data were then analyzed using clustering methods to determine which stems and roots were most likely to be connected. All cluster analyses were conducted in SYSTAT 8.0 (San Jose, CA, USA) (Wilkinson 1998). In the hierarchical clustering module, clusters were created using the per cent differences option in combination with the single, complete, average and Ward linkage options. All of these clustering methods produce a tree of relationships based upon the percentage dissimilarities between samples. Because the relationship between individuals in a population is often reticulate rather than simply tree-like, we also used the additive clustering module, which reconstructs a network of relationships and

then depicts it as a tree (Sattath & Tversky 1977). The strength of support for the clustering of roots and shoots was assessed using the bootstrapping feature, SAMPLE, in SYSTAT 8.0. One thousand bootstrap replicates with replacement were run for each type of analysis.

Microclimate, water potential, sap flow and calculated RHC

Microclimatic data were recorded at a weather station located at Powell's cave for comparison with sap flow and physiological measurements. Photosynthetically active radiation (PAR) (LI-190 sensor; Li-Cor, Lincoln, NE, USA), air temperature and relative humidity (RH) (HMP45C probe; Campbell Scientific, Logan, UT, USA) were continually recorded with a datalogger (model CR7, Campbell Scientific) throughout the duration of the studies. Changes in PAR, air temperature and RH during shading experiments (details described further below) were measured with a second set of sensors positioned under the shade cloth.

Diurnal fluctuations of leaf and root water potentials (Ψ) were measured throughout experiments using two different techniques. Leaf Ψ was measured diurnally using a Scholander pressure chamber (PMS, Albany, OR, USA), while deep woody root Ψ was measured using *in situ* stem hygrometers (Plant Water Status Instruments, Guelph, Ontario, Canada) linked to a second CR-7 datalogger located in the cave. To install the hygrometers, the outer surface of the roots was removed using a razor blade until wet xylem tissue was exposed. The exposed sapwood was then rinsed using distilled water; the hygrometers were clamped to each root, and the chambers were sealed using vacuum grease to prevent evaporative gradients (Dixon, Grace & Tyree 1984). The conditions in the cave were ideally suited for using the hygrometers because daily temperature fluctuations were <0.5 °C.

Sap flow in trunks and deep roots was measured using one of three different techniques. Granier-type, thermal dissipation probes, which are well suited for use in larger diameter axes, were used to measure sap flow in five *B. lanuginosa* trunks (mean diameter ≈ 11.6 cm). Each probe consisted of a heated (200 mW) and a non-heated reference needle, which were inserted radially into the xylem with the heated needle positioned ~ 10 cm above the reference needle. The temperature difference between the heated and non-heated needles was used to calculate sap flux density according to Granier (1987). Sap flux data were recorded every minute with the aboveground datalogger and were averaged over 15 min intervals. Probes were installed at breast height towards the north side of each tree and were covered with reflective shielding to protect the probes from direct radiation. Sapwood area was determined using increment cores extracted from each tree.

Sap flow sensors capable of measuring bidirectional flow were required for measurements in roots (Burgess *et al.* 2001), and we used two such techniques that work well on roots of different sizes. The constant-power heat-balance technique was used to measure sap flow in a small diameter

deep root of *B. lanuginosa* (see details in Kjelgaard *et al.* 1997; Sakuratani, Aoe & Higuchi 1999). Briefly, a 50Ω heater constructed of constantan wire (TFCC-003; Omega Engineering, Inc., Stamford, CT, USA) was wrapped tightly around the root in a narrow band and supplied constantly with 3.5–4.0 V using a voltage regulator (Dynamax, Inc., Houston, TX, USA) for the duration of the experiment. Axial heat transfer was measured using downstream and upstream thermocouples (Type T-36, Omega Engineering, Inc.) placed 15 mm above and below the heater. A third thermocouple, placed directly under the heater, was used to measure its output. Radial heat flux away from the heater was measured using a thermopile constructed with thermocouple junctions (TT-T-30, Omega Engineering, Inc.) linked across craft foam. The thermopile was wrapped around the root directly over the heater, and several layers of foam pipe insulation were wrapped around the entire sensor to limit radial heat loss. To avoid cutting the roots so that they could be used in future investigations, we calculated the apparent sheath conductance (k) by using the empirical technique described by Sakuratani *et al.* (1999).

Sap flow in deep roots of *Q. fusiformis* was measured using the heat ratio method (HRM) developed by Burgess *et al.* (2001) and with sensors designed by Thermal Logic, Inc. (Pullman, WA, USA). Briefly, two temperature sensor needles containing type-E thermocouples were installed symmetrically and in parallel in sapwood above and below (6 mm) a line heater probe containing a coiled heating element. The larger diameters of *Q. fusiformis* deep roots (relative to *B. lanuginosa* roots) allowed for insertion of the HRM sensor needles into the woody tissue. Any potential spacing errors between the thermocouple and heater needles were minimized by using a drill guide. A short pulse of heat was released into the sapwood, and the resultant velocity was calculated according to Burgess *et al.* (2001). We also used established protocols outlined in Burgess *et al.* (2001) to correct data for errors caused by incorrect probe spacing and wounding around drill holes. Sapwood properties were determined as for the other sap flow techniques.

We used sap flow and Ψ data from deep roots to calculate RHC with the following equation: $RHC = \text{deep root sap flow} / (\text{deep root } \Psi - \text{stream water } \Psi)$. Stream water Ψ was assumed to be constant and ~ 0 for calculation purposes. Ψ of the stream water was used for calculations because the deep tap roots were connected to networks of fine roots submerged in the underground stream.

Measurement of FRHC and AQP activity

An ultra-low flowmeter (ULFM) was constructed according to Tyree *et al.* (2002) to measure flow rates through fine roots of *B. lanuginosa* and *Q. fusiformis*. The ULFM measures the pressure drop (dP) across a standard PEEK capillary tube (diameter 0.13–0.18 mm; Upchurch Scientific, Oak Harbor, WA, USA), which was calibrated to quantify the linear relationship between flow rate and dP (Tyree *et al.* 2002). Water flows through the ULFM via two Omnifit 8-way manifolds (Cole-Parmer, Vernon Hills, IL, USA) with

flow between the manifolds occurring through PEEK tubing of various sizes. The pressure drop between the two manifolds was measured by a differential pressure transducer (PX26; Omega Engineering, Inc.) and recorded every two seconds using a datalogger (CR10X, Campbell Scientific). Once flow was determined using the ULFM, FRHC ($\text{m s}^{-1} \text{MPa}^{-1}$) was calculated using the following equation: $\text{FRHC} = (Q_v/P)(1/A)$, where Q_v is the volumetric flow rate ($\text{m}^3 \text{s}^{-1}$); P (MPa) is the pressure applied to the ULFM, and A (m^2) is the surface area of a cylinder calculated from fine root segment length and radius (North & Nobel 1991).

An underground lab with two ULFMs was established at depth in Powell's cave. A nitrogen gas cylinder (Catalina Cylinders, Garden Grove, CA, USA) provided a range of pressures to a captive air tank (Flotec, Delavan, WI, USA) filled with filtered distilled water, which in turn forced the water into the ULFM. An excised fine root was placed into 6 mm diameter polyethylene tubing and sealed with dental impression material (vinyl polysiloxane – Correct VPS Heavy Body; Pentron, Wallingford, CT, USA), providing a water barrier that forced water through, and prevented compression of, the delicate fine root. Once the epoxy solidified (~3 min while submerged in a container filled with stream water), the root and tube were placed into the outlet tube of the ULFM and clamped to prevent leaks. This configuration was then placed back into the stream water container. After the gas cylinder was pressurized and the fine root was given time to equilibrate to flow (~20–60 min), FRHC was determined as described earlier. Fine roots used in the FRHC measurements (mean diameter ≈ 0.45 mm, mean length ≈ 3.5 cm) were sampled from large root masses growing submerged in the stream water. *Bumelia lanuginosa* fine roots were primarily white and very fragile, while those of *Q. fusiformis* were dark reddish brown and more rigid (i.e. more woody). Fine roots from the growth chamber experiment (see further description) were similar in size and macroscopic structure to those sampled in the cave.

Henzler, Ye & Steudle (2004) and Ye & Steudle (2006) demonstrated the effectiveness of using hydroxyl radicals (*OH) to inhibit AQP and assess their contributions to root cell hydraulic conductivity. We used hydroxyl radicals on fine roots in the cave to determine if AQPs played an important role in water uptake. We chose this method because hydroxyl radicals are less toxic to roots, cave flora and fauna (and field researchers) than other means of AQP inhibition, such as mercuric chloride (HgCl_2). We compared the effectiveness of both inhibition techniques in the lab prior to use in the cave and showed similar levels of reversible AQP inhibition. Hydroxyl radicals were produced using the Fenton reaction by mixing equal parts of 0.6 mM H_2O_2 and 3 mM FeSO_4 in a total volume of 15 mL (see details in Henzler *et al.* 2004). After initial FRHC was determined, the fine root was subjected to hydroxyl radicals by injecting 10 mL of Fenton reaction mixture into the tube preceding the root sample. Inhibition was allowed to occur until FRHC measurements resettled. AQP contribution to FRHC (referred to as 'AQPC' throughout the rest of the

manuscript) was assessed as per cent loss between initial and inhibited FRHC.

All FRHC measurements were conducted at either midday (1100–1600 h) or midnight (2300–0400 h). Previous research has shown that root AQPC increases during the day and decreases at night (Henzler *et al.* 1999). To assess whether FRHC and AQPC change under varying canopy transpirational demands, we made seasonal comparisons during three field campaigns in Powell's cave; 15–21 May 2005, 9–19 July 2005 and 5–10 January 2006 to assess FRHC and AQPC in the evergreen *Q. fusiformis* and deciduous *B. lanuginosa*. During the July 2005 campaign, a 95% shade cloth was suspended above all five *B. lanuginosa* trees, effectively blocking solar radiation and limiting transpiration. During shading, PAR reaching the *B. lanuginosa* canopy approached zero, while the vapour pressure deficit (VPD) remained unchanged. The same shading treatment was used for the July 2002 experimental results summarized in Fig. 1.

Growth chamber experiment

Growth chamber experiments were used to investigate FRHC and AQPC under controlled conditions for both *Q. fusiformis* and *B. lanuginosa*. Twenty-five saplings of each species from Sunshine Nursery and Arboretum (Clinton, OK, USA) were grown in a hydroponic/pot system. The saplings remained in their original square pots (12.5 cm height \times 5.5 cm width), each of which had four square holes (4 cm^2) on the bottom that allowed fine roots to grow out of the bottom of the pot. Each pot was put into a 500 mL plastic tri-pour beaker, which had the bottom removed, and the beakers in turn were set into holes (8 cm diameter) on a high-density fiber board. The boards were then placed over one of several black plastic tubs filled with a modified half-concentration Hoagland solution, a conventional hydroponic solution (Hoagland & Snyder 1933), and the fine roots were allowed to grow into the solution for 30 d before taking measurements. This setup mimicked the cave system on a much smaller, controlled scale, with shallow roots in soil and deep roots submerged in a water solution. The pots were placed such that they did not touch the Hoagland solution to avoid wicking into the pot soil. A Meiko bubble bar (Meiko Pet Corp., Taichung, Taiwan) was placed on the bottom of each tub to prevent anoxia. Growth chambers were set on a 16/8 h light : dark cycle at a constant temperature of 26.7 °C. FRHC and AQPC measurements were gathered using the same protocol as the field measurements.

Statistical analysis

All statistical analyses were carried out using SPSS version 12.0 (SPSS, Chicago, IL, USA). All FRHC and AQPC measurements for both species in the field and *B. lanuginosa* in the growth chamber were analyzed using a two-way analysis of variance (ANOVA) testing for month, time of day and shading effects. FRHC and AQPC values for *Q. fusiformis* in the growth chamber were analyzed using independent *t*-tests.

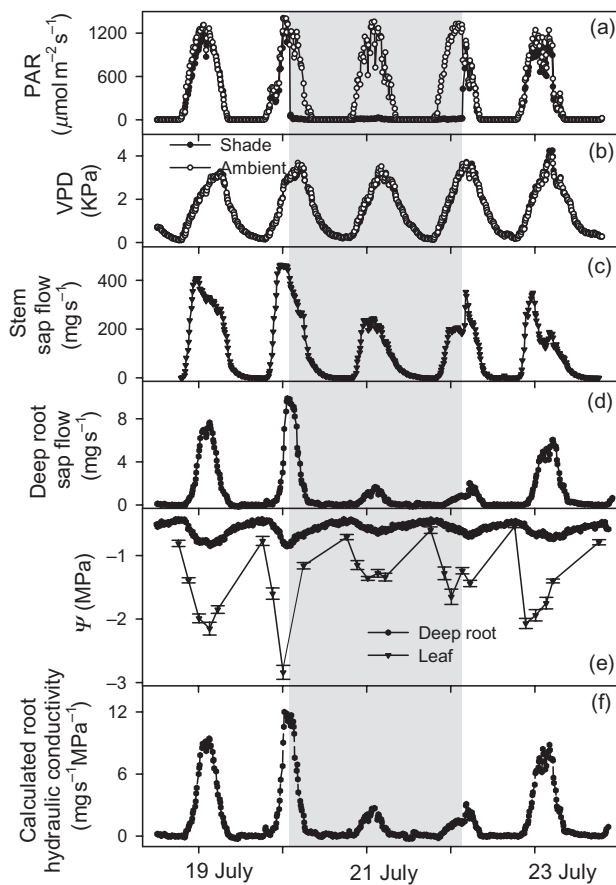


Figure 1. Water transport responses of *Bumelia lanuginosa* stems and roots to the shading treatment from 20–22 July 2002. Photosynthetically active radiation (PAR) (a), vapour pressure deficit (VPD) (b), stem and deep root sap flow (c,d), leaf and deep root water potential (Ψ) (e) were continually recorded. Root hydraulic conductivity (RHC) (f) was calculated using the following equation: $RHC = \text{deep root sap flow} / \text{deep root } \Psi$ (see Materials and Methods for details on this calculation). The light gray box in the centre of the figure represents the period during which the trees were shaded. See Materials and Methods for details of the shading experiment and physiological measurements.

RESULTS

Sap flow, water potential and calculated RHC

Sap flow for the stems and deep root of *B. lanuginosa* exhibited clear diurnal cycles, peaking at midday and returning to near zero at night (Fig. 1c,d). During the shading experiment, midday sap flow rates were reduced by ~48 and ~86% with respect to pre-treatment values during a period of largely clear cloudless weather for the *B. lanuginosa* stems and deep root, respectively. PAR was reduced to near zero under shading conditions, while the VPD did not differ from ambient conditions, suggesting that reduced canopy transpiration was most strongly influenced by shade-induced stomatal closure (Fig. 1a–c). Diurnal patterns in leaf and deep root Ψ closely tracked those of sap flow and increased (i.e. became less negative) during the shade treatment by ~44%

for leaves and ~24% for the deep root (Fig. 1e). Changes in xylem pressure in the canopy propagated quickly to deep roots, suggesting low capacitance of plant tissues and tight links to deep water resources for *B. lanuginosa* trees in this system (Fig. 1e).

RHC calculated using sap flow and Ψ data from the deep root of *B. lanuginosa* exhibited diurnal cycling and midday values that were reduced by ~79% during shading relative to full sun conditions (Fig. 1f). Similar diurnal cycling of calculated RHC was evident in deep roots of *Q. fusiformis* measured in the winter (Fig. 2). Diurnal cycling of calculated RHC for *Q. fusiformis* was also apparent during other measurement periods throughout the year (data not shown). Calculated RHC values were approximately 10-fold greater in *Q. fusiformis* than in *B. lanuginosa* as a result of greater sap flow in the larger diameter *Q. fusiformis* roots.

FRHC and AQP contribution in the cave

Overall, AQPC contributed significantly to FRHC of *Q. fusiformis* fine roots throughout the year (Fig. 3). *Quercus fusiformis* exhibited diurnal cycling in FRHC ($P < 0.05$) (as measured with the ULFM), with the highest values occurring during midday at each sampling period (Fig. 3). Midday FRHCs were 164, 103 and 33% higher than midnight FRHC in May, July and January, respectively (Fig. 3). AQPC showed a trend for diurnal cycling in May and July, with midday values 29 and 26% higher than midnight values, respectively. However, in January, AQPC was nearly equal for midday and midnight measurements (Fig. 3).

Changes in *B. lanuginosa* FRHC and AQPC occurred naturally on a diurnal and seasonal basis. High FRHC and AQPC corresponded with periods of high canopy water demands. In July, *B. lanuginosa* displayed a trend for diurnal

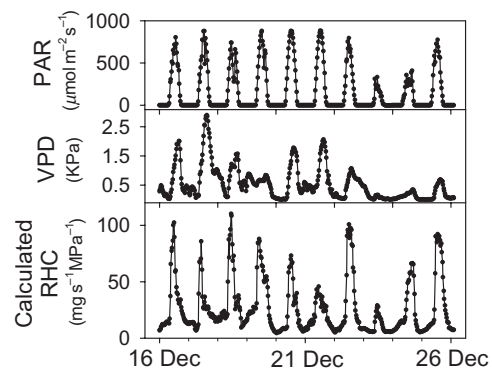


Figure 2. Diurnal fluctuations in calculated root hydraulic conductivity (RHC) for deep roots of *Quercus fusiformis*. Deep root sap flow and water potential were measured continually on tap roots at ~18–20 m depth in December 2003. Photosynthetically active radiation (PAR) and vapour pressure deficit (VPD) were recorded continually at an aboveground micro-meteorological station to illustrate changes in aboveground water demand during this period. RHC (bottom panel) was calculated using the same equation as Fig. 1.

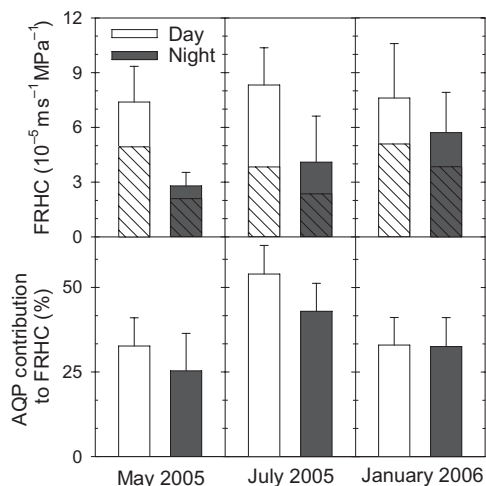


Figure 3. Fine root hydraulic conductivity (FRHC, top panels) and aquaporin contribution to FRHC (AQPC, lower panels) for deep roots of *Quercus fusiformis* at Powell's cave on three dates. The hatched portion of the bars in the top panels represents the mean FRHC after treatment with hydroxyl radicals. The difference between the native FRHC (entire bar top panels) and inhibited FRHC (hatched portion of bars in top panels) was used to calculate % AQPC (lower panels). This same format is used in all subsequent figures. Midday and midnight measurements are means (\pm SE) of replicates ($n = 8-12$) collected from various tap roots and compiled across several days. Replication and sampling were similar for data in all subsequent figures unless noted otherwise. ANOVA results: time of day effect, FRHC $P < 0.02$, AQPC $P = 0.373$; season effect, FRHC $P = 0.733$, AQPC $P = 0.100$; time of day \times season, FRHC $P = 0.725$, AQPC $P = 0.801$.

cycling in FRHC with values during the midday 47% higher compared to midnight values (Fig. 4). Summer AQPC was high both during the midday (46.1%) and midnight (39.9%) (Fig. 4). During January, FRHC ceased diurnal cycling and plunged by 85% throughout the day as compared to the summer values (Fig. 4). Likewise, winter AQPC fell by 65 and 47% during midday and midnight, respectively (Fig. 4).

Changes in *B. lanuginosa* FRHC and AQPC were also induced during the shading treatment. FRHC stopped cycling diurnally and plummeted 75% during midday and 35% during midnight when the five *B. lanuginosa* trees were shaded in July (Fig. 5). Similarly, AQPC dropped during both midday and midnight at 41 and 30%, respectively (Fig. 5).

Growth chamber results for FRHC and AQPC contribution

Quercus fusiformis saplings grown hydroponically in growth chambers also displayed diurnal cycling in both FRHC ($P < 0.05$) and AQPC ($P < 0.05$) (Fig. 6). FRHC during midday was nearly fivefold higher compared with midnight (Fig. 6), while AQPC displayed midday values that were greater than fourfold higher than midnight values (Fig. 6).

Fine roots of *B. lanuginosa* saplings grown hydroponically displayed similar response patterns to those collected in the cave. Both FRHC and AQPC decreased substantially during shading and at night, corresponding with periods of low canopy water demands (Fig. 7). Under pre-shaded conditions, there was diurnal cycling in both FRHC ($P < 0.05$) and AQPC ($P < 0.05$) (Fig. 7). FRHC and AQPC were 4.6 and 1.5 times higher during midday relative to night measurements (Fig. 7). When shaded, both FRHC and AQPC ceased diurnal cycling and plummeted by 92 and 69%, respectively, at midday (Fig. 7).

DISCUSSION

Our data show that dynamic regulation of AQPs contributes substantially to diurnal and seasonal changes in FRHC in two species of woody plants and partially minimizes the limitations associated with radial hydraulic conductivity in fine roots. These results are consistent with previous studies (e.g. Henzler *et al.* 1999), but to our knowledge represent the first such report from deep roots of plants in the field. Because radial water absorption by fine roots represents a large fraction of the resistance along the entire flow path (Stedde 2000), the changes in AQPC documented here effectively maximize the benefits achieved by increased axial hydraulic conductivity observed in deep root xylem of these same study species (McElrone *et al.* 2004). The close correspondence between maximum AQPC in deep roots of *B. lanuginosa* and *Q. fusiformis* and the times of highest canopy water loss suggest that this regulation minimizes the

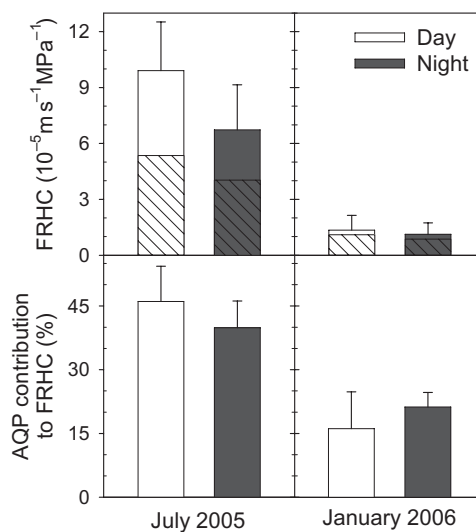


Figure 4. Fine root hydraulic conductivity (FRHC, top panels) and aquaporin contribution to FRHC (AQPC, lower panels) for *Bumelia lanuginosa* deep roots measured at Powell's cave in July 2005 and January 2006. The hatched portion of the bars in the top panels represents the mean FRHC after treatment with hydroxyl radicals (see Fig. 3 legend for details). ANOVA results: time of day effect, FRHC $P = 0.383$, AQPC $P = 0.578$; season effect, FRHC $P < 0.002$, AQPC $P < 0.002$; time of day \times season, FRHC $P = 0.447$, AQPC $P = 0.208$.

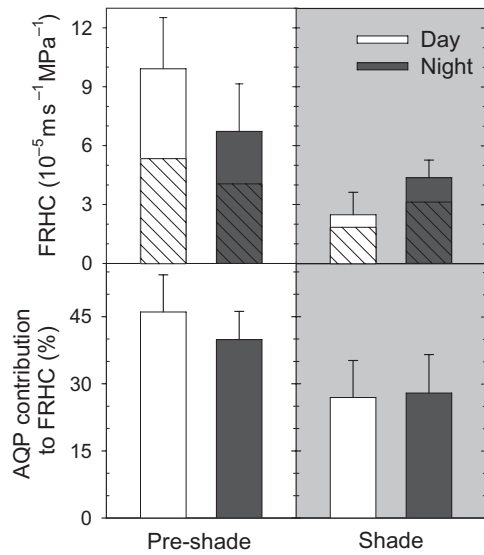


Figure 5. Fine root hydraulic conductivity (FRHC, top panels) and aquaporin contribution to FRHC (AQP contribution to FRHC, lower panels) for *Bumelia lanuginosa* deep roots measured at Powell's cave during pre-shade (white background) and shade (grey background) canopy conditions in July 2005. The hatched portion of the bars in the top panels represents the mean FRHC after treatment with hydroxyl radicals (see Fig. 3 legend for details). ANOVA results: time of day effects, FRHC $P = 0.845$, AQP $P = 0.942$; shade effect, FRHC $P < 0.02$, AQP $P < 0.05$; time of day \times shade, FRHC $P = 0.106$, AQP $P = 0.557$.

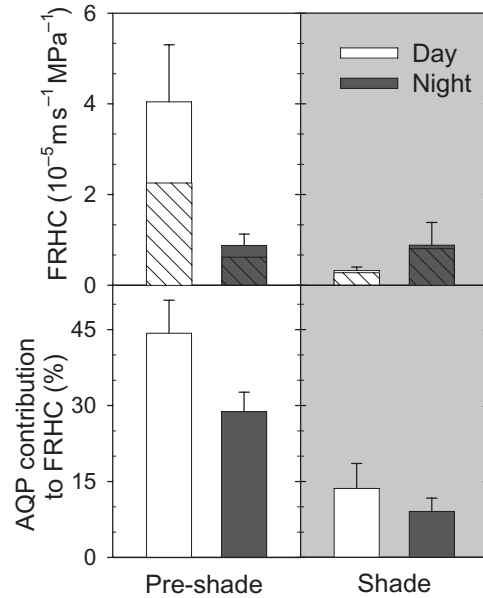


Figure 7. Fine root hydraulic conductivity (FRHC, top panels) and aquaporin contribution to FRHC (AQP contribution to FRHC, lower panels) for *Bumelia lanuginosa* saplings grown in hydroponic solution in growth chambers. The hatched portion of the bars in the top panels represents the mean FRHC after treatment with hydroxyl radicals (see Fig. 3 legend for details). ANOVA results: time of day effect, FRHC $P < 0.05$, AQP $P < 0.05$; shade effect, FRHC $P < 0.02$, AQP $P < 0.001$; time of day \times shade, FRHC $P = 0.153$, AQP $P = 0.475$.

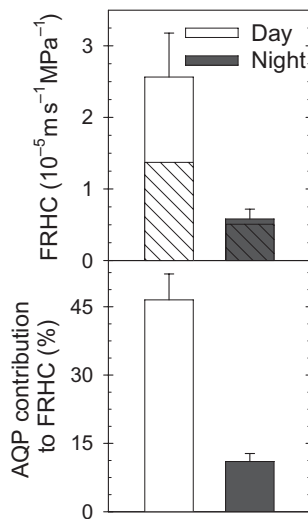


Figure 6. Fine root hydraulic conductivity (FRHC, top panel) and aquaporin contribution to FRHC (AQP contribution to FRHC, lower panel) for *Quercus fusiformis* saplings grown in hydroponic solution in growth chambers. The hatched portion of the bars in the top panel represents the mean FRHC after treatment with hydroxyl radicals (see Fig. 3 legend for details). Data represent means of 10–15 replicates. Independent t -test results: FRHC $P < 0.05$, AQP $P < 0.05$.

limitations of radial water absorption over a wide range of conditions and may represent a universal strategy among higher plants.

An alternative strategy that can be utilized to overcome water supply limitations imposed by fine root resistance is an increase in fine root absorptive surface area. In the underground stream in Powell's Cave, a 1 cm diameter tap root of *Q. fusiformis* or *B. lanuginosa* can support a network of absorbing fine roots that fills a volume roughly 5 m in length and 0.5 m in diameter (McElrone *et al.* 2004). Interestingly, both strategies (i.e. AQP regulation and increased fine root surface area) have been observed in *Arabidopsis* transformed with an antisense plasma membrane intrinsic AQP (*PIP1b*). Roots of transformed plants had three times lower water permeability coefficients and five times more root biomass than control plants, which suggests compensation for reduced AQP activity via increased root surface area (Kaldenhoff *et al.* 1998; see also Martre *et al.* 2002). Our current and previous work clearly shows how trees in this semi-arid system utilize both physiological (i.e. AQP regulation) and anatomical adjustments (i.e. tremendous fine root absorptive area and large xylem conduits; McElrone *et al.* 2004) to facilitate consistent uptake of reliable water resources from depth, which may contribute to their success in this habitat.

Radial water movement across fine roots occurs via three parallel pathways as described by the composite transport model (Steudle & Peterson 1998; Steudle 2000). Apoplastic

flow occurs outside of the cell membrane through the porous cell walls; symplastic flow occurs through cytoplasm of the cells connected by plasmodesmata, and transcellular flow takes place across plasma and vacuolar membranes. Studies of AQP inhibition have used HgCl_2 or OH^* to demonstrate the contribution of the transcellular pathway to RHC, finding contributions as high as 80–90% in sugar beet (Amodeo *et al.* 1999) and barley (Tazawa *et al.* 1997). In our study species, the maximum contribution of the transcellular pathway (i.e. AQP contribution to FRHC) was 45–55% at midday during the middle of the summer when canopy transpirational demand was at its peak (Figs 3 & 4, July data). Our field results are within the range of laboratory and greenhouse results for aspen, tomato, wheat and *Agave* where RHC decreased between 47–66% when treated with HgCl_2 (Maggio & Joly 1995; Carvajal, Cooke & Clarkson 1996; Wan & Zwiazek 1999; North & Nobel 2000). AQP contribution never dropped below 25% throughout the year for the evergreen *Q. fusiformis*, and contributed substantially to FRHC in the deciduous *B. lanuginosa* during the summer. Our work shows that AQPs can enhance the contribution of deep roots to total tree water use, which would be particularly important during extended periods of drought in a region with low soil water capacitance and shallow soils (frequently <20 cm depth) (e.g. Martre, North & Nobel 2001).

Diurnal cycling of RHC linked to AQP contribution has been documented in numerous species (Javot & Maurel 2002) and has been shown to continue for several daily cycles after roots are excised from shoots (e.g. Henzler *et al.* 1999). Such a response suggests the control of AQPs by a circadian rhythm. A recent study showed that sunflowers transferred from a 12 h photoperiod into continuous darkness maintained cycling of leaf hydraulic conductivity in phase with their previous light/dark cycles (Nardini, Salleo & Andri 2005). RHC diurnal cycling in *B. lanuginosa*, however, responded very rapidly to canopy shading and ceased significant diurnal cycling under the low light conditions in both field and growth chamber studies. Such a rapid response over an extensive vascular system (i.e. >25 m from top of canopy to deep roots) and the lack of diurnal cycling during shading suggest that RHC cycling in *B. lanuginosa* is not controlled by a circadian rhythm. We do not know whether the same is true for *Q. fusiformis*. However, our calculated RHC values for this species suggest a fine scale control of RHC in response to microclimatic-induced changes in canopy water demand. For example, calculated RHC dropped substantially on 23 December, with corresponding decreases in PAR and VPD that presumably reduced canopy transpirational water demand (Fig. 2). Therefore, control of RHC diurnal cycling in *B. lanuginosa* and *Q. fusiformis* likely involves AQP regulation by other mechanisms.

Regulation of plant AQP contribution has been attributed to a variety of mechanisms including (1) changes in AQP gene transcription and protein abundance and (2) gating of AQP channels (reviewed in Chaumont, Moshelion & Daniels 2005; Luu & Maurel 2005; Javot & Maurel 2002; Tyerman, Niemietz & Bramley 2002). Gene transcriptional control is

illustrated well in studies that have correlated diurnal cycling of RHC with changes in AQP mRNA abundance (e.g. Henzler *et al.* 1999; Lopez *et al.* 2003). Similar transcriptional and protein abundance regulation of AQPs is likely involved in the responses we documented here. In particular, long-term changes in light levels and canopy water demand (i.e. periods of extended shade and seasonally) would allow enough time for signalling between shoots and roots to alter AQP regulation by transcriptional and protein abundance (Javot & Maurel 2002; Luu & Maurel 2005). Our research group is currently studying patterns of transcription and protein abundance to understand their influence on daily and seasonal AQP regulation in deep fine roots of both study species.

AQP gating in plants can be controlled by several mechanisms that are linked to the characteristic protein structure, which consists of six membrane spanning α -helical domains connected by five loops (designated A–E), with the carboxy and amino termini bathing in the cytosol (Johansson *et al.* 2000; Chaumont *et al.* 2005; Luu & Maurel 2005; Törnroth-Horsefield *et al.* 2006). Gating can be controlled by reversible site-specific binding to amino acids within the loops and carboxy termini causing displacement of the loop and occlusion of the cytoplasmic side of the channel. For example, dephosphorylation of serine residues located on cytosolic loop B and the carboxy terminus (Johansson *et al.* 1998, 2000) leads to closure of some AQPs during drought stress (Törnroth-Horsefield *et al.* 2006), while protonation of a histidine residue located on loop D causes closure during flooding (Tournaire-Roux *et al.* 2003). Recent work has suggested that pressure pulses (i.e. hydraulic signals) transmitted in water columns can also lead to gating of AQPs either by reversible phosphorylation or by direct physical forces applied to the channel itself. Models describing the probable mechanisms by which pressure pulse stimuli are perceived are (1) energy input model where kinetic energy transferred to the channel constriction at the NPA motif causes a conformational change in the protein (Wan, Steudle & Hartung 2004) or (2) cohesion–tension model where tension on the water within the channel changes the open/closed state by reversible deformation or collapse of the channel (Ye, Wiera & Steudle 2004). Given the speed of the response over such a long distance, it is probable that short-term AQP regulation (i.e. diurnally or initial shading) in our deep fine roots may be governed by a pressure pulse stimulus. Stomatal opening/closing can produce sudden pressure pulses that are transmitted rapidly through the xylem and may trigger changes in AQP contribution in the deep roots. Evidence for rapid propagation of pressure signals from leaves to roots was demonstrated by other researchers through the simultaneous insertion of a pressure probe into the xylem of maize leaf middle vein and another probe into the xylem of the roots (Wegner & Zimmermann 1998). When light intensity was increased from 10 to 250 $\mu\text{mol m}^{-2} \text{s}^{-1}$, both probes read a nearly simultaneous drop in pressure after only 120 s. Re-irrigation is also known to induce rapid generation and propagation of electric and hydraulic signals in maize root systems that

enable leaves to respond rapidly to increasing soil moisture (Grams *et al.* 2007). Measurements of the kinetics involved in wound-induced hydraulic signalling have shown that the front of the hydraulic signal can travel through the plant at rates of at least 0.1 m s^{-1} (Malone 1992). If hydraulic signals move anywhere near this rapidly in our study species, stimuli induced by stomatal closure/opening could reach the AQPs in *B. lanuginosa* deep roots within 250 s. Further investigation is required to understand whether pressure pulse stimuli affect AQP gating directly or via cascading effects to other ion channels (e.g. calcium) or hormones (e.g. abscisic acid) Wan *et al.* (2004).

Fine roots studied in our cave system exhibited much higher FRHC than those in the growth chamber. Under full light, FRHC was on average 2.5 and 3.2 times higher in the field relative to the growth chamber for *B. lanuginosa* and *Q. fusiformis*, respectively. High FRHC in cave roots may be needed to accommodate higher transpirational water demands of a large tree canopy in a semi-arid environment compared with those in smaller saplings. Additionally, high FRHC may effectively buffer deep root Ψ above the critical threshold that induces xylem cavitation in these highly vulnerable organs (McElrone *et al.* 2004; Maherali *et al.* 2006). For example, if flow hypothetically increased 10-fold at midday relative to midnight, and FRHC remained constant, the Ψ gradient would become 10 times more negative with the increased flow (using the rearranged Ohm's law analogy, flow = conductivity \times Ψ gradient). Such drastic decreases in deep root Ψ could be potentially devastating for organs that exhibit as much as 24 times higher vulnerability to cavitation relative to *B. lanuginosa* stems (McElrone *et al.* 2004).

Both species exhibited very high midnight FRHC and AQPC in the field relative to the growth chamber. This pattern may be linked to hydraulic redistribution, a phenomenon where water moves passively via roots from moist to dry soil layers along Ψ gradients. Hydraulic redistribution has been found across numerous species and generally occurs during the night when transpiration-induced Ψ gradients have relaxed (Caldwell, Dawson & Richards 1998; Oliveira *et al.* 2005). Using sap flow measurements on deep roots in Powell's cave, we have found that *Q. fusiformis* and *B. lanuginosa* redistribute water at night to drying shallow soil via shallow roots (Bleby *et al.* unpublished data). During extended periods with little to no rainfall when shallow soils become very dry, deep roots exhibit increasing and continual nighttime flow. Nighttime flow induced by hydraulic redistribution may account for higher midnight FRHC and the smaller difference between midday and midnight measurements from the field site relative to the dramatic differences in the growth chamber.

CONCLUSION

To our knowledge, this study is unique in assessing the importance of AQPs for water uptake at depth. By using caves of the Edwards Plateau of central Texas, we were able to access intact and functioning tree roots at 18–20 m depth.

Our results indicate that AQPs contribute substantially to total water uptake, adjust according to canopy water demands and differ seasonally between evergreen and deciduous tree species. More investigation of this dynamic physiology is needed to understand its contribution to whole tree and ecosystem water cycling.

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