RESEARCH PAPER

An improved centrifuge method for determining water extraction curves and vulnerability curves in the long-vessel species Robinia pseudoacacia

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Abstract

Significant improvements to the centrifuge water-extraction method of measuring the percentage loss volume of water (PLV) and corresponding vulnerability curves (VCs) are reported. Cochard and Sperry rotors are both incapable of measuring the VCs of species with long vessels because of premature embolism induced by hypothetical nanoparticles that can be drawn into segments during flow measurement. In contrast, water extraction pushes nanoparticles out of the sample. This study focuses on a long-vessel species, Robinia pseudoacacia, for which many VCs have been constructed by different methods, and the daily water relations have been quantified. PLV extraction curves have dual Weibull curves, and this paper focuses on the second Weibull curve because it involves the extraction of water from vessels, as proven by staining methods. We demonstrate an improved water extraction method after evaporation correction that has accuracy to within 0.5%, shows good agreement with two traditional methods that are slower and less accurate, and is immune to nanoparticle artefacts. Using Poiseuille’s Law and the geometry of vessels, we argue that the percentage loss of conductivity (PLC) equals 2PLV–PLV² in a special case where all vessels, regardless of size, have the same vulnerability curve. In this special case, this equation predicts the data reasonably well.

Keywords: Nanoparticles, Robinia pseudoacacia, vulnerability curves, water extraction.

Introduction

Negative water pressure or tension, T, induces embolism in xylem conduits and reduces hydraulic conductivity (Kₜ) of the xylem. The Cohesion–Tension Theory (Dixon and Joly, 1985; Tyree and Zimmermann, 2002) states that the transport of water from roots to leaves via xylem conduits involves the movement of tensile water that is metastable. Different species of plants can sustain different levels of tension in metastable water without loss of Kₜ; loss of Kₜ happens when tensile water is replaced by embolisms in xylem conduits. A vulnerability curve (VC) plots the percentage loss of Kₜ versus T. Various methods have been used since 1985 to measure VCs, but different methods often produce different curves (Wang et al., 2014). The fastest method of measuring a VC is the centrifuge technique, and two types of rotors are currently used for this. In the Sperry rotor, samples are subjected to tensile water by spinning them to induce embolism, and Kₜ is measured with classic hydraulic methods after spinning; this method, sometimes called the static centrifuge method, was first described...
by Alder et al. (1997). The Cochard rotor permits the measurement of $K_b$ while samples are spinning; hence, it is an in situ flow technique (Cochard et al., 2005).

The Cochard rotor gives a proper VC for species with short vascular conduits (Cochard et al., 2013), but when vessels are longer than approximately half the rotor diameter, a ‘long-vessel artefact’ results in an over-estimate of $T_{50}$ by 1–4 MPa (Wang et al., 2014), where $T_{50}$ equals the tension causing 50% loss of $K_b$. Perhaps the reason for the long-vessel artefact is that embolisms are seeded by hypothetical nanoparticles or air-bubbles. These particles can be drawn into samples via the long vessels from the cuvettes to the axis of rotation when measuring $K_b$ using the flow technique in a Cochard rotor (Cochard et al., 2010; Sperry et al., 2012; Wang et al., 2014). If the vessels are short, pit membranes filter out the nanoparticles before they can reach the axis of rotation and seed embolisms. Experiments conducted by Wang et al. (2014) support the notion of nanoparticles, and the unusually high percentage loss of hydraulic conductivity (PLC) near the cut surface of samples of short-vessel species also supports anomalous cavitation due to nanoparticles (Cai et al., 2010). Du et al. (2019) unsuccessfully attempted to redesign the rotor to avoid nanoparticles being drawn into long vessels.

The Sperry rotor probably does not avoid these errors because repeated spins at the same tension cause a linear decline of conductivity with the increasing spin number. Torres-Ruiz et al. (2017) found that sapwood area-specific conductivities in samples from two long-vessel species (Olea europaea and Quercus palustris) were significantly decreased after a single spinning in the original rotor design (i.e. the static centrifuge method described by Alder et al., 1997); a similar conclusion was also reported in López et al. (2018), who measured Hakea dactyloides vulnerability curves by the static centrifuge method with 14 cm- and 27 cm-diameter rotors. Hence, an urgent need exists for an improved centrifuge technique free of the ‘open vessel artefact’ that can be used for long-vessel species.

Pivovaroff et al. (2016) have used a centrifuge to extract water from stem segments under tension in a Cochard rotor. The idea was to keep the water level equal in both cuvettes, so that no water flowed through the vessels; however, increasing the tension did centrifugally extract water from the stem segment as it drained from the vascular elements. Extraction was from the axis of rotation towards the cuvettes, and hence nanoparticles that might seed cavitation prematurely were pushed away. The amount of water extracted was quantified from the equal increase in water level in the two cuvettes. The extraction was due to cavitation of vessels, and some extraction occurred from other cellular components, such as water-filled wood-fibre tracheids or even living cells (Pivovaroff et al., 2016; Du et al., 2019). Pivovaroff et al. (2016) did not consider water evaporation during their extraction measurements; hence, the real water extraction volume was under-estimated.

Theory for a method to correct for evaporation

Evaporation occurs during water extraction, thereby causing an under-estimation of the amount of extracted water. Corrections for evaporation can be made by using the intercept of a regression line, as shown in Fig. 1. Typically, a sample is placed in a centrifuge rotor with water-filled cuvettes at each end. As the sample spins, the position of the meniscus, $x$, in the cuvettes is measured. Some water evaporates from various surfaces, such as the bark and the surface of the water in the cuvettes, making $x$ decline, and some water is extracted from the samples, making $x$ increase. We assume that the evaporation rate ($E_x$, mg min$^{-1}$) is constant at any given RPM but might increase with increasing RPM because of the increased air flow near the evaporation surfaces.

![Fig. 1. Theoretical plots of mass of water extracted from a stem sample versus time following a rapid tension increase at time zero. (A) Single exponential extraction curve with time constant $\tau$=5 min and (B) dual exponential extraction curve with $\tau$=1 min and $\tau$=5 min. The solid circles are the theoretical extraction mass versus time independent of evaporation. The solid triangles show the trend of mass loss with a constant evaporation rate without extraction. The open squares show the combined impact of extraction and evaporation together. The × symbols are theoretical experimental points corresponding to the open squares with a built-in error of ±1 mg, which was done to apply more realistic experimental errors for the extrapolation back to time zero. The linear extrapolation back to time zero provides the estimate of the maximum mass of water extracted according to the regression interception method (RIM).](image-url)
The volume change equals $\Delta x A_o$, where $\Delta x$ is the meniscus movement in mm and $A_o$ is the cross-sectional area of water in the cuvette in mm$^2$. The contribution of evaporation to the volume change equals $E_v t$, where $t$ is time in min. Water extraction is simultaneously approaching an equilibrium volume, $V_o$, as some function of time, $f(t)$, which for simplicity we make an exponential function in Fig. 1A, $f(t)=V_o[1-\exp(-t/\tau)]$, where $\tau$ is a time constant. In Fig. 1B we use a two-phase extraction curve: $f(t)=[V_o/2\times[2-\exp(-t/\tau)]-\exp(-t/\tau)]$. The movement of the meniscus equals the sum of extraction plus centrifugal extraction of water from the spinning sample. In the original paper, Pivovaroff et al. (2016) made no attempt to quantitatively account for evaporation. Instead, extraction was monitored until the meniscus momentarily stopped moving, as observed in the peak of the open squares in Fig. 1, and the rate of evaporation was assumed to have little impact on the peak value where the solid circles level off. The theory in Fig. 1 clearly shows that the level of evaporation observed in our experiments causes an underestimate of the total water extracted. In theory, the movement of the meniscus in the cuvette (the water level) will be the sum of both events (open squares in Fig. 1).

A peak extraction volume is observed when the evaporation rate equals the extraction rate, but after that peak (open squares at 8–10 min) evaporation dominates for the remaining time. In Fig. 1, we calculated the dynamics for two different exponential approaches to equilibrium for the extraction rate (solid circles). The relationships shown in the figure are robust, and when based on many simulations this method of calculation applies regardless of the kinetics by which the water extraction phase was approached; the $y$-intercept of the regression line always equals the extracted water volume given by the solid circles. This approach is designated as the regression intercept method (RIM).

In this paper, the theoretical approach in Fig. 1 (RIM) is compared to the Bordeux ‘peak’ method (Pivovaroff et al., 2016) to measure water extraction curves. We found that extraction curves were usually dual S-shaped or R- and S-shaped, and the first phase of the extraction curve occurred mostly between 0–2 MPa tension, which was followed by another S-shaped curve at high tension. We demonstrated that the first-phase curve in Robinia came from the extraction of water from nonvascular tissues in preliminary experiments. We provide more proof that the second-phase extraction curve came mostly from vessels and that the $T_{50}$ of the second curve represents the $T_{50}$ of the vulnerability curves measured by flow methods. Water loss from a single vessel will cause a loss of conductivity that is a function of the extraction volume (as derived in the Discussion). The results can also be corrected for the elastic deformation of the rotor and cuvettes.

Materials and methods

Plant materials and sampling

Robinia pseudoacacia L. was harvested near the Weihe River in Yangling, Shaanxi, China (34°16’ N, 108°24’ E) in the autumn of 2016. Measurements were carried out on current-year branches ($N>60$) from mature trees. Preliminary air injection experiments indicated a maximum vessel length of 60 cm; hence, sun-exposed branches of 100–150 cm length and 9 mm basal diameter were collected in the early morning when the water potential was maximum. Branches were sprayed with water and then enclosed in black plastic bags with wet paper and immediately transported to the laboratory within half an hour. The cut ends of the branches were kept under water, the leaves and thorns were removed, and the branches then were placed in water for at least 30 min to release the xylem tension. The samples used for spinning (~27.4 cm length) were flushed before the experiment. All experiments were performed using a Cochrad rotor inside an improved centrifuge to better control the temperature, called ‘ChinaTron’ centrifuge (model HR2100, Xiayi Centrifuge Instrument Co., Ltd., Changsha, China).

Measurements of rotor and cuvette deformation and evaporation from cuvettes versus RPM

The rotor and cuvette deformation were measured by following the movement of the meniscus of pure olive oil. Each cuvette was filled with 500 mg pure olive oil, inserted into the rotor, spun at 1000 RPM, and then increased to 9000 RPM in seven steps. The spinning was then decreased in steps at different RPMs back to 1000 RPM. The meniscus position (±0.01 mm relative to the axis of rotation) and spun at 2 MPa for 2 h. The rotational speed was increased in a stepwise manner. At each tension step, the meniscus position was recorded with a resolution of $\pm 0.01$ mm by 20× microscope every 1 min. Measurements typically lasted 1 h to get the regression line (evaporation rate) from the last 25–35 min. At each tension step, the y-intercept of the regression line was equated to the evaporation-corrected volume of the extracted water. We call this the regression intercept method (RIM, see Fig. 1). The stepwise rotational speed increase was repeated until a plateau was reached in the plot of extraction versus tension (or rotational speed). At the plateau, we can imagine that water has been extracted from virtually all vessels at the axis of rotation but not from the closed vessels near the cuvettes.

Xylem water extraction curves based on a regression intercept method

Water extraction curves were constructed using a Cochrad rotor (Cochard, 2002; Cochard et al., 2005) inside an improved centrifuge to better control the temperature but without water injection or $K_e$ measurements. Branch samples 27.35±0.05 cm long and 5.1–7.3 mm in diameter were cut under water, and the sample ends were cleanly cut with a fresh razor blade. The scars from the excised leaves and thorns were then sealed using super glue to reduce evaporation from the cut surface of the petioles and thorns. The samples were flushed with 0.01 M KCl (pre-prepared with ultrapure water) at an applied pressure of 150 kPa for 5–8 min to remove all native embolisms. Apical and basal sample diameters ($D$, ±0.01 mm) were measured with a digital caliper. Since cuvettes were 100 mm$^3$ in cross-section, the amount of water (in mg) needed to fill the two cuvettes to a height of 1 cm was calculated using $10(100–\pi D^2/4)$. The cuvettes were tared on an analytical balance, and water was added to the nearest mg (1 mm$^3$) with a syringe and fine needle. The amount of water added differed according to the apical versus basal stem diameters. The flushed samples were then placed in the rotor, and the rotational speed increased to 4763 RPM within 3 min (= 2.0 MPa tension at the axis of rotation) and spun at 2 MPa for 2 h.

The rotational speed was increased in a stepwise manner. At each tension step, the meniscus position was recorded with a resolution of 7.62 μm = ±0.01 div by 20× microscope every 1 min. Measurements typically lasted 1 h to get the regression line (evaporation rate) from the last 25–35 min. At each tension step, the y-intercept of the regression line was equated to the evaporation-corrected volume of the extracted water. We call this the regression intercept method (RIM, see Fig. 1). The stepwise rotational speed increase was repeated until a plateau was reached in the plot of extraction versus tension (or rotational speed). At the plateau, we can imagine that water has been extracted from virtually all vessels at the axis of rotation but not from the closed vessels near the cuvettes.
Normally, the two menisci became completely separated near the end of the water extraction curve, which indicated that enough vessels were embolized to prevent water flow between the two cuvettes.

Provovaroff et al. (2016) measured water extraction only until a peak of water extraction was reached a few minutes after the RPM increase. Hence, we refer to this as the ‘peak method’ in our experiments. However, the real volume extracted (obtained by the regression intercept method) was always more than the peak method value because evaporation occurred through the entire extraction process.

Weibull fits

Water extraction curves based on RIM and the ‘peak method’ were fitted to a Weibull curve (Eqn 1a), and the parameters were resolved by minimizing the sum of squares (or root mean square error, RMS). We fitted the absolute water extraction volume (V versus tension) and the percentage loss volume of water (PLV) curve. In the former, the absolute volume extraction, \( V_{\text{max}} \), maximum value, becomes a parameter in the PLV Weibull curve. If the PLV was plotted, then only the Weibull constants B and C were required.

\[
V = V_{\text{max}} \left( 1 - e^{-\left( \frac{T}{T_0} \right)^c} \right)
\]  

(1a)

\[
\text{PLV} = V/V_{\text{max}} = 1 - e^{-\left( \frac{T}{T_0} \right)^c}
\]  

(1b)

Where \( T \) is the tension, B is \( T_0 \), i.e. the value of \( T \) at 63% PLV, and \( C \) determines the curvature of the S- or R- shaped curve. The value of \( T \) at 50% PLV (\( T_{50} \)) was then computed from:

\[
T_{50} = B \left( \ln(2) \right)^{1/c}.
\]  

(2)

Vessel length measurement by the air-injection method

Vessel length was measured by the ‘air injection’ method described by Pan et al. (2015). Vessel lengths were measured on the same samples used for the water extraction curves. Briefly, the basal end of a sample was injected with compressed air (120±0.5 kPa) from an air-compressor (BB24V, Bambi Air-compressors Ltd, Birmingham, UK), while the distal end was immersed in water. Sample segments 1–2 cm long were sequentially excised, and the volume (\( V \)) of air bubbles flowing out from the distal end was collected in a volumetric cylinder during a time interval (\( \Delta t \)) based on the water displacement method; more details can be found in Wang et al. (2014). The air flow rate (\( Q, \text{ml min}^{-1} \)) was computed from \( V/\Delta t \). Hence, the pneumatic conductivity of the cut open vessels to the air (\( C \)) should be proportional to \( Qx \) (see eqn 4 in Cohen et al., 2003), where \( x \) is the sample length at which \( Q \) was measured. Using the theory of Cohen et al. (2003), the air conductivity was calculated as follows:

\[
C = C_0e^{(-kx)} \quad \text{or} \quad Qx = Q_0B_0e^{(-kx)}
\]  

(3)

where \( C_0 \) is the limiting conductivity when \( x=0 \), and \( k \) is an extinction coefficient. The natural log of \( C \) versus \( x \) results in a linear plot, from which \( k \) is evaluated by the slope of the regression line. Based on the theory of Cohen et al. (2003) and Sperry et al. (2005), the mean vessel length was calculated as \( L_{\text{mean}} = -2/k \).

Visualization of embolized vessels by a stain method and corresponding vulnerability curves

Samples used for staining were prepared using the same method as that for measuring the water extraction curves. Six to eight samples (27.3–27.4 cm long) were spun for each tension step and for the same time as was used for the water extraction curves to induce cavitation of the vessels. After spinning, the segments were cut progressively from each end to release tension until the last central 2 cm segment near the axis of rotation was left; the final cuts at each end of this segment were made with fresh razor blades. The staining method was similar to that described in Wang et al. (2014) and Cai et al. (2014), with only minor deviations. The 2-cm segments were immediately perfused with 0.02% (w/v) stain (basic fuchsin + 0.01 M KCl) at a pressure of 0.76 kPa (7.5 cm water height) for 2 h. Basic fuchsin binds strongly to the walls and travels much slower than the water; hence, preliminary experiments were done to choose the optimal pressure and timing for perfusion to ensure bubbles were not ejected from the embolized vessels and to allow enough time for staining of the non-embolized vessels. After staining the segments, they were flushed with 0.01 M KCl at 130 kPa for 5 min to remove excess stain.

Cross-sections of 18 μm thickness were cut from the middle of the 2-cm segments using a Leica microtome (RM 2235). Sections were mounted in glycerin on glass slides and photographed under a Zeiss microscope (Imager A.2) at 50× magnification with a digital camera (Infinity 1-SC, Regent Instruments Inc., Quebec, Canada). Then, the cross-sectional area (\( A_i \)) of each stained (conducting) and unstained (embolized) vessel in the whole cross-section was measured by digital analysis software (Image-Pro Plus version 6.0). Equivalent vessel diameters (\( D_i \)) were calculated as \( D_i = (A_i/n)^{1/2} \). The total vessel area in the cross-section of each segment was calculated from the sum of all stained and unstained vessel areas. The few vessels with tylose were not included in the measurement of unstained vessel area.

Vulnerability curves were plotted based on the unstained and stained vessel areas within the same cross-section at each tension. The percentage loss of conductivity was calculated from the percentage volumetric extraction from staining, which represent the stained vulnerability curves (PLV):

\[
\text{PLV} = 100 \frac{\sum A_{i,v}}{\sum A_{i,v} + \sum A_{i,u}}
\]  

(4)

where \( A_{i,u} \) is the area of the \( i \)-th unstained vessel and \( A_{i,v} \) is the area of the \( i \)-th stained vessel. The average volumetric PLV, for every tension (induced by centrifuging) were then calculated using the 6–8 replicate stems. Since the objective of the experiment was to obtain the relationship between water loss from vessels while excluding other sources such as bark or wood fibres, preliminary experiments with the stain and the peak method were performed to determine which tension steps corresponded to water loss from the vessels. After water was extracted from the samples that spun to tensions of 0–6 MPa in 1–MPa increments, the central 2-cm segment was examined for the presence or absence of stainable vessels in each tension step based on the method described above.

Silicone rubber-injection method to estimate extraction water volume

The rubber injection method is frequently used to measure vessel length (Cai et al., 2010, 2014; Pan et al., 2015). The replacement of water with rubber is analogous to the replacement of water with air because pit membranes can stop both.

Samples with diameters equal to those in previous experiments were flushed and then filled with rubber (see below). The samples were then sectioned at different distances (\( x \)) from the injection surface to obtain a measure of the total cross-sectional area of the rubber-filled vessels (\( A_i \)). We found that \( A_i \) was an exponential function of \( x \):

\[
A_i = A_i^0e^{-c(x/b)}
\]  

(5a)

where \( b \) and \( c \) are constants. The volume of the injected rubber can be calculated from the integral of Eqn 5a to \( x=\infty \). However, we need the integral only to \( x=x_m \), the distance from the axis of rotation to the location of the meniscus in a cuvette because air, unlike rubber, will pass only as far as the meniscus in the cuvette inside the centrifuge. Water in cut open vessels that are longer than the distance from the axis of rotation to the meniscus will be drained only to a distance \( x=x_m \); otherwise, the water will be drained to some shorter distance, \( x \). For each vessel, the volume of rubber will be \( xA_i \), where \( A_i \) is the area of the \( i \)-th vessel. The total volume of rubber (\( V_r \)) from the injection surface will be given by the integral of Eqn 5b:

\[
V_r = \int_0^{x_m} xA_i dx
\]
Volumes of silicone rubber were assessed to estimate the volume of extractable water as explained in the Results and Discussion. Six samples as used in the water extraction curve method were chosen with the same size. The samples were trimmed with a razor blade, sealed with super glue, and flushed with degassed 0.01 M KCl at 150 kPa for 5–8 min to remove all embolisms that would interfere with the injection of the silicone rubber solution. The samples were injected with rubber at 120 kPa for 24 h, then immersed in a water bath and placed in an oven at 45 °C for 3 d to cure (harden) the silicone. The rubber was freshly mixed from liquid silicone and hardener at a 10:1 ratio (10 RTV141 part A + 1 RTV141 part B; Bluesil RTV 141, Bluestar Silicones, Lyon, France). Uvitex, a fluorescent whitening agent (Ciba Uvitex OB; Ciba Specialty Chemical, Tarrytown, NY, USA), was added to make the silicone visible under UV light. The Uvitex was dissolved in chloroform (1% w/w), and 10 drops were added to the silicone mix.

The cured samples were cross-sectioned at several distances (0.2–27 cm) from the injection surface until fewer than 2% of the vessels were filled with rubber. Sections 18 μm thick were cut using a microtome and were mounted in water on a glass slide and examined under a microscope filled with rubber. Sections 18 μm thick were cut using a microtome and then summed to obtain $A_i$, at each distance $x$.

Data analysis

Student’s $t$-test was used to determine whether measured and deduced parameters of the water extraction experiments within each method were different before and after corrections for rotor and cuvette deformation. ANOVA was used to examine differences in the parameters in the ‘regression intercept method’ and ‘peak method’ for determining the water extraction curves, with or without rotor correction.

Results

Measurement of rotor and cuvette deformation

A significant negative relationship occurred between the meniscus movement of oil in the cuvette and the rotational speed of the centrifuge. The meniscus position ‘decreased’ with the increasing rotational speed, and then ‘increased’ with decreasing rotational speed (Fig. 2). Typically, a decrease means evaporation, but in terms of rotor deformation it means a stretching of the rotor as the RPM increases. Since the slopes of the meniscus position versus RPM for increasing and decreasing RPM were not significantly different, all data were pooled for the regression analysis. The slope was $-1.74 \times 10^{-5}$ mm RPM$^{-1}$ ($R^2=0.994$). If the oil had evaporated, the slopes for increasing and decreasing RPM would have been different; hence, the oil evaporation was negligible. If all the meniscus movement was due to radial stretch of the rotor, then the amount of deformation was 0.1% of the radius of the rotor when the RPM changed from 1000 to 9000.

Evaporation rate of water in cuvettes without samples

The meniscus position versus time at three different RPMs is shown in Fig. 3A, and the evaporation rate is equal to the absolute value of the slope. A positive relationship between the rate of evaporation from the water-filled cuvettes without samples and rotational speed was then found, with a slope of $2.46 \times 10^{-4}$ ($R^2=0.9945$; Fig. 3B). The increase in evaporation with increasing RPM may be a function of three factors: (1) the airflow rate near the meniscus; (2) the air temperature, which was thermostatically controlled at $25 \pm 0.1$ °C in all experiments; and (3) the humidity of the laboratory air drawn into the rotor.

Evaporation rate of water in cuvettes with samples

An example measurement of the water extraction volume using the regression intercept method is shown in Fig. 4A. At each RPM (or tension) step, the meniscus position generally increased for a few minutes, and then reached a peak, which was followed by a decrease. The peak occurred when the evaporation rate equaled the extraction rate; hence, extraction did not actually stop at the peak. When the water extraction rate fell to zero, a constant evaporation rate could be measured for the last 25–35 min within the measurement period. The slope and intercept of the meniscus position versus time was calculated by linear regression. As explained in the theory in the Introduction, the intercept should equal the maximum amount of water extracted, and the evaporation rate should equal the absolute value of the slope.

Based on the evaporation rate (i.e. the positive value) at different rotational speeds, a significant positive relationship between the mean evaporation rate and the rotational speed was found, with a slope of $3.84 \times 10^{-4}$ (Fig. 4B). The slope with the sample present was significantly greater than that without the sample, but the absolute evaporation rates were always less. When a sample of cross-sectional area $A_i$ was inserted into the cuvette of cross-sectional area 1 cm$^2$, the surface area of evaporation was reduced to $1-A_i$, and that explains the reduction between the dashed and the solid lines in Fig. 4B.
evaporation from the bark was apparently less than the evaporation from \( A \) cm\(^2\) of water displaced by the sample; otherwise the solid line would have exceeded the dashed line.

Water extraction curves after correction for rotor/cuvette deformation based on the \( y \)-intercept of the evaporation regression

Water extraction curves need to be corrected for rotor deformation and evaporation if the objective is to compare the volume of extraction to the volume of the vessels. Water extraction curves plotted as percentage loss of volume (PLV) before and after correction for rotor and cuvette deformation both tended to be typical S-shapes, and replicate curves were similar (Fig. 5). \( T_{50} \) was not significantly different between the values measured before and after correction for rotor and cuvette deformation (\( t \)-test, \( P = 0.906 \), Table 1).

Water extraction curves are associated with the second phase of extraction

Preliminary experiments on the peak versus the regression intercept method (RIM) indicated that the biphasic curve could be measured in less than 3 h by the peak method, and curves were similar to those in Pivovaroff et al. (2016); however, the RIM required 1–2 h per point, meaning that full experiments would last 20–26 h.

There was a baseline level of 15% unstained vessels that corresponded to permanently embolized vessels filled with tyloses, and this was apparent until tension exceeded 3 MPa (Fig. 6A), which corresponded quite well with the second phase of the PLV curve. In addition, when tension reached 2 MPa, after 2 h of spinning, the rate of evaporation became dominate in the combined curve (see open squares in Fig. 1), which means that extraction was also completed at this tension. Therefore, samples were spun for >2 h prior to starting experiments by the RIM.

Comparison of water extraction and Weibull curve parameters between RIM and ‘peak method’ before and after corrections

Weibull curves were fitted to the mean water extraction curves based on the ‘peak method’ (i.e. calculated from the accumulated absolute peak values of the meniscus movement of water).
and to the absolute water extraction curves of the RIM after correction for deformation of the rotor and cuvette (Fig. 6B). Both curves had typical S shapes.

For both the RIM and the ‘peak method’, there were no significant differences in the parameters of the Weibull curves ($V_o$, $B$, $C$), the calculated $T_{50}$, and the root mean square errors of the best-fitted curves before and after corrections for rotor and cuvette deformation (all $P>0.55$, $t$-tests; Table 1). In addition, the ANOVA demonstrated that $B$, $C$, and the calculated $T_{50}$ were not significantly different between the RIM and the ‘peak method’ with and without rotor and cuvette correction. However, the amount of water extracted, $V_o$, was significantly different according to the ‘peak method’, with values ~50% of those of the RIM (Fig. 6B, Table 1); therefore, half the water extracted was lost by evaporation.

The correction for distortion of the rotor and cuvettes increased the $V_o$ values by ~4% and ~8% in the RIM and ‘peak method’, respectively. In addition, the RMSerr of the fitted curves was significantly less for the RIM compared with the ‘peak method’.

**Comparison of ‘vulnerability curves’ between the stain method, and extraction curves based on the RIM and the ‘peak method’**

Vulnerability curves in the strict sense are plots of loss of hydraulic conductivity versus tension. But ‘vulnerability curves’ are often approximated by proxy values. In the staining method the conductive vessels are visualized by stain that passes through conductive vessels and hence stain the walls. But hydraulic conductivity is rarely computed from staining, rather vessel counts or stained and unstained vessel areas are more commonly reported by staining methods. In water extraction curves the volume of water extracted is measured, which is equal to the area of vessels extracted times the length of the water columns of the extracted vessels. Percentage loss of conductivity can be related to percentage loss of volume with certain assumptions (see below).

‘Vulnerability curves’ were computed from the measured area of stained (conducting) and unstained (embolized) vessels by measuring at least six samples at every selected tension (Fig. 7, open points ‘stained PLV’ and solid curve ‘stained Weibull’; $T_{50}$=4.364 MPa). The water extraction curves of the RIM and ‘peak method’ after correction for rotor and cuvette deformation had similar shapes and were close to the curve constructed from the stained vessels, with no differences in $T_{50}$ ($4.244\pm0.026$ and $4.369\pm0.068$ for RIM and ‘peak method’, respectively) (Table 1, Fig. 7). The lack of significant differences between the three ‘vulnerability curves’ indicated that the water-extraction method might be a good proxy for vulnerability curves in species such as *Robinia* with long vessels.

**Comparison of water extraction volumes and volumes of vessels in the samples**

The mean extracted water volume calculated using the RIM was $553\pm30$ mm$^3$. This water could come from the vessels and possibly from surrounding tissue, and it would be replaced

![Fig. 5](image_url). Water extraction curves plotted as percentage loss of volume (PLV) versus tension (xylem pressure) after correction for rotor and cuvette deformation in six samples of *Robinia*.

![Fig. 6](image_url). (A) Water extraction from vessels and non-vascular tissue is plotted as PLV, percentage loss volume by the ‘peak method’ ($N=8$). Vessels blocked by tyloses or embolisms are all unstained. Stained vessels are water-conducting vessels. The % unstained vessels equals the % of vessels that were newly embolized or filled with tyloses prior to the experiment ($N=3$). The conclusion is that the first S-shaped PLV curve from 0–2 MPa must originate from the extraction of water from non-vessels and the second S-shaped curve from 3–7 MPa must be from vessels. (B) Water extraction plotted as absolute extraction of water after rotor and cuvette correction for deformation using the ‘regression intercept method’ (RIM) and the ‘peak method’. Data are means (±SE) of $N=6$ samples. Fitted Weibull curves are also plotted.
Peng et al. by air. Based on the rubber injection method, the sum of the cross-sectional areas of rubber in the vessels, \(A_r\), versus the distance from the injection surface is shown in Fig. 8. The log-transformed data proved that the relationship from which the rubber volume was calculated by a simple integral was exponential (Fig. 8A). This integral volume to \(x_m = 12.5\) was calculated, where 12.5 cm is the approximate distance from the axis of rotation to the water level in the cuvette. Then since air flows both ways from the axis of rotation, the volume of water extracted (\(V_w\)) should equal \(2V_r\) at the minimum. The computed volume of rubber was 256 mm\(^3\). The mean area of injected vessels (\(A_r/\text{number of vessels}\)) increased with distance from the injection surface (Fig. 8B), which proved that larger diameter vessels were longer than smaller diameter vessels.

**Table 1.** Parameters of Weibull curves, \(T_{50}\) values, and the root mean square (RMS) error of the best-fit curves for the ‘regression intercept method’ (RIM) and ‘peak method’ before and after correction for rotor and cuvette deformation.

| \(V_o (\text{mm}^3)\) | \(B\) | \(C\) | \(T_{50} (\text{MPa})\) | RMS error |
|----------------------|--------|--------|----------------------|-----------|
| RIM                  |        |        |                      |           |
| Original             | 533.63±30.38\(^a\) | 4.643±0.031\(^a\) | 4.171±0.184\(^a\) | 4.249±0.027\(^a\) | 0.0186±0.0012\(^b\) |
| Corrected            | 552.98±30.11\(^a\) | 4.643±0.030\(^a\) | 4.119±0.177\(^a\) | 4.244±0.026\(^a\) | 0.0187±0.0013\(^a\) |
| Peak method          |        |        |                      |           |
| Original             | 272.21±22.98\(^b\) | 4.708±0.080\(^a\) | 5.268±0.486\(^a\) | 4.381±0.070\(^a\) | 0.0321±0.0039\(^b\) |
| Corrected            | 291.83±22.55\(^b\) | 4.710±0.078\(^a\) | 5.010±0.419\(^a\) | 4.369±0.068\(^b\) | 0.0300±0.0035\(^b\) |

Data are means (±SE) for \(N=6\) samples. Different letters indicate significant differences as determined by ANOVA followed by Tukey's honest significant difference test for multiple comparisons (\(P<0.05\)).

Fig. 7. Mean ‘vulnerability curves’ for stem samples of *Robinia* based on data using the stained vessels method and percentage water extraction constructed using the ‘regression intercept method’ (RIM) and the ‘peak method’. Data are means (±SE). Corrected refers to Weibull curves corrected for rotor and cuvette deformation in the water extraction method.

**Fig. 8.** Estimation of extracted water volume and vessel area using the silicone rubber injection method in stems samples from *Roninia*. (A) A plot of total cross-sectional area of injected vessels, \(A_r\), versus the distance from the injection surface, \(x\). (B) Plot of mean vessel area computed from the area of rubber divided by the number of vessels filled with rubber versus the distance from the injection surface, \(x\).

**Discussion**

The water extraction methods used in this paper are much improved over those reported by Pivovaroff et al. (2016) and are probably most useful in obtaining vulnerability curves (VCs) from species with long vessels. Some of the species studied in Pivovaroff et al. (2016) had either short vessels or were conifers with only 2-mm-long conduits (tracheids), but all the extraction curves were biphasic; further, the second phase roughly corresponded to the classical, bench-top-dehydration VCs, regardless of the conduit length. Wang et al. (2014) used the three most common methods to measure VCs in *Robinia*: bench-top-dehydration, the pressure sleeve, and the Cochard cavitation method. They argued that the bench-top-dehydration method, often considered the ‘gold standard’, produced the only VC that agreed with the water relations of *Robinia*. 
Our lowest estimate of volume of water extraction came from the rubber injection method, which gave a value of 256 mm$^3$ (Fig. 8A, Eqn 5b), but that analysis was likely to be a minimum value because the calculation assumed that all embolisms only occurred in the sample vessels passing through the axis of rotation. Nonetheless, the rubber volume equalled 46% of the water extraction (553 mm$^3$). At the other extreme, if we assumed that all vessel lumens drained their water, then the volume would equal the intercept of Fig. 8A (237 mm$^3$) multiplied by the length of the sample between the cuvette water levels (~250 mm), which would yield a volume of extraction that was 592 mm$^3$, or 107% of the mean extraction volume. Hence, it is plausible that the water extraction of the second-phase curve is mostly from the vessels. Below, we discuss how Poiseuille’s Law and xylem anatomy may be used to compute a relationship between the PLV curves and PLC curves (the percentage loss of water conductivity, same as VC).

Derivation of PLC as a function of PLV

Imagine a sample initially having $V_0$, volume of water in the vessel lumens, and that a volume $V_e$ is extracted as it spins in a centrifuge. By definition, the percentage loss of volume $PLV=(V_e/V_0)$ if we consider only vessel water. In a sample segment cut from 1–2 cm to each side of the axis of rotation, the volume of water extracted from the vessels is $V_e=\Delta A \times \Delta x$, where $\Delta x$ is the length of the sample segment and $A_e$ is the area of vessels from which the water is extracted. Similarly, $V_0=\Delta x \times A_m$, where $A_m$ is the cross-sectional area of all vessels; hence, in the special case where the average length of vessels is much more than $\Delta x$, $PLV=1-(A_e/A_m)$, where $A_i$ is the sum of the area of all full vessels. By definition $PLC=1-(K_i/K_m)$, where $K_m$ is the maximum hydraulic conductivity, and PLC ought to be proportional to $1-(A_e/A_m)^2$. This prediction follows from Poiseuille’s Law that states that the conductivity of one vessel is $\pi D_i^4/128\eta$, where $D_i$ is the diameter of the $i$th full vessel, and $\eta$ is the viscosity of water. By applying algebra to the special case where the vessel length is more than the segment length, it follows that:

$$PLC = 2PLV – PLV^2$$  \hspace{1cm} (6)

for all the vessels in a sample. This means that loss of conductivity of the vessel lumen can be computed from $2\times$ the loss of the vessel lumen volume minus the loss of the vessel lumen volume squared. If all vessels have the same loss of conductivity at any given tension regardless of vessel diameter, Eqn (6) would arguably apply to the collective behaviour of all vessels in a branch sample.

This equation is likely to apply to the hydraulics of sample segments harvested (cut under water) ±2 cm each side the axis of rotation where the tension is highest and relatively uniform. In *Robinia*, the largest diameter vessels contribute the most to flow and are also the longest vessels (Cai et al., 2010), so Eqn (6) is likely to apply over the entire length of the sample spinning in the ChinaTron centrifuge that we used (modified Cochard centrifuge). Hence, Eqn (6) may be valid for computing PLC from the PLV curves and for correctly quantifying the vulnerability curves of ‘recalcitrant species’, i.e. those with long vessels that cavitate too soon according to the hypothetical nanoparticle effect. The weakness of the above argument is that the PLV of the sample segment used to measure PLC will not be the same as the PLV of the whole sample unless most of the vessels are 24 cm or 25 cm long, which is equal to the distance between the water levels in the cuvettes. Nevertheless, the prediction in Eqn (6) is qualitatively useful because vessel length is usually a linear function of vessel diameter, and the biggest vessels in a *Robinia* contribute the most to both conductivity and water volume (Cai et al., 2010).

Comparing PLV and PLC curves

The Cochard rotor (Cavitron technique) measures water flow and hydraulic conductivity through samples simultaneously while spinning them. Wang et al. (2014) demonstrated that this technique generates premature embolisms when the vessel length is longer than ~8 cm. Even in species with vessels <3 cm, extensive embolisms occur near the water surface of the cuvettes, more than can be accounted for by the vulnerability curves (Cai et al., 2010). Since most *Robinia* stems have a mean vessel length of ~14 cm, the Cochard rotor produced a $T_{50}$ of 0.3 MPa (Wang et al., 2014). This impossibly low value disagreed with the bench-top-dehydration value of 3.74 MPa and the regression intercept method (RIM) water extraction value of 4.24 MPa (Table 1). We used Eqn (6) to compare the theoretical PLC to the bench-top-dehydration PLC curves (Fig. 9). The theoretical PLC value calculated from 2PLV–PLV$^2$ yielded $T_{50}=3.59$ MPa, which was not significantly different from the 95% confidence intervals for $T_{50}$ of the best-fit vulnerability curve of the bench-top method (solid line in Fig. 9). The bench-top-dehydration method generally has
a large scatter of points (diamonds in Fig. 9), which explains the large 95% confidence intervals indicated by the horizontal bar in the figure. Notably, the $B$ values of the Weibull fits ($=T_{50}$) were nearly identical in the theoretical and best-fit lines of bench-top dehydration method, which intersect at 66%.

In Robinia, the first phase of water extraction came from non-vessel tissues as indicated by the staining with basic fuchsin, and the first phase was separated by 2 MPa from the second, where water was extracted by cavitation of the vessels (Fig. 6A). Our proposed regression intercept method (RIM) may not work well in long-vessel species where the $T_{50}$ for cavitation is much below 2 MPa because the two phases will have too much overlap to separate them with mathematical certainty. Pivovaroff et al. (2016) assumed, without experimental proof, that extracted water in the first phase came from the bark (see methods section in their paper), but our results proved that it did not come from the vessels in our experiments. The biggest quantitative improvement in the methods in our paper was to estimate the impact of evaporation on the extraction kinetics (Fig. 1), and this doubled the water extraction volume identified as being from the vessels, bringing the data more in line with anatomical estimates (Fig. 6). An additional benefit of our improved method was a reduction by a factor of two in the RMS error in the Weibull fits (Fig. 6B, and Table 1).

We demonstrated that no evaporation occurred when olive oil was placed in the cuvettes. We thought of placing a layer of olive oil of 3–5 mm on top of the water in the cuvettes while spinning the samples, but we were concerned about how much might be drawn into the vessels as the RPM increased from 0–1000 RPM because at the start of the spinning the sample ends would be bathed in a mixture of oil and water. Preliminary experiments with oil on top of water resulted in inconsistent results, so we abandoned this approach.

Conclusions

The preliminary dye experiments (Fig. 6A) proved that all vessels of Robinia remained stained and conductive during the first phase of the extraction curve (from 0–2 MPa); hence, this phase can be ignored as unimportant to the loss of hydraulic conductivity.

The PLV curves obtained in this paper, together with the bench-topdehydration curves presented by Wang et al. (2014), gave us more confidence that the $T_{50}$ of Robinia was somewhere between 3.74–4.24 MPa. When the PLV was converted to a theoretical PLC, the $T_{50}$ value obtained, 3.59 MPa, was not significantly different from the bench-top value, which was 3.74 MPa (Fig. 9). In comparison, the Cochard Cavitron technique gave the unlikely value of 0.3 MPa (Wang et al., 2014), which was inconsistent with the water relations of Robinia. It will be interesting if future studies find that all long-vessel species have higher $T_{50}$ values by use of water extraction curves than previously reported. However, we strongly recommend the use of dye experiments to determine what part of the extraction curve involves water loss from vessels. In our case, the dye data weighted by the unstained vessel cross-sectional area agreed very well with the improved water extraction method PLV values (Fig. 7). Pivovaroff et al. (2016) stated in their methods that ‘Water extraction curves were calculated by first removing points between 0 and 0.8 MPa to exclude elastic water storage (Tyree and Yang, 1990)’. We do not agree with this characterization of the first extraction phase because elastic extraction from living cells should follow the same curve for dehydration and rehydration, but Tyree and Yang (1990) clearly demonstrated hysteresis that was characteristic of non-elastic water storage (Tyree and Yang, 1992; Yang and Tyree, 1992).

In our experiments, the Weibull curves fitted to the first and second phases of extraction were distinctly separated (Fig. 6A), allowing a simple subtraction (Du et al., 2019). However, if the $T_{50}$ for the second extraction phase is too close to the first phase, then, a simple subtraction will not work. Instead, we suggest fitting the entire extraction curve to a dual Weibull equation and subtracting the first Weibull curve from the total extraction curve. This analysis method would probably provide more accurate estimates of the second-phase $T_{50}$.

The ‘peak method’ resulted in a water extraction volume that was approximately half that estimated by the regression intercept method (RIM), but when the curves were normalized to PLV the differences in $T_{50}$ and the B and C Weibull constants were negligible (Table 1). However, the advantage of the RIM was a significant reduction in the SE of these parameters and the RMS error on the Weibull fits. Overall, we believe that this improved water extraction method produces a better estimate of all parameters for gross volume extraction and Weibull values.

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