Rapid determination of comparative drought tolerance traits: using an osmometer to predict turgor loss point

Megan K. Bartlett1*, Christine Scoffoni1, Rico Ardy1, Ya Zhang2, Shanwen Sun2, Kunfang Cao2 and Lawren Sack1

1Department of Ecology and Evolution, University of California Los Angeles, 621 Charles E. Young Drive South, Los Angeles, CA 90095, USA; and 2Key Laboratory of Tropical Forest Ecology, Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, Mengla, Yunnan 666303, China

Summary

1. Across plant species, drought tolerance and distributions with respect to water availability are strongly correlated with two physiological traits, the leaf water potential at wilting, that is, turgor loss point (πtlp), and the cell solute potential at full hydration, that is, osmotic potential (πo). We present methods to determine these parameters 30 times more rapidly than the standard pressure–volume (p–v) curve approach, making feasible community-scale studies of plant drought tolerance.

2. We optimized existing methods for measurements of πo using vapour-pressure osmometry of freeze-thawed leaf discs from 30 species growing in two precipitation regimes, and developed the first regression relationships to accurately estimate pressure–volume curve values of both πo and πtlp from osmometer values.

3. The πo determined with the osmometer (πosm) was an excellent predictor of the πo determined from the p–v curve (πpv, \( r^2 = 0.80 \)). Although the correlation of πosm and πpv enabled prediction, the relationship departed from the 1 : 1 line. The discrepancy between the methods could be quantitatively accounted for by known sources of error in osmometer measurements, that is, dilution by the apoplastic water, and solute dissolution from destroyed cell walls. An even stronger prediction of πpv could be made using πosm, leaf density (ρ), and their interaction (\( r^2 = 0.85 \), all \( P < 2 \times 10^{-10} \)).

4. The πosm could also be used to predict πtlp (\( r^2 = 0.86 \)). Indeed, πosm was a better predictor of πtlp than leaf mass per unit area (LMA; \( r^2 = 0.54 \)), leaf thickness (T; \( r^2 = 0.12 \)), ρ (\( r^2 = 0.63 \)), and leaf dry matter content (LDMC; \( r^2 = 0.60 \)), which have been previously proposed as drought tolerance indicators. Models combining πosm with LMA, T, ρ, or LDMC or other p–v curve parameters (i.e. elasticity and apoplastic fraction) did not significantly improve prediction of πtlp.

5. This osmometer method enables accurate measurements of drought tolerance traits across a wide range of leaf types and for plants with diverse habitat preferences, with a fraction of effort of previous methods. We expect it to have wide application for predicting species responses to climate variability and for assessing ecological and evolutionary variation in drought tolerance in natural populations and agricultural cultivars.

Key-words: climate change, functional traits, leaf traits, survival, water deficit, water relations

Introduction

The bulk leaf turgor loss point (πtlp), the water potential at which wilting occurs, is typically strongly related to plant drought tolerance and, therefore, species distributions with respect to water supply (Abrams & Kubiske 1990; Engelbrecht, Velez & Tyree 2000; Baltzer et al. 2008; Bartlett, Scoffoni & Sack 2012). This parameter is generally estimated from a pressure–volume (p–v) curve, which measures the decline of leaf water potential (\( \Psi_{\text{leaf}} \)) with leaf dehydration (Koide et al. 1989). Physiologically, the πtlp is the \( \Psi_{\text{leaf}} \) at which the average cell turgor pressure is lost; at this point, \( \Psi_{\text{leaf}} \) equals osmotic potential and subsequent \( \Psi_{\text{leaf}} \) declines are because of increasing osmotic concentration (with π the symbol for osmotic potential). Across species, πtlp is correlated with other important drought tolerance parameters, including \( \Psi_{\text{leaf}} \) at 50% loss.
of hydraulic and stomatal conductances and the lethal $\Psi_{\text{leaf}}$ (Auge et al. 1998; Brodribb & Holbrook 2003; Sack et al. 2003; Bucci et al. 2004; Lenz, Wright & Westoby 2006; Scoffoni et al. 2012). Recent analyses have shown that osmotic potential at full hydration ($\pi_o$) is the key trait driving both $\pi_{\text{tp}}$ across species, and the shifts in $\pi_{\text{tp}}$ for given species during seasonal and experimental droughts, and thus that $\pi_o$ and $\pi_{\text{tp}}$ are powerful traits for predicting drought tolerance and distributions with respect to water supply (Bartlett, Scoffoni & Sack 2012). However, the standard $p\rightarrow v$ curve method for determining $\pi_o$ and $\pi_{\text{tp}}$ is highly time-consuming for measuring large species sets. We present a method for rapid $\pi_{\text{tp}}$ and $\pi_o$ determination, based on osmometer measurement of $\pi_o$.

The $p\rightarrow v$ curve has been the most commonly used method for measuring $\pi_o$ because it allows estimation of a number of physiological parameters, including $\pi_{\text{tp}}$ (Tyree & Hammel 1972; Turner 1988; Koide et al. 1989). Methods have been described for measuring $\pi_o$ using a thermocouple psychrometer or osmometer (i.e. a psychrometer with Peltier cooling) (Turner 1981) for samples of extracted (expressed) sap from crushed leaf tissue (Wenkert 1980; Eldredge & Shockey 1990; Morgan 1992), hot water extractions from dried leaf tissue (Kohl 1996, 1997) or discs of leaf tissue that have been rapidly frozen and thawed to break cell walls and release protoplasmic contents (Kikuta & Richter 1992a; Ball & Oosterhuis 2005; Callister, Arndt & Adams 2006). Previous work towards cross-validating $\pi_o$ measurement methods found correlations between measurements made with the $p\rightarrow v$ curve and estimates based on psychrometry measurements of vacuolar fluid (Shackel 1987), and osmometer measurements of freeze-thawed tissue, wherein leaf tissue is frozen to rupture cells and allow vapour pressure measurements based on evaporation from the cytoplasm (Nonami & Schulze 1989), although the choice of method influenced $\pi_o$ values (Ball & Oosterhuis 2005). At least two sources of error have been proposed to influence osmometer methods: (i) apoplastic dilution, wherein symplastic fluid released from crushed cells is diluted by apoplastic water with low solute concentration, resulting in less negative $\pi_o$ values; and (ii) dissolution of cell wall solutes from destroyed cell walls, which makes $\pi_o$ more negative (Shepherd 1975; Turner 1981; Grange 1983; Kikuta & Richter 1992a).

Among osmometer methods, measurement of freeze-thaw discs is most robust to these errors, especially when first- and second-order veins are excluded (Kikuta & Richter 1992a; Callister, Arndt & Adams 2006), although values for $\pi_o$ may be more negative (Grange 1983; Kikuta, Kyriakopoulous & Richter 1985; Callister, Arndt & Adams 2006), less negative (Meinzer et al. 1986; Ball & Oosterhuis 2005) or equal to (Auge, Hickok & Stodola 1989) those from the $p\rightarrow v$ curve. Notably, there have been no standard protocols and experimental techniques, which may have contributed to discrepancies.

The first purpose of this study was to develop an osmometry method for prediction of $p\rightarrow v$ curve values of $\pi_o$ and $\pi_{\text{tp}}$. Because previous studies showed a strong relationship across species between $p\rightarrow v$ curve values of $\pi_{\text{tp}}$ and $\pi_o$ (Sack et al. 2003; Blackman, Brodribb & Jordan 2010; Scoffoni et al. 2011), we aimed to estimate $\pi_{\text{tp}}$ from $\pi_o$ values determined from osmometry for diverse species varying strongly in leaf construction and physiology. We used freeze-thaw discs because of their lower susceptibility to error and easier processing than expressed sap and hot water extractions (Kikuta & Richter 1992a). We also tested whether including other leaf functional traits would improve $\pi_o$ and $\pi_{\text{tp}}$ prediction. The second purpose of this study was to evaluate the sources of method discrepancies. We estimated cell wall investment using functional traits to determine the relative contribution of cell wall dissolution and apoplastic dilution to differences between the two methods. We thus provide an efficient and accurate alternative to the $p\rightarrow v$ curve for determining $\pi_o$ and $\pi_{\text{tp}}$ for comparative studies at scales from physiology to community ecology.

**Materials and methods**

**EXPERIMENTS TO OPTIMIZE OSMOMETER MEASUREMENTS**

Osmotic potential was measured with a VAPRO 5520 vapour pressure osmometer (Wescor, Logan, UT), a newer model of the VAPRO 5500, shown to be accurate and precise in previous studies of expressed sap osmotic potential (Ball & Oosterhuis 2005). Because there is no published standard method, we first conducted several experiments to optimize methodology. One sun-exposed branch was collected from each of nine Hedera canariensis (Araliaceae) and 14 Heteromeles arbutifolia (Rosaceae) individuals growing adjacent to the University of California, Los Angeles campus. Excised branches were kept in humid, opaque bags, recut underwater at least two nodes distal to the original cut and then rehydrated overnight in bags. One leaf disc was sampled from one mature, fully expanded leaf per branch, centrally between the midrib and margin, using an 8-mm-diameter cork borer.

Tests were carried out of the potential impacts on $\pi_o$ measurement of (i) disc freezing time, (ii) thawing time and (iii) reduction of evaporation during thawing. All discs were tightly wrapped in foil to limit condensation or frost after freezing and evaporation prior to processing. To test for an effect of disc freezing time, discs were submerged in liquid nitrogen (LN$_2$) for 2, 5 or 15 min. To test for an effect of thawing time, upon removal from the LN$_2$, the disc was either immediately measured or allowed to thaw for 1 h. To test the effectiveness of reducing evaporation during thawing, foil-wrapped discs were thawed either exposed on a laboratory bench, or placed inside a sealed plastic bag humidified with moist paper, and compared to discs measured immediately after freezing. After each treatment, the disc was punctured 10–15 times with sharp-tipped forcepts to facilitate evaporation through the cuticle and decrease equilibration time (Kikuta & Richter 1992b) immediately before sealing in the osmometer chamber, using the standard 10-µL chamber well. A measurement was recorded approximately every 2 min without opening the chamber, until the equilibrium was indicated by an increase between measurements of $< 0.001$ MPa.

If a given set of treatments did not affect the equilibration time or the final $\pi_o$ value, data were pooled for subsequent comparisons. Thus, for example, given no effect of LN$_2$ exposure time, the $\pi_o$ data for different exposure times were pooled before testing for the effect of thawing time.

SPECIES AND METHOD COMPARISON

To evaluate the utility of the osmometer method in determining \( \pi_o \) and \( \pi_{tlp} \) for comparative studies, we tested 30 woody species that varied strongly in their drought tolerance, at two locations with different precipitation regimes. First, we selected 15 diverse tree and shrub species cultivated in gardens adjacent to the University of California, Los Angeles campus, including the two used in the optimization experiments (Table 1). These species originate from a range of native habitats, from chaparral to tropical wet forest, and currently experience a mean annual temperature of 17.3°C and annual precipitation of 450 mm (National Weather Service). We also selected 15 forest tree species at the Center for Tropical Forest Science long-term research plot in Xishuangbanna, Yunnan, China, a tropical rainforest with a mean annual temperature of 21°C, and annual precipitation of 1532 mm, with over 80% of annual precipitation occurring from May to October (Cao et al. 2006). Trees in this forest show strong topographic habitat associations, which are hypothesized to reflect variation in soil preferences (Lan et al. 2009). Our sampling was conducted during the wet season.

One branch from each of three to six individuals was collected for osmometer measurements as described above. Leaf discs were treated with a 2 min submersion time in LN2, 10 min equilibration time, and no thawing time outside of the osmometer chamber, given the results of the optimization experiments (see Results). P–v curves were produced and analysed according to the bench drying method (Sack, Pasquet-Kok & PrometheusWiki 2010) with a pressure chamber (Plant Moisture Stress Model 1000, Corvallis, Oregon), and turgor loss point (\( \pi_{tlp} \)), osmotic potential (\( \pi_o \)), apoplastic fraction (\( \alpha_a \)), and modulus of elasticity (\( \alpha_o \)) were determined according to standard methods (Turner 1988; Koide et al. 1989; Sack, Pasquet-Kok & PrometheusWiki 2010). P–v curve data were determined within 4 weeks of the osmometer data from the same individuals of Bauhinia galpinii at UCLA and all the XTBO species; for the remaining 14 species at UCLA, previously published p–v data were used that had been determined for the same individuals within the previous 2 years (Scoffoni et al. 2008, 2011, 2012). We selected individuals at UCLA that are irrigated year-round and collected leaves for both approaches during the same times of year to minimize potential differences in seasonal osmotic adjustment.

Prior to measurement, leaves were rehydrated overnight, which is a standard pre-treatment in the literature for p–v curve determination to ensure all measurements are made at full hydration and are therefore comparable across studies with differences in water availability. Failing to rehydrate may instead produce leaf values at arbitrary relative water contents below saturation. We note that rehydration prior to measurement can cause solute leakage from cells into the apoplast, such that p–v curve analyses find less negative values of \( \pi_{tlp} \) and \( \pi_o \) and lower values of \( \alpha_a \) (Kubiske & Abrams 1990, 1991a; b, Sack, Pasquet-Kok & PrometheusWiki 2010). Additionally, rehydration prior to measurement can cause solute leakage from cells into the apoplast, such that p–v curve analyses find less negative values of \( \pi_{tlp} \) and \( \pi_o \) and lower values of \( \alpha_a \) (Kubiske & Abrams 1990, 1991a; b). Such effects can reduce resolution for determining seasonal shifts in p–v parameters for given species (Kubiske and Abrams 1990, 1991a, 1991b). Even so, using a standard rehydration treatment does not preclude species-comparisons and is arguably necessary to produce comparable measurements. Our analysis of data from previous studies indicated that species-differences in p–v parameters are largely robust to rehydration effects after one corrects data for the plateau effect; p–v parameters determined with and without rehydration were strongly correlated across species, although the relationships were not 1:1, and measurements on rehydrated material underestimated the most negative osmotic potentials (\( \rho^2 = 0.61 \) for \( \pi_o \) and 0.77 for \( \pi_{tlp} \), \( P < 0.001 \); data from Kubiske & Abrams 1990, 1991a; b; Fig. S1). These potential effects on solute concentration and p–v parameters, as well as the need for standardization, warrant further consideration to develop best measurement practices. However, explicitly recommending a pre-measurement rehydration method is outside the scope of our study, as it would not affect the method proposed here. A rehydration pre-treatment should not affect the relationship between osmometer and p–v curve estimates of osmotic potential, as long as the pre-treatment is consistent between the two methods, as was applied here.

Leaf fresh mass, leaf area (LI-COR 3000C area metre), thickness (T, mm) and dry mass after oven drying for 72 h at 70°C were determined for calculation of leaf dry mass per unit area (LMA; g m⁻²), leaf dry matter content (LDMC; dry mass/fresh mass) and leaf density (\( \rho \), LMA/T; g cm⁻³). Thickness was averaged from the top, middle and bottom of each leaf.

STATISTICS

We first tested the \( \pi_o \) values determined using the osmometer (\( \pi_{osm} \)) against those from p–v curve analysis (\( \pi_{tlp} \)) using a paired r-test. Next, we used regression analysis to test how well \( \pi_{osm} \) and \( \pi_{tlp} \) could be predicted from \( \pi_{osm} \) (R; version 2.120, http://www.r-project.org/). We additionally tested a range of linear models for predicting \( \pi_{osm} \) and \( \pi_{tlp} \) from \( \pi_{osm} \) when including additional p–v parameters and leaf functional traits (\( \alpha_a \), \( \rho \), LMA, T, p, and LDMC; Table S1). We also tested the ability to predict \( \pi_{osm} \) from \( \pi_{tlp} \) as an estimate based on a previously derived analytical solution for the p–v equations giving \( \pi_{osm} \) as a function of \( \pi_{tlp} \) and \( \rho \) (Bartlett, Scoffoni & Sack 2012):

\[
\pi_{osm} = \frac{\pi_{tlp} \times \rho}{\pi_{osm} + \rho} \tag{eqn 1}
\]

Model selection was performed within a maximum likelihood framework. Maximum likelihood parameters were determined for each model applied to the data for all species; the \( r^2 \) and slope of expected vs. observed values, forced through the origin, was used as an index of goodness of fit. Models were compared using the Akaike information criterion corrected for low n (AICc); the model with the lowest AICc value has best support, and differences > 2 in AICc values are considered meaningful (Burnham & Anderson 2002, 2004). Parameters were estimated using the simulated annealing procedure for global optimization and then used as the initial values in Nelder–Mead simplex search procedure for local optimization; standard errors for the parameters were generated from the Hessian matrix (R version 2.14.0; RDCT, 2005; code available on request). For the best-fit models, we calculated the 95% confidence intervals and 95% prediction intervals assuming sample sizes of 3, 6, or 10 leaves per species (Sokal & Rohlf 1995; Royer et al. 2007).

To determine whether the prediction of drought tolerance parameters would differ between the two sampled locations, the two data sets (UCLA and Xishuangbanna) were compared in their parameter values, and in the best-fit relationship of \( \pi_{osm} \) and \( \pi_{tlp} \) against predictor variables, using analysis of covariance to compare the slopes and intercepts (SMATR software; Falster, Warton & Wright 2006; Warton et al. 2006).

The second purpose of our study was to investigate the source of discrepancies between osmometer and p–v curve measurements of \( \pi_o \).
Table 1. Woody species tested, origin, leaf type (evergreen or deciduous, E or D, respectively) and pressure-volume curve parameters and osmotic potential at full turgor measured using osmometry, with mean ± standard error values for each parameter. Species nomenclature and biomes and continents of origin from Scoffoni et al. (2008, 2011) and (Fang, Wang & Tang 2011). Species of the Xishuangbanna Botanic Garden (XTBG) were from native forest plots

<table>
<thead>
<tr>
<th>Family</th>
<th>Biome, continent of origin</th>
<th>Leaf type</th>
<th>Turgor loss point (MPa)</th>
<th>Osmotic potential (MPa)</th>
<th>Elasticity (MPa)</th>
<th>Apoplastic fraction</th>
<th>Osmometer osmotic potential (MPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>UCLA species</strong></td>
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</tr>
<tr>
<td><em>Alberta magna</em></td>
<td>Fabaceae</td>
<td>E</td>
<td>-1.97 ± 0.07</td>
<td>-1.39 ± 0.05</td>
<td>0.80 ± 0.17</td>
<td>0.45 ± 0.02</td>
<td>-1.45 ± 0.01</td>
</tr>
<tr>
<td><em>Bauhinia galpinii</em></td>
<td>Fabaceae</td>
<td>D</td>
<td>-1.41 ± 0.07</td>
<td>-1.15 ± 0.08</td>
<td>0.78 ± 0.16</td>
<td>0.08 ± 0.04</td>
<td>-0.95 ± 0.05</td>
</tr>
<tr>
<td><em>Camellia sasanqua</em></td>
<td>Theaceae</td>
<td>E</td>
<td>-2.12 ± 0.28</td>
<td>-1.61 ± 0.13</td>
<td>0.71 ± 0.11</td>
<td>0.23 ± 0.17</td>
<td>-1.39 ± 0.08</td>
</tr>
<tr>
<td><em>Cercocarpus betuloide</em></td>
<td>Rosaceae</td>
<td>E</td>
<td>-2.59 ± 0.02</td>
<td>-1.64 ± 0.04</td>
<td>1.10 ± 0.70</td>
<td>0.59 ± 0.08</td>
<td>-2.08 ± 0.07</td>
</tr>
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<td><em>Comarostaphylis diversifolia</em></td>
<td>Ericaceae</td>
<td>E</td>
<td>-2.60 ± 0.14</td>
<td>-2.23 ± 0.12</td>
<td>3.41 ± 0.77</td>
<td>0.47 ± 0.10</td>
<td>-2.66 ± 0.06</td>
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<td><em>Eucalyptus erythrocorys</em></td>
<td>Myrtaceae</td>
<td>E</td>
<td>-2.24 ± 0.10</td>
<td>-1.67 ± 0.06</td>
<td>2.15 ± 0.28</td>
<td>0.63 ± 0.05</td>
<td>-1.54 ± 0.05</td>
</tr>
<tr>
<td><em>Holodendron canariensis</em></td>
<td>Araliaceae</td>
<td>E</td>
<td>-2.06 ± 0.09</td>
<td>-1.16 ± 0.07</td>
<td>1.28 ± 0.79</td>
<td>0.43 ± 0.07</td>
<td>-1.54 ± 0.05</td>
</tr>
<tr>
<td><em>Heteromeles arbutifolia</em></td>
<td>Rosaceae</td>
<td>E</td>
<td>-2.34 ± 0.10</td>
<td>-1.89 ± 0.10</td>
<td>1.64 ± 0.49</td>
<td>0.28 ± 0.06</td>
<td>-1.96 ± 0.04</td>
</tr>
<tr>
<td><em>Hymenoporum florum</em></td>
<td>Pittosporaceae</td>
<td>D</td>
<td>-2.06 ± 0.05</td>
<td>-1.38 ± 0.04</td>
<td>0.58 ± 0.48</td>
<td>0.36 ± 0.03</td>
<td>-1.75 ± 0.07</td>
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<td><em>Lantana camara</em></td>
<td>Verbenaceae</td>
<td>E</td>
<td>-1.37 ± 0.04</td>
<td>-1.10 ± 0.04</td>
<td>4.85 ± 0.33</td>
<td>0.23 ± 0.12</td>
<td>-0.64 ± 0.01</td>
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<td><em>Magnolia grandiflora</em></td>
<td>Magnoliaceae</td>
<td>E</td>
<td>-2.06 ± 0.05</td>
<td>-1.43 ± 0.02</td>
<td>9.14 ± 1.31</td>
<td>0.16 ± 0.01</td>
<td>-1.68 ± 0.04</td>
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<td><em>Pterocarya racemosa</em></td>
<td>Platanaceae</td>
<td>D</td>
<td>-2.03 ± 0.06</td>
<td>-1.54 ± 0.04</td>
<td>1.81 ± 0.53</td>
<td>0.36 ± 0.04</td>
<td>-1.55 ± 0.06</td>
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<td><em>Quercus agrifolia</em></td>
<td>Fagaceae</td>
<td>E</td>
<td>-3.00 ± 0.12</td>
<td>-2.31 ± 0.12</td>
<td>2.08 ± 1.28</td>
<td>0.44 ± 0.09</td>
<td>-3.03 ± 0.12</td>
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<td><em>Raphiolepis indica</em></td>
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<td>E</td>
<td>-2.07 ± 0.18</td>
<td>-1.37 ± 0.15</td>
<td>1.15 ± 0.79</td>
<td>0.69 ± 0.05</td>
<td>-1.99 ± 0.14</td>
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<td>Lamiaceae</td>
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<td>-1.18 ± 0.07</td>
<td>-0.92 ± 0.05</td>
<td>0.54 ± 0.21</td>
<td>0.22 ± 0.02</td>
<td>-0.79 ± 0.02</td>
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<tr>
<td><strong>XTBG species</strong></td>
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</tr>
<tr>
<td><em>Baccaurea ramiflora</em></td>
<td>Euphorbiaceae</td>
<td>E</td>
<td>-1.11 ± 0.10</td>
<td>-0.83 ± 0.07</td>
<td>2.53 ± 0.20</td>
<td>-0.34 ± 0.20*</td>
<td>-0.70 ± 0.007</td>
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<td>Lecythidaceae</td>
<td>E</td>
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<td>-0.77 ± 0.02</td>
<td>3.28 ± 0.72</td>
<td>-0.15 ± 0.12*</td>
<td>-0.74 ± 0.02</td>
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<tr>
<td>* Diospyros nigrococca*</td>
<td>Ebenaceae</td>
<td>E</td>
<td>-1.63 ± 0.09</td>
<td>-1.42 ± 0.06</td>
<td>0.94 ± 0.37</td>
<td>-0.50 ± 0.39*</td>
<td>-1.61 ± 0.04</td>
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<td>E</td>
<td>-1.51 ± 0.05</td>
<td>-1.31 ± 0.04</td>
<td>0.96 ± 0.90</td>
<td>-0.08 ± 0.08*</td>
<td>-1.04 ± 0.11</td>
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<td><em>Harpullia expanoides</em></td>
<td>Sapindaceae</td>
<td>E</td>
<td>-1.70 ± 0.38</td>
<td>-1.19 ± 0.34</td>
<td>6.35 ± 1.96</td>
<td>-0.23 ± 0.23*</td>
<td>-1.58 ± 0.08</td>
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<td><em>Koeppen globularia</em></td>
<td>Myristicaceae</td>
<td>E</td>
<td>-1.39 ± 0.13</td>
<td>-1.10 ± 0.08</td>
<td>8.14 ± 0.90</td>
<td>0.28 ± 0.10</td>
<td>-0.98 ± 0.08</td>
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<td><em>Macopa transpenns</em></td>
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<td>-1.49 ± 0.15</td>
<td>-1.25 ± 0.08</td>
<td>0.79 ± 0.40</td>
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<td>0.42 ± 0.04</td>
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<td>-1.46 ± 0.12</td>
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<td>0.42 ± 0.08</td>
<td>-1.24 ± 0.04</td>
</tr>
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<td><em>Pterospermum mangnunense</em></td>
<td>Acanthaceae</td>
<td>E</td>
<td>-1.82 ± 0.25</td>
<td>-1.43 ± 0.20</td>
<td>1.12 ± 0.32</td>
<td>0.20 ± 0.28*</td>
<td>-1.26 ± 0.14</td>
</tr>
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<td><em>Saprosma ternata</em></td>
<td>Rubiaceae</td>
<td>E</td>
<td>-1.25 ± 0.06</td>
<td>-1.07 ± 0.05</td>
<td>1.69 ± 0.94</td>
<td>-0.24 ± 0.14*</td>
<td>-0.91 ± 0.12</td>
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<td><em>Parashorea chinensis</em></td>
<td>Dipterocarpaceae</td>
<td>E</td>
<td>-1.52 ± 0.04</td>
<td>-1.12 ± 0.03</td>
<td>0.41 ± 1.17</td>
<td>-0.12 ± 0.13*</td>
<td>-1.36 ± 0.10</td>
</tr>
<tr>
<td><em>Sloanea tomentosa</em></td>
<td>Elaegnaceae</td>
<td>E</td>
<td>-1.45 ± 0.05</td>
<td>-1.12 ± 0.05</td>
<td>0.76 ± 0.92</td>
<td>0.21 ± 0.07</td>
<td>-1.14 ± 0.12</td>
</tr>
<tr>
<td><em>Symbiopsis albicans</em></td>
<td>Euphorbiaceae</td>
<td>E</td>
<td>-2.18 ± 0.22</td>
<td>-1.52 ± 0.23</td>
<td>0.48 ± 1.87</td>
<td>-0.06 ± 0.12*</td>
<td>-1.70 ± 0.18</td>
</tr>
<tr>
<td><em>Trigonobium thymoides</em></td>
<td>Euphorbiaceae</td>
<td>E</td>
<td>-1.19 ± 0.19</td>
<td>-0.99 ± 0.19</td>
<td>0.69 ± 1.64</td>
<td>-0.32 ± 0.14*</td>
<td>-0.82 ± 0.05</td>
</tr>
</tbody>
</table>

*Species marked with an asterisk had an extrapolated apoplastic fraction not significantly different from 0 (t-test, P > 0.1).
We tested the influence of the opposing biases of apoplastic dilution and cell wall dissolution, considered the most significant biases in osmometer methods (see Introduction). We compared the measured $\pi_{\text{osm}}$ with an estimated value ($\hat{\pi}_{\text{osm}}$), determined from $\pi_{\text{pv}}$ and adjusted for these effects. We assumed that the amount of apoplastic dilution would be proportional to $a_{t}$, and assumed an apoplastic solution concentration of 0 for non-halophytic species (Gabriel & Kesselmeier 1999; James et al. 2006), and that additional solute from the cell walls would be proportional to wall investment. Thus, we fitted the following equation, which includes both the apoplastic dilution effect and the cell wall dissolution effect, and their interaction:

$$
\hat{\pi}_{\text{osm}} = a \times \pi_{\text{pv}} \times (1 - a_{t}) + b \times \text{wall investment} + c \times \text{wall investment} \times \pi_{\text{pv}} \times (1 - a_{t}) + d \quad \text{eqn 2}
$$

We used LMA, $T$, $p$, $e$, and LDMC as estimates of cell wall investment. In particular, $e$, $p$, and LDMC should be strongly related to the proportion of leaf tissue occupied by cell walls (Garnier & Laurent 1994; Lenz, Wright & Westoby 2006).

The determination of $a_{t}$ by p–v analysis involves extrapolation beyond the range of data and thus can be imprecise (Andersen, Jensen & Losch 1991; Wardlaw 2005), and 11 species measured here had $a_{t}$ values not significantly different from 0, including 10 species with negative $a_{t}$ values ($t$-test; $P > 0.10$). The apoplastic dilution and cell wall investment analyses were conducted including all species, setting to 0 those $a_{t}$ values that did not differ significantly from 0 (see Table 1). Notably, determination of other p–v parameters is robust to uncertainty in $a_{t}$ (Andersen, Jensen & Losch 1991).

**Results**

**OPTIMIZING THE OSMOMETER METHOD FOR $\pi_{\text{osm}}$ DETERMINATION**

The method optimization experiments indicated reliable approaches to rapidly determine osmotic potential from leaf discs in the osmometer. First, there was no effect of freezing time for *Hedera canariensis* or *Heteromeles arbutifolia*. The minimum time used, 2 min, was adequate to completely freeze leaf tissue and fracture the cell walls (Fig. 1a). Notably, Kikuta & Richter (1992a,b) allowed discs to thaw for 1 h before measuring, but we found complete thawing occurs within chamber equilibration time and additional thawing time was unnecessary (Fig. 1b).

Leaf discs must be shielded from evaporation prior to measurement. Discs exposed on the bench for 1 h had inaccurate low $\pi_{\text{cv}}$ values, whereas discs could be stored in humidified bags for 1 h with no change in measured $\pi_{\text{osm}}$ (Fig. 1b). The equilibration time of approximately 10 min varied little among individuals, treatments or species.

**PREDICTION OF $\pi_{\text{pv}}$ FROM OSMOMETRY MEASUREMENTS**

Across the 30 measured species, the values of $\pi_{e}$ measured by osmometry ($\pi_{\text{osm}}$) and p–v curves ($\pi_{\text{pv}}$) were equivalent on average (species-mean ± standard error were $-1.38 ± 0.10$ and $-1.41 ± 0.07$ MPa respectively; paired t-test; $P = 0.31$). Further, we found strong correlation between $\pi_{\text{osm}}$ and $\pi_{\text{pv}}$ (Fig. 2). However, while the 1 : 1 line forced through the origin fitted the data with statistical significance ($P < 1 \times 10^{-5}$), it had low goodness of fit ($r^2 = 0.47$), that the $\pi_{\text{osm}}$ overestimated $\pi_{\text{pv}}$ at less negative values and underestimated $\pi_{\text{pv}}$ at more negative values. The best-fit model for predicting $\pi_{\text{pv}}$ included both $\pi_{\text{osm}}$ and $e$, estimated functional trait $p$, and their interaction term ($r^2 = 0.85; P < 2 \times 10^{-12}$) (Table S1). Notably, $\pi_{\text{osm}}$ alone was also an excellent predictor of $\pi_{\text{pv}}$ ($r^2 = 0.80; P < 2 \times 10^{-10}$; Fig. 2). The 95% prediction intervals were $±18\%$, $±13.5\%$ and $±11\%$ for the univariate model, if estimating species values from sample sizes of 3, 6 and 10 leaves, respectively, compared to $±14.5\%$, $±107\%$, and $±9\%$ for the model incorporating $p$ and $±14\%$, $±10.5\%$, and $±9\%$ for the best-fit model based on $\pi_{\text{osm}}$ and $e$. Thus, $\pi_{pv}$ can be estimated accurately from osmometry measurements alone or from $\pi_{\text{osm}}$ and $p$.

**IDENTIFYING THE TRAITS THAT AFFECT METHOD COMPARISON FOR OSMOTIC POTENTIAL**

We tested whether the deviation of the $\pi_{\text{osm}}$ vs. $\pi_{\text{pv}}$ relationship from the 1 : 1 line could be accounted for by the opposing effects of apoplastic dilution and cell wall dissolution by fitting eqn 2. This model estimated the bias in the $\pi_{\text{osm}}$ vs. $\pi_{\text{pv}}$ relationship; $\hat{\pi}_{\text{osm}}$ was correlated with $\pi_{\text{pv}}$ with a slope statistically indistinguishable from 1 (slope ± standard error = 0.954 ± 0.14; $r^2 = 0.65; P < 2 \times 10^{-4}$; Table S1; Fig. 3). In applying eqn 2, LDMC and LMA were significantly better metrics for cell wall investment than $p$, $T$ or $e$ (DAIACC > 2; Table S1). The bias in the original relationship, wherein $\pi_{\text{osm}}$ becomes increasingly more negative relative to $\pi_{\text{pv}}$, as both decrease, and *vice versa* as they approach 0, is thus associated with the negative correlations of LDMC and LMA with $\pi_{\text{pv}}$ ($r^2 = 0.56, 0.49$; both $P < 1 \times 10^{-4}$, respectively); species with higher osmotic concentrations tend to have greater cell wall investment. For species with $\pi_{e}$ values closer to zero, cell wall dissolution only weakly offsets apoplastic dilution, whereas for species with more negative $\pi_{e}$, cell wall dissolution increasingly offsets dilution, accounting for the method discrepancy across $\pi_{e}$ values.

**PREDICTION OF $\pi_{\text{tip}}$ FROM OSMOMETRY MEASUREMENTS**

Osmometer measurements enabled accurate prediction of the turgor loss point (Fig. 4). The $\pi_{\text{tip}}$ was strongly correlated with $\pi_{\text{osm}}$ ($r^2 = 0.86; P < 1 \times 10^{-12}$), as expected, given the close correlation of $\pi_{\text{tip}}$ with $\pi_{\text{pv}}$ ($r^2 = 0.91; P < 2 \times 10^{-12}$) (Fig. 2; Table S1).

We tested whether $\pi_{\text{tip}}$ could be predicted from other leaf functional traits alone or whether these improved the predic-
tion from \( \pi_{\text{osm}} \). We considered physiological traits \( a_l \) and \( \varepsilon \), and \( \rho \), \( T \), \( \text{LMA} \) and \( \text{LDMC} \), frequently measured traits representing structural investment (Sack et al. 2003). Across species, the \( \pi_{\text{tp}} \) was significantly negatively correlated with \( \varepsilon \) \((r^2 = 0.57; P < 2 \times 10^{-8})\), \( \text{LMA} \) \((r^2 = 0.56; P < 2 \times 10^{-8})\), \( \text{LDMC} \) \((r^2 = 0.61; \ P < 2 \times 10^{-8})\), \( \rho \) \((r^2 = 0.63; \ P < 2 \times 10^{-8})\), \( T \) \((r^2 = 0.12; \ P = 0.03)\) and \( a_l \) \((r^2 = 0.22; \ P = 0.00)\). The best-fit models from the osmometer method, that is, those with AICc values within two units of the most negative value, predicted \( \pi_{\text{tp}} \) from \( \pi_{\text{osm}} \) alone and from both \( \pi_{\text{osm}} \) and \( \rho \) (Table S1; \( P < 2 \times 10^{-12} \), \( r^2 = 0.86-0.89 \)). The observed \( \pi_{\text{tp}} \) was also correlated, although not as strongly, with \( \pi_{\text{tp}} \) predicted from equation 1, \( \hat{\pi}_{\text{tp}} \), calculated from \( \varepsilon \) and \( \pi_{\text{osm}} \) \((P < 2 \times 10^{-10} ; r^2 = 0.78)\). The leaf construction traits thus did not add significant predictive power to the relationship between \( \pi_{\text{tp}} \) and \( \pi_{\text{osm}} \), and the univariate relationship is more parsimonious. The 95% prediction intervals of the univariate relationship of \( \pi_{\text{tp}} \) to \( \pi_{\text{osm}} \) were \( \pm 23\% \), \( \pm 174\% \) and \( \pm 148\% \), if estimating species values from sample sizes of 3, 6, and 10 leaves, respectively. The \( \pi_{\text{tp}} \) can therefore be reliably predicted from osmometer measurements, even given wide variation in other pressure-volume parameters and leaf construction traits.

As expected, the values of \( \pi_{\text{osm}} \) and \( \pi_{\text{tp}} \) for species from the wetter XTBG site (–1.19 and –1.51 MPa, respectively) were significantly less negative than those for the UCLA site (–1.55 and –2.09, respectively; \( t \)-tests; both \( P < 0.001 \)). The recommended models for \( \pi_{\text{pv}} \) and \( \pi_{\text{tp}} \) gave excellent predictions for these mean parameters at each site (predicted \( \pi_{\text{osm}} = -1.20 \) for XTBG and –1.55 for UCLA; predicted \( \pi_{\text{tp}} = -1.59 \) and –2.02, respectively). Further, there were no statistically significant differences between the regression lines for the two sites, relating observed \( \pi_{\text{tp}} \) to \( \pi_{\text{osm}} \) predicted from \( \pi_{\text{osm}} \); observed \( \pi_{\text{pv}} \) to \( \pi_{\text{pv}} \) predicted from \( \pi_{\text{osm}} \) observed \( \pi_{\text{pv}} \) to \( \pi_{\text{pv}} \) predicted from \( \rho \), \( \pi_{\text{osm}} \) and their interaction; or observed \( \pi_{\text{tp}} \) to \( \pi_{\text{tp}} \) predicted from \( \pi_{\text{osm}} \) and \( \varepsilon \) (SMATR ANCOVA, all \( P > 0.3 \)). These regression relationships and the osmometer measurements themselves are therefore robust across ecosystems with different water availabilities.

Fig. 3. Accounting for the discrepancy between measurement of osmotic potential at full turgor with a pressure-volume curve ($\pi_{\text{osm}}$) and that measured with osmometry ($\pi_{\text{osm}}$), as was seen in the departure of the data in Fig. 2 from the 1 : 1 line. This bias could be accounted for by the effects of apoplastic dilution and cell wall dissolution in the osmometry measurement. Here, $\pi_{\text{osm}}$ predicted from $\pi_{\text{osm}}$ using eqn 2, with leaf dry matter content as a proxy for cell wall investment was tightly correlated with measured $\pi_{\text{osm}}$ with no bias (slope ± standard error = 0.954 ± 0.14; $r^2$ = 0.65; $P < 2 \times 10^{-4}$). For this analysis, apoplastic fraction values not significantly different from 0 were set as 0 (see Table 1), and data for species from both locations were pooled ($n = 30$).

**Discussion**

This study provides an approach to estimating key water relations parameters rapidly, which should enable the standardized assessment of many species for drought tolerance. The optimized freeze-thaw disc osmometer measurements ($\pi_{\text{osm}}$) were tightly correlated with p–v curve estimates of $\pi_{\text{osm}}$ ($\pi_{\text{osm}}$) and also $\pi_{\text{osm}}$ with the $\pi_{\text{osm}}$ estimation improved by including leaf density as a predictor, whereas the $\pi_{\text{osm}}$ estimation was independent of both leaf structure and habitat preferences. We propose our optimized osmometer method for determining $\pi_{\text{osm}}$ as a standard method. The minimum equilibration time, however, should be confirmed for instruments with different well sizes.

Earlier studies have used osmometer methods for measuring $\pi_{\text{osm}}$ and compared them with expressed sap and p–v curve methods, but the largest previous study showed relationships of $\pi_{\text{osm}}$ and $\pi_{\text{osm}}$ for five species (Callister, Arndt & Adams 2006). We expanded on that work, refining the methodology by evaluating the effects of freezing time, thawing time and thawing conditions and providing equations for the relationship of $\pi_{\text{osm}}$ and $\pi_{\text{osm}}$ for 30 species. Additionally, while previous studies have shown a correlation of $\pi_{\text{osm}}$ with $\pi_{\text{osm}}$ (Sack et al. 2003; Lenz, Wright & Westoby 2006; Scoffoni et al. 2011; Bartlett, Scoffoni & Sack 2012), we are the first to our knowledge to show that $\pi_{\text{osm}}$ can be used to predict $\pi_{\text{osm}}$ as a rapid alternative to p–v curves.

Notably, $\pi_{\text{osm}}$ and $\pi_{\text{osm}}$ were tightly correlated but not equal. The $\pi_{\text{osm}}$ was higher than $\pi_{\text{osm}}$ for species with less negative values and lower than $\pi_{\text{osm}}$ for species with more negative values. Our analysis indicated that this discrepancy may relate to both apoplastic dilution and wall solute enrichment. A high LDWC, which reflects the proportion of cell wall material in the leaf tissue, correlates across species with more negative $\pi_{\text{osm}}$ values, possibly because greater cell wall investment enables maintenance of a high relative water content at $\pi_{\text{osm}}$ and/or because drought tolerant plants construct leaf tissue with a high density of relatively smaller cells to increase the efficiency of osmotic adjustment (Cutler, Rains & Loomis 1977; Bartlett, Scoffoni & Sack 2012). Therefore, for species with more negative $\pi_{\text{osm}}$ wall solute enrichment would play a more important role than apoplastic dilution, increasing the discrepancy between the two methods. However, the $\pi_{\text{osm}}$ and $\pi_{\text{osm}}$ were equivalent on average across species, and the discrepancies between the two methods were accounted for in our regression model

$$\pi_{\text{osm}} = 0.587\pi_{\text{osm}} - 0.546 \quad \text{eqn 3}$$

which can be used to reliably estimate $\pi_{\text{osm}}$ ($r^2 = 0.80$). We recommend this regression approach to estimate and present $\pi_{\text{osm}}$ rather than simply determining $\pi_{\text{osm}}$, because $\pi_{\text{osm}}$ values are most common in the literature. However, the regression equation

$$\pi_{\text{osm}} = 0.466\pi_{\text{osm}} - 9.31 \times 10^{-5}\pi_{\text{osm}} \rho - 9.26 \times 10^{-4}\rho - 0.455 \quad \text{eqn 4}$$

provided the most accurate estimate from the osmometer method ($r^2 = 0.87$). We recommend further validation of
these models in species with closely spaced large veins that cannot be avoided when sampling leaf discs.

To our knowledge, this is the first study to produce a regression equation allowing prediction of $\pi_{olp}$ from osmometer measurements:

$$\pi_{olp} = 0.832\pi_{osm} - 0.631$$ \hspace{1cm} \text{eqn 5}

This approach can be applied in other systems. This regression equation was highly significant ($r^2 = 0.86$; $P < 2 \times 10^{-12}$) for diverse species with a wide range of drought tolerances, leaf characteristics and $p_v$-parameter values (Table 1, Fig. 4). The prediction intervals for the estimation of $\pi_{olp}$ and $\pi_{pw}$ were reasonably narrow, <15% given sampling of 10 leaves per species or 14–17% for sampling of 6 leaves. We propose that the osmometer method and regressions developed here are an accurate proxy for $p_v$-curve measurements of $\pi_o$ and $\pi_{olp}$. This approach will continue to improve as comparative data become available for more species and a wider range of $p_v$-parameter values. However, this species set already encompasses 40%, 48%, 52% and 78% of the total range of $\pi_o$, $\pi_{olp}$ and $\pi_{pw}$, respectively, found in a global meta-analysis of $p_v$ data, suggesting that these regressions will be robust across the range of $p_v$-parameter variation (Bartlett, Scoffoni & Sack 2012).

The method presented here for determining $\pi_o$ and $\pi_{olp}$ has several advantages over generating $p_v$-curves. Osmometer measurements require approximately 10–15 min per individual leaf and an hour for six, which is typically sufficient replication for reliable determination of species means (Sack et al. 2003; Hulshof & Swenson 2010), compared to the approximately one or 2 days required to generate a $p_v$ curve for 4–6 leaves. Thus, this method involves a thirty- to fiftyfold increase in measuring speed or reduction in effort by >95%. This reduction in effort makes feasible sampling across a wide range of taxa, even potentially an entire community. Indeed, for communities experiencing strongly seasonal climates, repeated sampling for given species may be necessary to determine the role of $\pi_o$ and $\pi_{olp}$ adjustment in conferring ecological drought tolerance. Notably, osmometer measurements had similar or lower standard errors for estimates of $\pi_o$ for given species than $p_v$-curves (paired t-test; $P = 0.08$; $n = 30$). The osmometer is likely to have greater precision because it directly measures $\pi_o$ whereas $p_v$-curve determination requires extrapolation from the solute potential vs. relative water content relationship. Osmometer measurements are also more feasible than $p_v$-analysis for fragile, large or succulent leaves, or leaves with short or no petioles.

Given the significance of $\pi_{olp}$ and $\pi_o$ in estimating drought adaptation and acclimation, and thus potentially for predicting species’ distribution across soil moisture gradients, rapid surveys would be useful for community-level studies of this functional trait and for drought tolerance screening of agricultural cultivars (cf. Kraft, Valencia & Ackerley 2008). Notably, $\pi_o$ and $\pi_{olp}$ are much better predictors of leaf drought tolerance than LMA, $\rho$, and LDMC (Poorter & Markesteijn 2008; Bartlett, Scoffoni & Sack 2012), leaf traits that have been frequently suggested as proxies for the $p_v$ curve parameters or as indices for drought tolerance mainly because of the convenience with which they can be determined (e.g. Niinemets 2001; Kraft, Valencia & Ackerly 2008; Violle & Jiang 2009). However, the method described here is equally rapid and convenient, given access to the instrument, and, having greater predictive power and mechanistic relevance, should have considerable value for study of the comparative physiology and ecology of drought tolerance.

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**References**


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Supporting Information

Additional Supporting Information may be found in the online version of this article.

Figure S1. Testing the robustness of species values for osmotic potential at full turgor (τo) and at turgor loss point (τtlp) as estimated with the pressure-volume curves (p-v curves) to standard rehydration treatment, based on published data.

Table S1. Regression equations predicting pressure-volume curve parameters.

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