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

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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 (π_{tlp}), and the cell solute potential at full hydration, that is, osmotic potential (π_o). 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 π_o 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 π_o and π_{tlp} from osmometer values

3. The π_o determined with the osmometer (π_{osm}) was an excellent predictor of the π_o determined from the p–v curve (π_{pv}, $r^2 = 0.80$). Although the correlation of π_{osm} and π_{pv} 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 π_{pv} could be made using π_{osm}, leaf density (ρ), and their interaction ($r^2 = 0.85$, all $P < 2 \times 10^{-10}$).

4. The π_{osm} could also be used to predict π_{tlp} ($r^2 = 0.86$). Indeed, π_{osm} was a better predictor of π_{tlp} 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 π_{osm} with LMA, T, ρ, or LDMC or other p–v curve parameters (i.e. elasticity and apoplastic fraction) did not significantly improve prediction of π_{tlp}.

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 (π_{tlp}), 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 (Ψ_{leaf}) with leaf dehydration (Koide *et al.* 1989). Physiologically, the π_{tlp} is the Ψ_{leaf} at which the average cell turgor pressure is lost; at this point, Ψ_{leaf} equals osmotic potential and subsequent Ψ_{leaf} declines are because of increasing osmotic concentration (with π the symbol for osmotic potential). Across species, π_{tlp} is correlated with other important drought tolerance parameters, including Ψ_{leaf} at 50% loss

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of hydraulic and stomatal conductances and the lethal Ψ_{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 (π_o) is the key trait driving both π_{tlp} across species, and the shifts in π_{tlp} for given species during seasonal and experimental droughts, and thus that π_o and π_{tlp} are powerful traits for predicting drought tolerance and distributions with respect to water supply (Bartlett, Scoffoni & Sack 2012). However, the standard p–v curve method for determining π_o and π_{tlp} is highly time-consuming for measuring large species sets. We present a method for rapid π_{tlp} and π_o determination, based on osmometer measurement of π_o.

The p–v curve has been the most commonly used method for measuring π_o because it allows estimation of a number of physiological parameters, including π_{tlp} (Tyree & Hammel 1972; Turner 1988; Koide *et al*. 1989). Methods have been described for measuring π_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 & Shock 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 π_o measurement methods found correlations between measurements made with the p–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 π_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 π_o values; and (ii) *dissolution of cell wall solutes* from destroyed cell walls, which makes π_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 π_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–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–v curve values of π_o and π_{tlp}. Because previous studies showed a strong relationship across species between p–v curve values of π_{tlp} and π_o (Sack *et al*. 2003; Blackman, Brodribb & Jordan 2010; Scoffoni *et al*. 2011), we aimed to estimate π_{tlp} from π_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 π_{pv} and π_{tlp} 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–v curve for determining π_o and π_{tlp} 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 π_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 forceps 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 < 0·01 MPa. If a given set of treatments did not affect the equilibration time or the final π_o value, data were pooled for subsequent comparisons. Thus, for example, given no effect of LN_2 exposure time, the π_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 π_o and π_{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 21·0°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 LN$_2$, 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 (π_{tlp}), osmotic potential (π_{pv}), apoplastic fraction (a_f), and modulus of elasticity (ε) 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 XTBG 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 Ψ_{leaf} values at arbitrary relative water contents below saturation. We note that rehydration before measurement can lead to hydration of the airspaces by capillarity uptake and/or exudation of water from cells. During p–v curve determination, we used the standard correction method to remove data points representing an oversaturated symplastic water content; these points appear in the curve as a 'plateau' of points with constant Ψ_{leaf} despite a decreasing relative water content (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 π_{tlp} and π_o, and lower values of a_f (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 param-

eters 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 ($r^2 = 0·61$ for π_o, and 0·77 for π_{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 (ρ; LMA/T; g cm^{-3}). Thickness was averaged from the top, middle and bottom of each leaf.

STATISTICS

We first tested the π_o values determined using the osmometer (π_{osm}) against those from p–v curve analysis (π_{pv}) using a paired *t*-test. Next, we used regression analysis to test how well π_{pv} and π_{tlp} could be predicted from π_{osm} (R; version 2·12·0, http://www.r-project.org/). We additionally tested a range of linear models for predicting π_{pv} and π_{tlp} from π_{osm} when including additional p–v parameters and leaf functional traits (a_f, ε, LMA, T, ρ, and LDMC; Table S1). We also tested the ability to predict π_{tlp} from $\hat{\pi}_{tlp}$, an estimate based on a previously derived analytical solution for the p–v equations giving π_{tlp} as a function of π_o and ε (Bartlett, Scoffoni & Sack 2012):

$$\hat{\pi}_{tlp} = \frac{\pi_{osm} \times \varepsilon}{\pi_{osm} + \varepsilon} \qquad \text{eqn 1}$$

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 π_{pv} and π_{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 π_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

	Family	Biome, continent of origin	Leaf type	Turgor loss point (MPa)	Osmotic potential (MPa)	Elasticity (MPa)	Apoplastic fraction	Osmometer osmotic potential (MPa)
UCLA species								
Alberta magna	Rubiaceae	Temperate Forest, Africa	E	-1·97 ± 0·07	-1·39 ± 0·05	8·08 ± 0·17	0·45 ± 0·02	-1·45 ± 0·01
Bauhinia galpinii	Fabaceae	Temperate Forest, Africa	D	-1·41 ± 0·07	-1·15 ± 0·08	7·81 ± 1·61	0·08 ± 0·04	-0·95 ± 0·05
Camelia sasanqua	Theaceae	Temperate Forest, Asia	E	-2·12 ± 0·18	-1·61 ± 0·13	7·71 ± 1·11	0·23 ± 0·17	-1·39 ± 0·08
Cercocarpus betuloides	Rosaceae	Mediterranean, N. Am.	E	-2·59 ± 0·02	-1·64 ± 0·04	11·0 ± 0·70	0·59 ± 0·08	-2·08 ± 0·07
Comarostaphylis diversifolia	Ericaceae	Mediterranean, N. Am.	E	-2·60 ± 0·14	-2·23 ± 0·12	34·1 ± 9·77	0·47 ± 0·10	-2·66 ± 0·06
Eucalyptus erythrocorys	Myrtaceae	Temperate Forest, Austral.	E	-2·24 ± 0·10	-1·67 ± 0·06	21·5 ± 2·48	0·63 ± 0·05	-1·54 ± 0·05
Hedera canariensis	Araliaceae	Temperate Forest, Africa	E	-2·06 ± 0·09	-1·16 ± 0·07	12·8 ± 0·79	0·43 ± 0·07	-1·54 ± 0·05
Heteromeles arbutifolia	Rosaceae	Mediterranean, N. Am.	E	-2·34 ± 0·10	-1·89 ± 0·10	16·4 ± 0·49	0·28 ± 0·06	-1·96 ± 0·04
Hymenosporum flavum	Pittosporaceae	Tropical Rainforest, Austral.	D	-2·06 ± 0·05	-1·38 ± 0·04	5·88 ± 0·48	0·36 ± 0·03	-1·75 ± 0·007
Lantana camara	Verbenaceae	Tropical Dry Forest, Pantropical	E	-1·37 ± 0·04	-1·10 ± 0·04	4·85 ± 0·33	0·23 ± 0·12	-0·64 ± 0·01
Magnolia grandiflora	Magnoliaceae	Temperate Forest, N. Am.	E	-2·06 ± 0·05	-1·43 ± 0·02	9·14 ± 1·31	0·16 ± 0·01	-1·68 ± 0·04
Platanus racemosa	Platanaceae	Temperate Riparian, N. Am.	D	-2·03 ± 0·06	-1·54 ± 0·04	8·81 ± 0·53	0·36 ± 0·04	-1·55 ± 0·06
Quercus agrifolia	Fagaceae	Mediterranean, N. America	E	-3·00 ± 0·12	-2·31 ± 0·12	20·8 ± 1·28	0·44 ± 0·09	-3·03 ± 0·12
Raphiolepis indica	Rosaceae	Temperate Forest, Asia	E	-2·07 ± 0·18	-1·37 ± 0·15	11·5 ± 0·79	0·69 ± 0·05	-1·99 ± 0·14
Salvia canariensis	Lamiaceae	Temperate Forest, Africa	E	-1·18 ± 0·07	-0·92 ± 0·05	5·49 ± 0·21	0·22 ± 0·02	-0·79 ± 0·02
XTBG species								
Baccaurea ramiflora	Euphorbiaceae	Tropical Rainforest	E	-1·11 ± 0·10	-0·83 ± 0·07	2·53 ± 0·20	-0·34 ± 0·20*	-0·70 ± 0·007
Barringtonia pendula	Lecythidaceae	Tropical Rainforest	E	-1·02 ± 0·09	-0·77 ± 0·02	3·28 ± 0·72	-0·15 ± 0·12*	-0·74 ± 0·02
Diospyros nigrocortex	Ebenaceae	Tropical Rainforest	E	-1·63 ± 0·09	-1·42 ± 0·06	9·94 ± 0·37	-0·50 ± 0·39*	-1·61 ± 0·04
Eurya austroyunnanensis	Theaceae	Tropical Rainforest	E	-1·51 ± 0·05	-1·31 ± 0·04	9·69 ± 0·90	-0·08 ± 0·08*	-1·04 ± 0·11
Harpullia cupaniodes	Sapindaceae	Tropical Rainforest	E	-1·70 ± 0·38	-1·19 ± 0·34	6·35 ± 1·96	-0·23 ± 0·23*	-1·58 ± 0·08
Knema globularia	Myristicaceae	Tropical Rainforest	E	-1·39 ± 0·13	-1·10 ± 0·08	8·14 ± 0·90	0·28 ± 0·10	-0·98 ± 0·08
Macropanax dispermus	Araliaceae	Tropical Rainforest	E	-1·49 ± 0·15	-1·25 ± 0·08	7·94 ± 0·40	-0·20 ± 0·20*	-1·18 ± 0·06
Mallotus garrettii	Euphorbiaceae	Tropical Rainforest	E	-1·62 ± 0·31	-1·27 ± 0·20	12·0 ± 1·11	0·42 ± 0·04	-1·01 ± 0·09
Mezzettiopsis creaghii	Annonaceae	Tropical Rainforest	E	-1·82 ± 0·14	-1·46 ± 0·12	17·7 ± 5·48	0·42 ± 0·08	-1·24 ± 0·04
Pterospermum menglunense	Acanthaceae	Tropical Rainforest	E	-1·82 ± 0·25	-1·43 ± 0·20	11·2 ± 3·92	0·20 ± 0·28*	-1·26 ± 0·14
Saprosma ternata	Rubiaceae	Tropical Rainforest	E	-1·25 ± 0·06	-1·07 ± 0·05	6·91 ± 0·94	-0·24 ± 0·14*	-0·91 ± 0·12
Parashorea chinensis	Dipterocarpaceae	Tropical Rainforest	E	-1·52 ± 0·04	-1·12 ± 0·03	4·19 ± 1·17	-0·12 ± 0·13*	-1·36 ± 0·10
Sloanea tomentosa	Elaeocarpaceae	Tropical Rainforest	E	-1·45 ± 0·05	-1·12 ± 0·05	6·72 ± 0·96	0·21 ± 0·07	-1·14 ± 0·12
Sumbaviopsis albicans	Euphorbiaceae	Tropical Rainforest	E	-2·18 ± 0·22	-1·52 ± 0·23	4·84 ± 1·87	-0·06 ± 0·12*	-1·70 ± 0·18
Trigonostemon thrysoideum	Euphorbiaceae	Tropical Rainforest	E	-1·19 ± 0·19	-0·99 ± 0·19	6·95 ± 1·84	-0·32 ± 0·14*	-0·82 ± 0·005

*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 π_{osm} with an estimated value ($\hat{\pi}_{osm}$), determined from π_{pv} and adjusted for these effects. We assumed that the amount of apoplastic dilution would be proportional to a_f, and assumed an apoplastic solute 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:

$$\hat{\pi}_{osm} = \underbrace{a \times \pi_{pv} \times (1 - a_f)}_{\text{apoplastic dilution}} + \underbrace{b \times \text{wall investment}}_{\text{wall dissolution}}$$
$$+ \underbrace{c \times \text{wall investment} \times \pi_{pv} \times (1 - a_f)}_{\text{interaction}} + d \qquad \text{eqn 2}$$

We used LMA, T, ρ, ε, and LDMC as estimates of cell wall investment. In particular, ε, ρ 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_f 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_f values not significantly different from 0, including 10 species with negative a_f values (*t*-test; $P > 0.10$). The apoplastic dilution and cell wall investment analyses were conducted including all species, setting to 0 those a_f values that did not differ significantly from 0 (see Table 1). Notably, determination of other p–v parameters is robust to uncertainty in a_f (Andersen, Jensen & Losch 1991).

Results

OPTIMIZING THE OSMOMETER METHOD FOR π_o 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 π_o values, whereas discs could be stored in humidified bags for 1 h with no change in measured π_o (Fig. 1b). The equilibration time of approximately 10 min varied little among individuals, treatments or species.

PREDICTION OF π_{pv} FROM OSMOMETRY MEASUREMENTS

Across the 30 measured species, the values of π_o measured by osmometry (π_{osm}) and p–v curves (π_{pv}) were equivalent on average (species-mean $\pm$ 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 π_{osm} and π_{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 ($r^2 = 0.47$), such that the π_{osm} overestimated π_{pv} at less negative values and underestimated π_{pv} at more negative values. The best-fit model for predicting π_{pv} included both π_{osm} and ε (Table S1) and eliminated this bias (r^2 for predicted value against observed value, forced through the origin $= 0.86$; $P < 2 \times 10^{-11}$). The second most strongly supported model for predicting π_{pv} included π_{osm}, the easily measured functional trait ρ, and their interaction term ($r^2 = 0.85$; $P < 2 \times 10^{-12}$) (Table S1). Notably, π_{osm} alone was also an excellent predictor of π_{pv} ($r^2 = 0.80$; $P < 2 \times 10^{-10}$; Fig. 2). The 95% prediction intervals were $\pm 18\%$, $\pm 13.5\%$ and $\pm 11\%$ for the univariate model, if estimating species values from sample sizes of 3, 6 and 10 leaves, respectively, compared to $\pm 14.5\%$, $\pm 10.7\%$, and $\pm 9\%$ for the model incorporating ρ and $\pm 14\%$, $\pm 10.5\%$, and $\pm 9\%$ for the best-fit model based on π_{osm} and ε. Thus, π_{pv} can be estimated accurately from osmometry measurements alone or from π_{osm} and ρ.

IDENTIFYING THE TRAITS THAT AFFECT METHOD COMPARISON FOR OSMOTIC POTENTIAL

We tested whether the deviation of the π_{osm} vs. π_{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 eliminated the bias in the π_{osm} vs. π_{pv} relationship; $\hat{\pi}_{osm}$ was correlated with π_{pv} with a slope statistically indistinguishable from 1 (slope $\pm$ standard error $= 0.954 \pm 0.14$; $r^2 = 0.65$; $P < 2 \times 10^{-4}$; Table S1; Fig. 3). In applying eqn 2, LDMC and LMA were significantly better metrics for cell wall investment than ρ, T or ε (ΔAICc > 2; Table S1). The bias in the original relationship, wherein π_{osm} becomes increasingly more negative relative to π_{pv} as both decrease, and *vice versa* as they approach 0, is thus associated with the negative correlations of LDMC and LMA with π_{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 π_o values closer to zero, cell wall dissolution only weakly offsets apoplastic dilution, whereas for species with more negative π_o, cell wall dissolution increasingly offsets dilution, accounting for the method discrepancy across π_o values.

PREDICTION OF π_{tlp} FROM OSMOMETRY MEASUREMENTS

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

We tested whether π_{tlp} could be predicted from other leaf functional traits alone or whether these improved the predic-
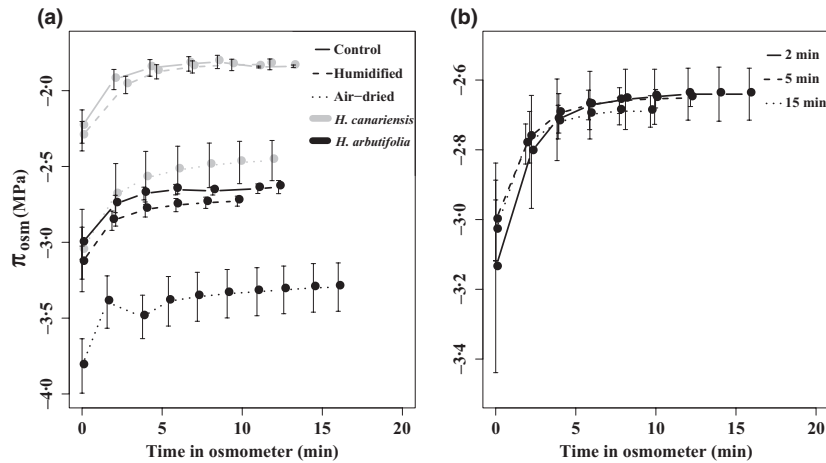
Fig. 1. Effects of different treatments on the measurement of osmotic potential at full turgor by osmometry (π_{osm}) of freeze-thawed leaf discs for *Hedera canariensis* and *Heteromeles arbutifolia*. The π_{osm} was repeatedly measured approximately every 2 min once the disc was sealed in the chamber, with stability (i.e. equilibrium) achieved when the change between two sequential measurements was <0·01 MPa. Equilibration required 10 min or less for all individuals across species. (a). Providing leaf discs with a 1 h thawing time did not affect their equilibration pattern or π_{osm} relative to a control sample measured immediately after freezing, as long as the discs were prevented from dehydrating (bars = standard errors). (b). Varying the immersion time in liquid nitrogen between 2, 5 and 15 min did not affect π_{osm} at equilibrium for *Heteromeles arbutifolia*.

tion from π_{osm}. We considered physiological traits a_f and ε, and ρ, T, LMA and LDMC, frequently measured traits representing structural investment (Sack *et al.* 2003). Across species, the π_{tlp} was significantly negatively correlated with ε ($r^2 = 0·57$; $P < 2 \times 10^{-8}$), LMA ($r^2 = 0·56$; $P < 2 \times 10^{-5}$), LDMC ($r^2 = 0·61$; $P < 2 \times 10^{-5}$), ρ ($r^2 = 0·63$; $P < 2 \times 10^{-5}$), T ($r^2 = 0·12$; $P = 0·03$) and a_f ($r^2 = 0·22$; $P = 0·02$). The best-fit models from the osmometer method, that is, those with AICc values within two units of the most negative value, predicted π_{tlp} from π_{osm} alone and from both π_{osm} and ρ (Table S1; $P < 2 \times 10^{-12}$, $r^2 = 0·86–0·89$). The observed π_{tlp} was also correlated, although not as strongly, with π_{tlp} predicted from equation 1, $\hat{\pi}_{tlp}$, calculated from ε and π_{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 π_{tlp} and π_{osm}, and the univariate relationship is more parsimonious. The 95% prediction intervals of the univariate relationship of π_{tlp} to π_{osm} were ±23%, ±17·4% and ±14·8%, if estimating species values from sample sizes of 3, 6, and 10 leaves, respectively. The π_{tlp} 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 π_o and π_{tlp} 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 π_{pv} and π_{tlp} gave excellent predictions for these mean parameters at each site (predicted $\pi_o = -1·20$ for XTBG and −1·55 for UCLA; predicted $\pi_{tlp} = -1·59$ and −2·02, respectively). Further, there were no statistically significant differences between the regression lines for the two sites, relating observed π_{tlp} to π_{tlp} predicted from π_{osm}; observed π_{pv} to π_{pv} predicted from π_{osm}; observed π_{pv} to π_{pv} predicted from ρ,
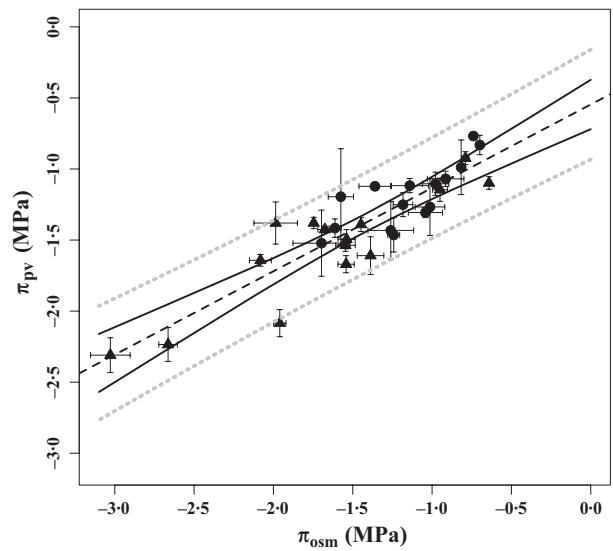
Fig. 2. Measurements of osmotic potential at full turgor from pressure-volume (π_{pv}) curve analysis plotted against measurements made with the osmometer (π_{osm}) for species of a wide range of leaf structure and drought tolerances (circles = Xishuangbanna Tropical Botanical Garden species, triangles = University of California, Los Angeles species; see Table S1). Results from the two methods were strongly correlated ($r^2 = 0·80$; $P < 2 \times 10^{-10}$); fitted line is $\pi_{pv} = 0·587\pi_{osm} - 0·546$. Black solid lines are 95% confidence intervals, grey dashed lines are 95% prediction intervals, and error bars represent standard errors.

π_{osm}, and their interaction; or observed π_{pv} to π_{pv} predicted from π_{osm} and ε (SMATR ANCOVA, all $P > 0·3$). These regression relationships and the osmometer measurements themselves are therefore robust across ecosystems with different water availabilities.
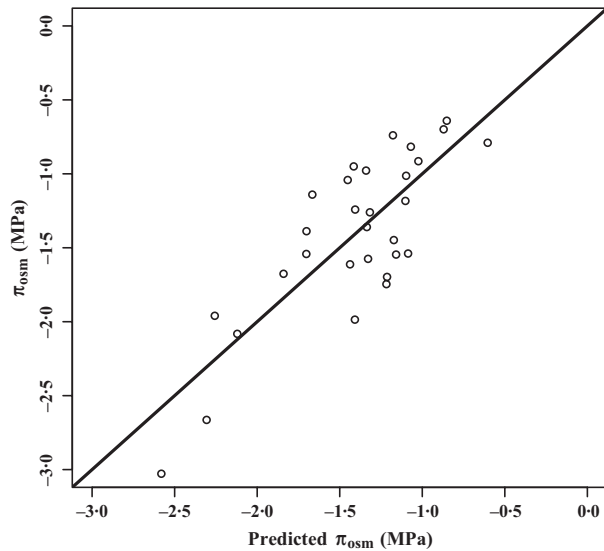
Fig. 3. Accounting for the discrepancy between measurement of osmotic potential at full turgor with a pressure-volume curve (π_{pv}) and that measured with osmometry (π_{osm}), as was seen in the departure of the data in Fig. 2 from the 1 : 1 line. This bias could be accounted for by the effects of apoplastic dilution and cell wall dissolution in the osmometry measurement. Here, π_{osm} predicted from π_{pv} using eqn 2, with leaf dry matter content as a proxy for cell wall investment was tightly correlated with measured π_{osm} with no bias (slope $\pm$ standard error $= 0.954 \pm 0.14$; $r^2 = 0.65$; $P < 2 \times 10^{-4}$). 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 (π_{osm}) were tightly correlated with p–v curve estimates of π_o (π_{pv}) and also π_{tlp}, with the π_{pv} estimation improved by including leaf density as a predictor, whereas the π_{tlp} estimation was independent of both leaf structure and habitat preferences. We propose our optimized osmometer method for determining π_o 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 π_o and compared them with expressed sap and p–v curve methods, but the largest previous study showed relationships of π_{osm} and π_{pv} 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 π_{osm} and π_{pv} for 30 species. Additionally, while previous studies have shown a correlation of π_{pv} with π_{tlp} (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 π_{osm} can be used to predict π_{tlp} as a rapid alternative to p–v curves.

Notably, π_{osm} and π_{pv} were tightly correlated but not equal. The π_{osm} was higher than π_{pv} for species with less negative
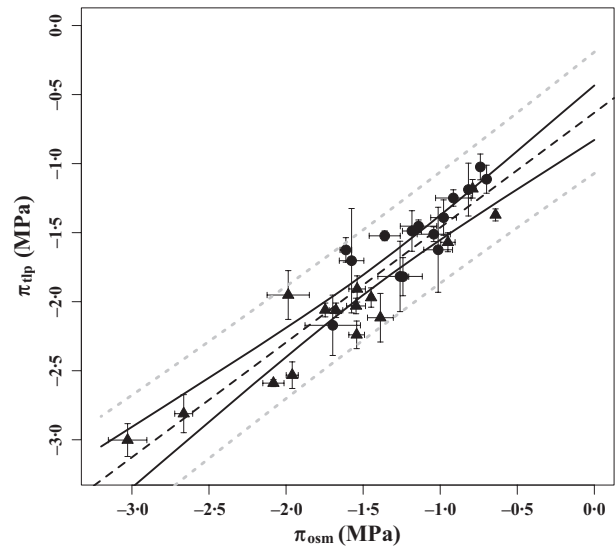
Fig. 4. The prediction of turgor loss point of pressure-volume curve analysis (π_{tlp}) using the osmotic potential at full turgor determined using an osmometer (π_{osm}) for species of a wide range of leaf structure and drought tolerance (circles = Xishuangbanna Tropical Botanical Garden species, triangles = University of California, Los Angeles species; see Table S1). The π_{osm} and π_{tlp} were strongly correlated ($r^2 = 0.86$; $P < 1 \times 10^{-12}$); fitted line is eqn 4. Black solid lines are 95% confidence intervals, grey dashed lines are 95% prediction intervals, and error bars represent standard errors.

values and lower than π_{pv} for species with more negative values. Our analysis indicated that this discrepancy may relate to both apoplastic dilution and wall solute enrichment. A high LDMC, which reflects the proportion of cell wall material in the leaf tissue, correlates across species with more negative π_o values, possibly because greater cell wall investment enables maintenance of a high relative water content at π_{tlp} 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 π_o, wall solute enrichment would play a more important role than apoplastic dilution, increasing the discrepancy between the two methods. However, the π_{osm} and π_{pv} were equivalent on average across species, and the discrepancies between the two methods were accounted for in our regression model

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

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

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

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 π_{tlp} from osmometer measurements:

$$\pi_{tlp} = 0{\cdot}832\pi_{osm} - 0{\cdot}631 \qquad\qquad \text{eqn 5}$$

This approach can be applied in other systems. This regression equation was highly significant ($r^2 = 0{\cdot}86$; $P < 2 \times 10^{-12}$) 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 π_{tlp} and π_{pv} 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 π_o and π_{tlp}. 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 π_o, ε, π_{tlp} and a_f, 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 π_o and π_{tlp} 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 π_o and π_{tlp} adjustment in conferring ecological drought tolerance. Notably, osmometer measurements had similar or lower standard errors for estimates of π_o for given species than p–v curves (paired *t*-test; $P = 0{\cdot}08$; $n = 30$). The osmometer is likely to have greater precision because it directly measures π_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 π_{tlp} and π_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 & Ackerly 2008). Notably, π_o and π_{tlp} are much better predictors of leaf drought tolerance than LMA, ρ, 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.

Acknowledgements

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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 (π_o) and at turgor loss point (π_{tlp}) 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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