Journal of Plant Hydraulics

INRAE

Journal of Plant Hydraulics 10: e-001

Resolving micro-scale water potential gradients within leaves

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Date of submission: 20.5.2024
Date of publication: 9.07.2024

Even the greatest experts in leaf water relations, and leaf physiology more generally, will admit to discomfort when considering how the estimation of important properties often relies on a melange of bulk-leaf measurements and inferential models. For example, the estimation of photosynthetic capacity and its variation across taxa and conditions is based on applying leaf gas exchange measurements to a Russian doll of nested models and assumptions, including those required to estimate stomatal conductance (Gaastra, 1959). The estimation of leaf hydraulic conductance and its xylem and outside-xylem components likewise depends on whole-leaf measurements and assumptions based on simplified models, including treating highly dispersed locations (the sites where water exits the leaf xylem, and where water potential during transpiration equals the value measured by a pressure chamber) as single, well-defined points.

Indeed, some of the most important open questions in leaf water relations involve differences in water potential at very small scales: What are the pathways of water movement distal to the leaf xylem? How are the resistances to water movement in these pathways regulated in response to dehydration and other environmental factors? Where precisely is water status "sensed" in the leaf and transduced into stomatal responses? What is the role of vapor transport in moving water through the leaf? Rigorous understanding and reliable prediction of leaf responses to environmental change require clear answers to these questions, which in turn require resolution of water potential gradients within intact, transpiring leaves: laterally (across the lamina), transdermally (among cell layers), among tissues (from bundle sheath to mesophyll to epidermis to guard cells), and across cell membranes (between mesophyll symplast and apoplast). Yet, research to date has relied on leaf-scale measurements and/or assumptions and computational models at best calibrated with anatomy.

One major question exemplifies how the understanding of diverse leaf processes, from stomatal biology to photosynthesis, ultimately rest on understanding of water relations at small scales: namely, What is the relative humidity of water vapor in the leaf intercellular airspaces (Buckley and Sack, 2019; Rockwell *et al.*, 2022)? Answering this question is critical to accurate estimation of leaf internal CO_2 concentration and stomatal conductance based on typical gas exchange measurements—which have previously assumed leaf airspace saturation, an idea that pervades the last 50 years of leaf ecophysiology literature, as well as current textbooks (e.g., Nobel, 2020). An approximately equivalent question is, What is the water potential of liquid water at the sites of evaporation adjacent to the leaf intercellular airspaces? This question has stymied experimental approaches. The reason is simply that it involves gradients of water potential at microscopic scales within leaves, locations that have eluded existing methods of direct experimental measurement. Even the largest scale relevant to water potential gradients outside the xylem – namely the scale of a leaf areole (the smallest vein-bounded leaf region) – is typically on the order of a few hundred micrometers in size, which is far smaller than any established non-disruptive method for directly measuring water potential. Worse still, it is possible (and likely, as discussed below) that extreme gradients of water potential do occur at far smaller scales, between the symplasm and adjacent apoplasm of mesophyll cells.

Tools exist for cell-scale measurements of some water relations parameters, including the cell pressure probe (Meidner & Edwards, 1975) and the nanoliter osmometer (Shackel, 1987), which can measure turgor pressure and osmotic pressure, respectively, of

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individual cells. However, these methods are extremely technically challenging, impossible to apply to cells in the leaf interior without disturbing the sensitive biophysical context in which micro-scale gradients of water potential and temperature occur, and difficult or impossible to apply in species with thick cuticles or dense trichome layers. Thus, although these methods have produced valuable insights about micro-scale water potential gradients in leaves, such insights are thin on the ground and poorly replicated across species.

Due to these methodological challenges, the best available methods to study micro-scale water potential gradients have, until recently, been heavily model-laden and empirically constrained only by bulk-leaf measurements. Early considerations focused on physical models and very simple computational models (reviewed by Tyree & Yianoulis (1980)). Based in part on that work, more recent spatially-explicit computational models of water movement in leaves have assumed that water potential is locally homogeneous; that is, large gradients in water potential do not occur at very small scales, such as across membranes (e.g., Rockwell *et al.,* 2014; Buckley *et al*., 2017). This assumption allows water flows in adjacent pathways (within cells, through cell walls, and through the intercellular airspaces) to be modeled using a single field for water potential, rather than two or more distinct fields reflecting sharp discontinuities in water potential between adjacent pathways (Scoffoni *et al*., 2023). Similarly, recent methods to quantify leaf intercellular airspace humidity (Cernusak *et al*., 2018; Wong *et al*., 2022) combine bulk-leaf measurements of gas exchange with mathematically elaborate, yet spatially simplified, models of processes in the leaf interior. For instance, the Cernusak method infers airspace relative humidity by assuming an initial value for this parameter – which is an input for a model of gas exchange and stable isotope discrimination for ^{18}O in CO_2 ($\delta^{18}O$) – and then adjusting it until the value of $\delta^{18}O$ predicted at the chloroplast surface agrees with the value inferred from measured whole-leaf discrimination. The Wong method is conceptually similar in that it forces two independent estimates of an unknown variable (in this case the CO_2 concentration in the intercellular airspaces near one surface of an amphistomatous leaf) to converge by adjusting the assumed relative humidity in the airspaces.

Both the Cernusak and Wong methods lead to inferred values of airspace relative humidity that imply very low water potential in the apoplast of mesophyll cells near the stomatal cavity: below -30 MPa in some cases (Cernusak *et al*., 2018). Given the apparent lack of turgor loss in the adjacent mesophyll cells, and the fact that turgor loss occurs at comparatively high bulk leaf water potentials (generally above -3 MPa; e.g., Bartlett *et al*., (2012)), those results suggest that extremely large water potential gradients – on the order of tens of megapascals – occur across the cell walls and/or the membranes of mesophyll cells near the stomatal cavity (Buckley & Sack, 2019). Those studies also suggested that the degree of unsaturation increases as the leaf-to-air vapor gradient increases. However, because both methods treat the leaf interior as, in effect, comprising a single exchange site and resistor for each diffusing gas species (water vapor and CO_2), by definition they cannot further resolve spatial gradients in water potential. Nor can they conclusively determine whether, as Wong *et al*. inferred, the intercellular humidity is close to saturation at locations deep within the leaf and declines steeply due to vapor-phase resistance through the mesophyll, or if instead the humidity is strongly unsaturated throughout the leaf airspaces.

Thus, although recent creative approaches have generated valuable insights and helped to sharpen the underlying questions about micro-scale leaf water relations, these and other established methods for resolving water potential gradients in leaves remain hamstrung by reliance either on models, on measurements that occur at too coarse a spatial scale, and/or on techniques that are exceedingly difficult (Table 1). A new experimental tool, AquaDust (Jain *et al*., 2021), has potential to circumvent these limitations, and to provide near-direct measurements of micro-scale gradients in apoplastic water potential in intact leaves. AquaDust contains FRET (Forster Resonance Energy Transfer) reporters – fluorescent dyes whose emission spectra depend on the distance between adjacent covalently-linked dye molecules. These dyes are embedded within hydrogel nanoparticles, which in turn are infiltrated into a leaf, where they settle on the outer surface of the apoplast of cells in the leaf interior and, presumably, equilibrate with the water potential in those locations. As apoplastic water potential increases or decreases, the hydrogel particles swell or shrink, respectively, altering the spacing of dye molecules. This leads to a relationship between emission spectrum and apoplastic water potential. Confocal fluorescence microscopy can then be used to map the spatial distribution of apoplastic water potential.

Published experiments using AquaDust (Jain *et al*., 2024a,b) confirm that mesophyll apoplastic water potential can be substantially lower than bulk-leaf water potential measured with the pressure chamber, and also lower than the bulk-leaf turgor loss point. For example, in tomato, apoplastic water potential near the transpiring abaxial surface was about -0.83 MPa when bulk leaf water potential was near the turgor loss point (-0.65 MPa) (Jain *et al*., 2024a); in maize, apoplastic water potential was about -2.8 MPa when bulk leaf water potential was about -1.3 MPa (Jain *et al*., 2024b). Moreover, the drawdown of water potential below that of the leaf xylem was 3-5 times greater for the apoplast than for the bulk leaf. Assuming bulk-leaf water potential largely reflects the condition of water in the mesophyll symplast, these results imply a very large

resistance between the symplast and the evaporating site in the apoplast – either across the mesophyll cell membrane, across the cell wall matrix itself, or both (Buckley and Sack, 2019). This finding is consistent with previous inferences, based on whole-leaf approaches, that transmembrane resistances distal to the xylem can be both large and sensitively responsive to environmental conditions (e.g., Scoffoni *et al*., 2017).

AquaDust results published to date have not reported apoplastic water potentials low enough to confirm dramatic unsaturation of the leaf intercellular airspaces; the lowest reported apoplastic water potential, which occurred in maize, was about -3.75 MPa (Jain *et al*., 2024b), which is equivalent to relative humidity of about 97.3%. However, more recent AquaDust experiments, not yet published, have indeed confirmed severe unsaturation on par with the findings of Wong *et al*. and Cernusak *et al*. (Abe Stroock, personal communication, 05 May 2024).

AquaDust has an unprecedented combination of features that make it uniquely well-suited for resolving micro-scale water potential gradients in intact, transpiring leaves (Table 1). For one, its use can be minimally disruptive. AquaDust might be expected to alter gas phase water relations at a local scale by interfering with vapor exchange at the outer surface of the apoplast; however, any such effects are apparently negligible, given that infiltration with AquaDust has no discernible direct effect on either gas exchange rates or stomatal conductance (Jain *et al*., 2021, 2024b,a). Infiltration does cause localized mechanical damage to the cuticle, but the damaged area is small and can be avoided during measurement by only interrogating unaffected areas. The minimal disruption caused by AquaDust contrasts greatly with the pressure chamber (which stops transpiration entirely, collapsing all water potential gradients in the leaf), and to some degree even with the method of Wong *et al*. for inferring airspace unsaturation (which requires reducing $[CO_2]$ to the compensation point at one surface). Inferring apoplastic water potential using AquaDust also requires few assumptions – only that the material comes to chemical potential equilibrium with the water in the apoplast, and that the calibration of emission spectrum vs water potential is robust. And finally, AquaDust provides the prospect of similar spatial resolution as the cell pressure probe and nanoliter osmometer, but with far more extensive coverage and without disrupting native in vivo conditions.

At present, the demonstrated spatial resolution of water potential measurements using AquaDust depends on which tissue gradient and spatial axis one considers. Comparing across tissues, the spatial resolution corresponds to the minimum bulk tissue volume in which the substance's emission spectrum has been examined in results reported thus far; this volume corresponds to the area of leaf surface that is interrogated using the fiber optic point probe for quantifying emission spectrum (ca. 10 mm^2), and the depth of tissue that dominates emission from AquaDust following excitation (ca. 25-30 µm; cf. Figure 2 in Jain *et al*. 2024b). Thus, the resolution is "micro-scale" with respect to depth within the leaf (below the leaf surface), but not with respect to position along the leaf surface. When comparing different water compartments, however, the resolution is well below a micron: because AquaDust localizes to the apoplast, it effectively interrogates water potential in a region that is only a few hundred nanometers thick, while being unaffected by water potential in the immediately adjacent symplastic zones. Moreover, techniques exist to interrogate emission spectra at micron scales using confocal microscopy; such 'spectral imaging' methods could be applied with AquaDust to quantify gradients in apoplastic water potential at micron scales.

The ecophysiology community urgently needs a resolution to the question of airspace unsaturation, and preferably a resolution that will enable other investigators to confidently infer stomatal conductance from traditional gas exchange measurements. More generally, we need methods to resolve the three-dimensional distribution of water potential in intact, transpiring leaves, to address a range of other questions about hydraulic and stomatal function (Buckley, 2019; Earles *et al*., 2019; Scoffoni *et al*., 2023). For example, such methods could help clarify precisely where in the leaf dehydration leads to observed declines in leaf hydraulic conductance (K_{leaf}) (Scoffoni *et al*., 2017), and where loss of tissue volume during dehydration is transduced into signals that lead to stomatal closure (Sack *et al*., 2018). To promote experimental progress on these and related topics using AquaDust, we strongly recommend (1) that the investigators who have developed AquaDust make the material widely available – possibly on a (compensated) contract basis, or through a broader commercialization effort – so that other scientists can confirm and extend these findings in other species and conditions, and (2) that AquaDust be directly combined with other approaches, on the same leaf, to cross-validate the methods, including (3) existing methods based on gas exchange (Cernusak *et al*., 2018; Wong *et al*., 2022), (4) within-cell water potential measurements, using intracellular protein biosensors (Cuevas-Velazquez *et al*., 2021), and (5) leaf water transport models that are explicitly resolved at the fine structural scale needed to allow inference of symplastic-apoplastic water potential gradients, analogous to that already achieved for roots (e.g., MECHA; Couvreur *et al*., 2018).

Method → *Measure of suitability ↓*	transport models[1]	stable isotopes[2]	2-surface gas exchange[3]	pressure chamber	thermocouple psychrometer	P probe + nL osmometer	AquaDust
freedom from assumptions	1	3	4	5	4	5	5
spatial resolution	4	3	3	1	2	5	3-5
non-disruptiveness	5	5	4	1	1	2	5
feasibility/ ease of use	4	3	4	5	5	1	3
TOTAL SCORE	14	14	15	12	12	13	**16-18**

Table 1. Qualitative comparison of different methods for resolving water potential gradients in leaves.
Methods are listed in order of increasing freedom from assumptions. Scores are given on a scale of 1 – 5, with 5 being best (most free from assumptions, finest spatial resolution, least disruptive, easiest to use). [1]e.g., Buckley et al. (2017), Rockwell et al., (2014); [2] Cernusak et al., (2018); [3] Wong et al., (2022).

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