Video Article

Measurement of Leaf Hydraulic Conductance and Stomatal Conductance and Their Responses to Irradiance and Dehydration Using the Evaporative Flux Method (EFM)

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Abstract

Water is a key resource, and the plant water transport system sets limits on maximum growth and drought tolerance. When plants open their stomata to achieve a high stomatal conductance (g_s) to capture CO_2 for photosynthesis, water is lost by transpiration[1,2]. Water evaporating from the airspaces is replaced from cell walls, in turn drawing water from the xylem of leaf veins, in turn drawing from xylem in the stems and roots. As water is pulled through the system, it experiences hydraulic resistance, creating tension throughout the system and a low leaf water potential (Ψ_{leaf}). The leaf itself is a critical bottleneck in the whole plant system, accounting for on average 30% of the plant hydraulic resistance[3]. Leaf hydraulic conductance (K_{leaf} = 1/ leaf hydraulic resistance) is the ratio of the water flow rate to the water potential gradient across the leaf, and summarizes the behavior of a complex system: water moves through the petiole and through several orders of veins, exits into the bundle sheath and passes through or around mesophyll cells before evaporating into the airspace and being transpired from the stomata. K_{leaf} is of strong interest as an important physiological trait to compare species, quantifying the effectiveness of the leaf structure and physiology for water transport, and a key variable to investigate for its relationship to variation in structure (*e.g.*, in leaf venation architecture) and its impacts on photosynthetic gas exchange. Further, K_{leaf} responds strongly to the internal and external leaf environment[3]. K_{leaf} can increase dramatically with irradiance apparently due to changes in the expression and activation of aquaporins, the proteins involved in water transport through membranes[4], and K_{leaf} declines strongly during drought, due to cavitation and/or collapse of xylem conduits, and/or loss of permeability in the extra-xylem tissues due to mesophyll and bundle sheath cell shrinkage or aquaporin deactivation[5-10]. Because K_{leaf} can constrain g_s and photosynthetic rate across species in well watered conditions and during drought, and thus limit whole-plant performance they may possibly determine species distributions especially as droughts increase in frequency and severity[11-14].

We present a simple method for simultaneous determination of K_{leaf} and g_s on excised leaves. A transpiring leaf is connected by its petiole to tubing running to a water source on a balance. The loss of water from the balance is recorded to calculate the flow rate through the leaf. When steady state transpiration (E, mmol • m^{-2} • s^{-1}) is reached, g_s is determined by dividing by vapor pressure deficit, and K_{leaf} by dividing by the water potential driving force determined using a pressure chamber (K_{leaf}= E /- $\Delta\psi_{leaf}$, MPa)[15].

This method can be used to assess K_{leaf} responses to different irradiances and the vulnerability of K_{leaf} to dehydration[14,16,17].

Video Link

The video component of this article can be found at http://www.jove.com/video/4179/

Protocol

1. Measuring Leaf Hydraulic Conductance for Hydrated Leaves (K_{max})

1. Collect shoots preferably in the evening or night such that xylem tensions are low, cut in air and place in dark plastic bag filled with wet paper towels. In the lab, recut at least two nodes from the end of every shoot under water. Let shoot rehydrate overnight in pure water, or other solution to be used for K_{leaf} measurements. Ultrapure water is recommended as a flow solution unless the impacts of different solutions are to be investigated as a factor.
2. Cut the leaf from the shoot with a fresh razor blade under partially degassed flow solution. Ultrapure water is recommended as a flow solution unless the impacts of different solutions are to be investigated as a factor. We recommend degassing the solution overnight in a flask using a vacuum pump, but other methods may be employed.
3. Wrap pre-stretched parafilm around the petiole to ensure a good seal between petiole and tubing. This step is especially helpful for non-round petioles, as wrapping extra parafilm will help round the petiole to fit inside the tubing. Dental paste can be applied to the petiole before wrapping the parafilm to fill grooves in petioles.

4. Connect the leaf to silicone tubing under ultrapure water to prevent air entering the system. As the leaf is lifted above the source of water, the water in the tubing is under subatmospheric pressure, and thus the seal is good if no air is drawn into the tubing during the measurement. Alternatively to sealing the petiole directly into silicone tubing, a compression fitting with rubber gasket can be used (*e.g.*, (Omnifit A2227 bore adaptor; Omnifit, Cambridge, UK); this approach is useful for petioles with especially complex shapes, or for grass blades, which can be wrapped around a glass rod and sealed with the compression fitting. When feasible, sealing the petiole directly into silicone tubing has the advantage of being rapid and leaving a petiole-tubing junction that is transparent, allowing bubbles to be more easily seen. The tubing connects the leaf to hard tubing running to a graduated cylinder on a balance (±10 µg resolution), which logs data every 30 sec to a computer (*e.g.* using "Balance Link" Software; Mettler-Toledo GmbH, Greifensee, Switzerland) for the calculation of flow rate through the leaf.
5. Leaves should be held adaxial surface upwards in wood frames strung with fishing line above a large box fan and under a light source (> 1,000 µmol • m^2 • s^{-1} photosynthetically active radiation for high-irradiance measurements), slightly above the level of the meniscus in the graduated cylinder. The leaf should be placed above the water in the graduated cylinder to ensure that water moving to the leaf is driven by transpiration, and not entering the leaf under pressure due to gravity, as would be the case if the leaf were placed below the meniscus of the water in the cylinder. Small containers filled with wet paper towel are placed inside the balance chamber to ensure the air in the balance is water-saturated, thus avoiding evaporation from the water source to the atmosphere in the balance. This possibility can be checked by halting flow into the leaf using a four-way stopcock valve in the tubing, and checking that the mass of the water cylinder on the balance does not decline.
6. Place a clear Pyrex container filled with water above the leaf to absorb the heat of the lamp.
7. Maintain leaf temperature between 23 °C and 28 °C throughout the experiment by adding more cold water to the Pyrex container such that the heat lamp does not heat the Pyrex container sufficiently to warm the air around the leaf below.
8. Allow the leaf to transpire on the apparatus for at least 30 min, thus giving sufficient time to acclimate to high irradiance, and additionally until flow rate has stabilized, with no upward or downward trend, and with a coefficient of variation <5% for at least 5-10 measurements made at 30 sec flow intervals. It is essential for the flow rate to reach a steady state because this method assumes a stable Ψ_{leaf}. Previous studies found these criteria to be sufficient for stabilization of flow rate (E), Ψ_{leaf} and K_{leaf}; tests with longer measurement periods after stable flow was established showed no relationship of K_{leaf} to measurement time for each of seven species of a wide range of leaf capacitance[17,18] . Stabilization generally takes 30 min or less, but in some species may require more than an hour for some species.

***Note**: When flow rate is very low (<8 µg s^{-1}), stability can be determined using the running average of the last five 30 sec intervals.*

9. Discard measurements if the flow suddenly changes, either due to sudden stomatal closure, or apparent leakage from the seal or blockage in the system by particles or air bubbles.
10. When flow stabilizes, record leaf temperature with a thermocouple.
11. Quickly remove the leaf from the tubing, dab dry the petiole and place the leaf into a sealable bag which had been previously exhaled in, to halt transpiration.
12. Let the leaf equilibrate in its bag for at least 20 min.
13. Average the final 10 flow rate measurements. This will be your E measurement.
14. When the leaf is equilibrated, measure the final leaf water potential (Ψ_{final}) with a pressure chamber.
15. Measure leaf area with a flatbed scanner and image processing software, or with a leaf area meter.
16. K_{leaf} is calculated as E / -$\Delta\Psi_{leaf}$ (where $\Delta\Psi_{leaf} = \Psi_{final}$ - 0 MPa) and further normalized by leaf area. Units are in mmol m^{-2} s^{-1} MPa^{-1}. Because the leaf is positioned just above the level of water in the graduated cylinder, the effect of gravity in reducing the pressure of water entering the leaf would be negligible, especially relative to the Ψ_{final}, which is always > -0.1 MPa.
17. To correct for changes in K_{leaf} induced by temperature dependence of water viscosity[15,19,20] , standardize K_{leaf} values to 25 °C by using the following equation:

$$K_{\mathrm{leaf}}(25^{\circ}\mathrm{C}) = \frac{K_{\mathrm{leaf}}(t)}{\left(0.88862 \times \left(\frac{1}{10^{\left((1.3272 \times (20-t) - 0.001053 \times (t-20)^2)/(t+105)\right)}}\right)\right)}$$

18. Stomatal conductance (g_s) can be determined as E divided by the leaf-to-air vapor pressure difference, equivalent to the vapor pressure deficit (VPD), determined using a temperature and relative humidity sensor adjacent to the leaf. VPD is calculated using the following equation[21]

$$VPD = \left(1 - \frac{RH}{100}\right) \times \left(\frac{VP}{AP}\right)$$

where RH is the relative humidity, VP is the saturation vapor pressure of the air (in kPa; a function of temperature, according to the Arden Buck equation[22]), and AP the atmospheric pressure (in kPa).

2. Measuring Leaf Hydraulic Conductance for Dehydrated Leaves

1. Collect shoots preferably in the evening or night such that xylem tensions are low, cut in air and place in dark plastic bags filled with wet paper towels. In the lab, recut at least two nodes from the end of every shoot under water. Let shoot rehydrate overnight in pure water, or other solution to be used for K_{leaf} measurements. Ultrapure water is recommended as a flow solution unless the impacts of different solutions are to be investigated as a factor.
2. Cut shoots into segments with at least three leaves under ultrapure water and dehydrate shoots with a fan for different periods of time to a range of Ψ_{leaf} values.
3. Once shoots are dehydrated, place sealable bags (Whirl-Pak; Nasco, Fort Atkinson, WI, USA) which have been previously exhaled in) around each leaf of the shoot, and then place the whole shoot in a large sealable bag with wet paper towel.
4. Let the shoot equilibrate for at least 30 min (for strongly dehydrated shoots longer equilibration times may be necessary).

5. After equilibration, excise the top and bottom leaf and measure initial Ψ_{leaf} (=Ψ_o) using a pressure chamber.
6. Discard the shoot if the difference in the Ψ_{leaf} of those two leaves is greater than 0.1 MPa (for strongly dehydrated leaves, this range can be extended to 0.3 MPa).
7. The third leaf is then used to determine K_{leaf} and g_s with the EFM, following steps 1.2 -1.18 above. Notably, steady state E can be obtained even for leaves that have been severely dehydrated leaves, as stomata still open and generate g_s values substantially higher than cuticular conductance[17].
8. To construct hydraulic vulnerability curves, calculate K_{leaf} using the Ψ_{final} as in step 1.16, and then plot K_{leaf} values against whichever is lowest, Ψ_o or Ψ_{final}, *i.e.*, the strongest dehydration experienced by the leaf during the experiment (=Ψ_{lowest}). Similarly, the decline of g_s in response to leaf dehydration can be determined by plotting g_s against Ψ_{lowest}. Previous work has shown that dehydration induces at least partially irreversible declines in K_{leaf} and g_s[14,16].
9. We recommend obtaining at least 6 K_{leaf} values per 0.5 MPa interval of Ψ_{lowest} (this interval can be reduced to 0.25 MPa if the species you are working with is really vulnerable to dehydration).
10. Perform Dixon outlier test on K_{leaf} values for each 0.5 MPa water potential interval of Ψ_{lowest} to remove outliers from your vulnerability curve[23].
11. Because species differ in their responses of K_{leaf} and g_s to dehydration[14,16], fit different functions for each species and select the maximum likelihood (lowest AIC value; see [14] for details). We recommend fitting at least four functions previously used in the literature: linear ($K_{leaf} = a\,\Psi_{leaf} + y_o$), sigmoidal ($K_{leaf} = \frac{a}{1+e^{-\left(\frac{\Psi_{leaf}-x_0}{b}\right)}}$), logistic ($K_{leaf} = a/(1+(\frac{\Psi_{leaf}}{x_0})^b)$) and exponential ($K_{leaf} = y_0 + ae^{-b\Psi_{leaf}}$).

3. Representative Results

The decline of leaf hydraulic conductance with dehydration varies strongly across species (**Fig. 1**). Drought sensitive species experience a stronger decline at less negative water potentials than more drought tolerant species, when measured under high irradiance (PAR > 1,000 µmol • m^{-2} • s^{-1}). The water potential at 80% loss of hydraulic conductance (P_{80}) for mesic herb *Helianthus annuus* was 3 MPa less negative than that of *Heteromeles arbutifolia*, native to California chaparral. *Heteromeles arbutifolia* responded linearly to the decline in water potential, whereas *Helianthus annuus* showed a non-linear decline.

Both stomatal conductance (g_s) and leaf hydraulic conductance (K_{leaf}) can also respond dramatically to irradiance (**Fig. 2**). For *Raphiolepis indica*, the stomatal conductance declined strongly with dehydration for leaves measured under high irradiance (PAR > 1,000 µmol • m^{-2} • s^{-1}) and under lab light (PAR < 6 µmol • m^{-2} • s^{-1}). Under high irradiance, leaves measured with the EFM that had been dehydrated to the water potential that is typical for well-lit leaves on intact plants at mid-day showed similar g_s as for leaves measured on intact plants with a porometer (star in **Fig. 2**). Notably, for leaves at maximum hydration, K_{leaf} was four-fold higher for leaves exposed to high than low irradiance (11 vs 2.8 mmol m^{-2} s^{-1} MPa^{-1}), and therefore K_{leaf} showed a stronger light response in well hydrated leaves than g_s. Further, under high irradiance, K_{leaf} declined more strongly with dehydration. The P_{80} was 0.93 MPa less negative under high than low irradiance.

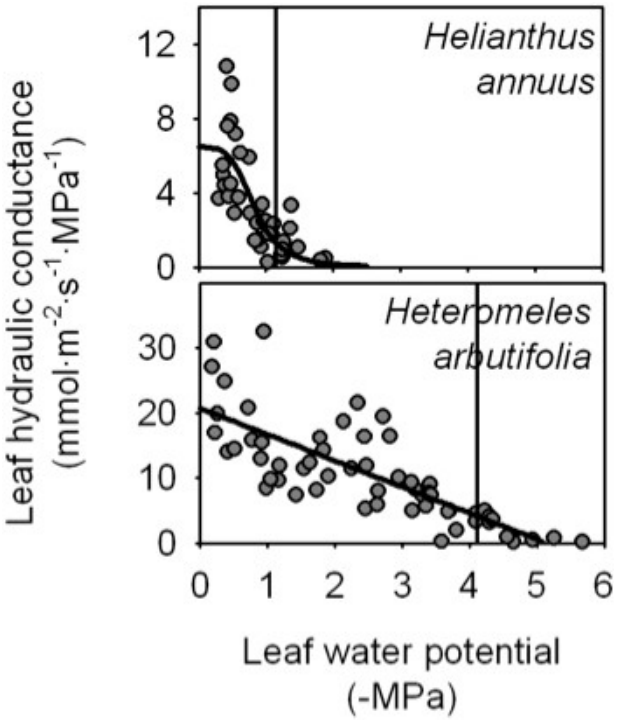

Figure 1. Leaf hydraulic vulnerability curves for a drought sensitive (top) and a drought tolerant (bottom) species (from [14,24]). Each point represents a single measurement of leaf hydraulic conductance (K_{leaf}) on a detached leaf using the evaporative flux method (EFM). The vertical bars represent the water potential at which the species lost 80% of initial leaf hydraulic conductance (P_{80}). Best fit functions using maximum likelihood are plotted for both species.

$$Helianthus\ annuus\ K_{\mathrm{leaf}}\ 6.5/(1\left(\frac{\Psi_{\mathrm{leaf}}}{0.83}\right)^{4.15})(n=39)$$

$$\text{and } Heteromeles\ arbutifolia\ K_{\mathrm{leaf}}=-4.02\ \Psi_{\mathrm{leaf}}\ +20.7(n=61)$$

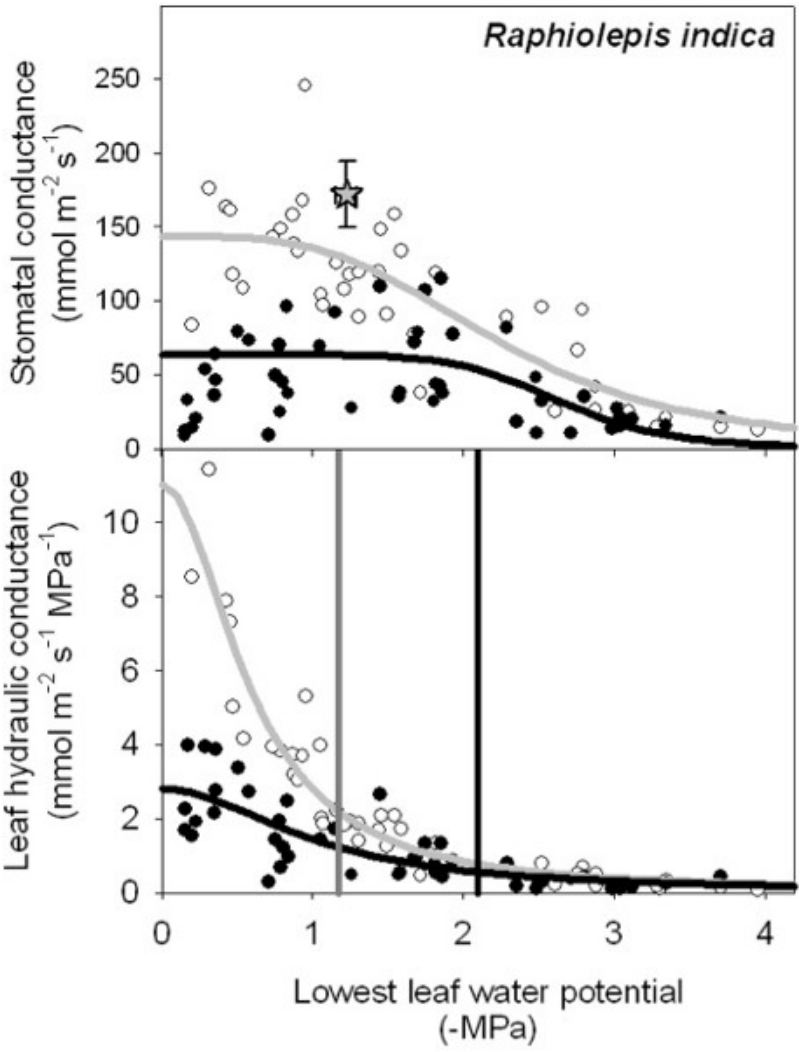

Figure 2. The response of stomatal conductance (g_s) and of leaf hydraulic conductance (K_{leaf}) to declining leaf water potential for *Raphiolepis indica* measured under high vs low irradiance (from [16]). (PAR > 1,000 and < 6 mmol • m^{-2} • s^{-1}). Each point represents a single measurement on a detached leaf using the evaporative flux method (EFM), with white and black dots representing leaves measured under high and low irradiance respectively. Equations for stomatal conductance curves in high vs low irradiance: $K_{leaf} = 11/(1 + (\frac{\Psi_{leaf}}{0.59})^{2.03})$ (n = 42) and $K_{leaf} = 2.8/(1 + (\frac{\Psi_{leaf}}{1.03})^{1.96})$ (n = 47) and $g_s = 63/\left[1 + (\frac{\Psi_{leaf}}{2.7})^{7.3}\right]$ $(n = 35)$. Equations for vulnerability curves in high vs. low irradiance: $K_{leaf} = 2.8/(1 + (\frac{\Psi_{leaf}}{1.03})^{1.96})(n = 47)$ and $K_{leaf} = 11/(1 + (\frac{\Psi_{leaf}}{0.59})^{2.03})$. The grey star in the upper panel represents the mean ± standard error for g_s and leaf water potential measured on leaves of intact plants at mid-day with a porometer. Grey and black vertical bars in the lower panel represent the water potential at which the species lost 80% of initial leaf hydraulic conductance (P_{80}) under high and low irradiance respectively.

Discussion

The evaporative flux method presented here allows relatively rapid (30 min) determination of leaf hydraulic conductance in the laboratory with simultaneous measurement of stomatal conductance.

The EFM is so far the method that most closely follows the natural pathway of water in leaves, given that water evaporates in the leaf airspaces and diffuses from the stomata[15]. A number of other experimental methods for K_{leaf} determination exist[25], with three being especially common. (1) In the high pressure flow method (HPFM), water is pushed through the leaf under high pressure[15,26] . However, vulnerability curves cannot be obtained with this method because applied positive pressures may refill emboli and rehydrate mesophyll tissue. (2) In the rehydration kinetics method (RKM), K_{leaf} is calculated using the analogy between the rehydration of dehydrated leaves with the charging of a capacitor in series with a resistors [27,28]. This method is rapid, but does not reproduce the entire system of natural transpirational pathways in the leaf and is not easily modified to allow measurement of leaves acclimated to high irradiance. (3) In the vacuum pump method (VPM) the leaf is placed in a

chamber, its petiole connected to a water source on a balance, while vacuums of different intensities are applied to the leaf driving water loss from the leaf[15,26] . This method can be time consuming. Measurements using all three methods have been found to give consistent results[15,28,29] , and thus the EFM is frequently used given its being based on transpirational water movement, and its relative rapidity, and its modification for measuring K_{leaf} under different conditions.

All of the typical methods used for determining K_{leaf} involve a level of uncertainty in quantifying accurately the driving force corresponding to transpiration[25]. In the EFM, the $\Delta\Psi_{leaf}$ used to calculate K_{leaf} is lower than the true driving force, as it is based on the bulk Ψ_{leaf} after the leaf is equilibrated for the pressure bomb. At this point, the bulk Ψ_{leaf} does not necessarily represent the water potential of the cells at the end of the hydraulic pathway, where the water evaporates, but rather a volume-weighted average for the relatively few cells of low water potential where water is moving and evaporating, and for the more numerous cells of high water potential that may be isolated from the transpiration stream. How much the Ψ_{leaf} differs from the true driving force thus depends on where the water is evaporating in the leaf. If water evaporates throughout the leaf, the bulk Ψ_{leaf} may be close to the true driving force, and if water evaporates only from relatively few cells near the stomata, the the bulk Ψ_{leaf} may substantially underestimate the true driving force, *i.e.*, the water potential of those cells may in fact be much lower, and thus actual K_{leaf} would be lower than that determined with the EFM. Thus, the degree that K_{leaf} determined by the EFM represents the true K_{leaf} is sensitive to the water flow pathways, which are still not well understood. While the EFM provides data that match those of other methods for measuring K_{leaf} (see above), and is excellent for comparative use, the possibility of species-variation in water flow pathways through the mesophyll needs further investigation. Indeed, even for leaves of given species, these water pathways might vary under different conditions, *e.g.*, under different irradiances and heat loads[4,30] or of different water status (given tissue shrinkage)[31]. Further knowledge of the water transport pathways would thus allow the EFM to be used to resolve the effects of the changing pathways on overall leaf hydraulic transport.

The measurement of stomatal conductance in the EFM has the advantages of allowing determination of responses to irradiance and dehydration, and providing matched responses of g_s with K_{leaf}. Additionally, the effect of previous leaf dehydration followed by rehydration on g_s can be assessed with this method. However, this method has two potential drawbacks. First, this g_s measurement is made on an excised leaf, rather than on an intact plant, and second, the determination of stomatal conductance is based on climate measurement some distance from the leaf, rather than close to the leaf as in a porometer chamber, though the ventilation of leaf with the fan equilibrates the leaf surface with surrounding air. For some species, excised leaves may show different stomatal responses than for leaves on an intact plant [16,32] . However, for several species g_s estimated using the EFM was similar to values measured on intact plants with a porometer for leaves at mid-day Ψ_{leaf}[16] (**Fig. 2**).

The EFM can be applied for physiological insight, *i.e.*, investigating the dynamics of K_{leaf}, its basis in structure and anatomy, and its responses to environmental factors. Additionally, the method can be used to compare plants of given species grown under different conditions, or of different ages, or to compare plants of different species adapted to different environments.

By constructing leaf vulnerability curves, one can gain insight into the mechanisms of species tolerances to drought. During drought, cavitation and cell collapse have been shown to occur in the leaf xylem of some species, and cell shrinkage in the mesophyll during dehydration could also impact water movement (reviewed in [14]). Future studies will be needed to determine the relative importance of each of these processes in driving the decline of K_{leaf} and stomatal closure.

Irradiance is also a strong factor influencing K_{leaf} and the EFM is highly suited for measurements under high irradiance. The combined response of aquaporins to irradiance and drought should gain much interest. High irradiance leads to increased expression and/or activation of aquaporins that permit faster water flow through living cells, increasing K_{leaf}, whereas dehydration leads to reduced expression and/or de-activatation of aquaporins, reducing K_{leaf}. The combined impacts of irradiance and water supply on K_{leaf} require further investigation[4,10,16,33] .

Further, across species, K_{leaf} and its vulnerability has been related to species distributions with respect to the supply of light water and other resources[3,13,24] . Species from dry areas tend to show greater resistance to hydraulic decline, facing more negative soil water potentials, and by maintaining a high K_{leaf} at more negative water potentials, they can maintain high g_s and continue capturing CO_2 for photosynthesis[13,14]. Thus, the study of leaf hydraulic properties provides insights at the level of cell, leaf, and whole plant and its responses to the environment, and is likely to yield numerous important new discoveries at all scales.

Disclosures

No conflicts of interest declared.

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