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TECHNICAL FOCUS

Improving xylem hydraulic conductivity measurements by correcting the error caused by passive water uptake

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Xylem hydraulic conductivity (K) is typically defined as K = F/(P/L), where F is the flow rate through a xylem segment associated with an applied pressure gradient (P/L) along the segment. This definition assumes a linear flow–pressure relationship with a flow intercept (F_0) of zero. While linearity is typically the case, there is often a non-zero F_0 that persists in the absence of leaks or evaporation and is caused by passive uptake of water by the sample. In this study, we determined the consequences of failing to account for non-zero F_0 for both K measurements and the use of K to estimate the vulnerability to xylem cavitation. We generated vulnerability curves for olive root samples (*Olea europaea*) by the centrifuge technique, measuring a maximally accurate reference K_{ref} as the slope of a four-point F vs P/L relationship. The K_{ref} was compared with three more rapid ways of estimating K. When F_0 was assumed to be zero, K was significantly under-estimated (average of −81.4 ± 4.7%), especially when K_{ref} was low. Vulnerability curves derived from these under-estimated K values overestimated the vulnerability to cavitation. When non-zero F_0 was taken into account, whether it was measured or estimated, more accurate K values (relative to K_{ref}) were obtained, and vulnerability curves indicated greater resistance to cavitation. We recommend accounting for non-zero F_0 for obtaining accurate estimates of K and cavitation resistance in hydraulic studies.

Introduction

Studies on hydraulic conductivity (K) in plants are crucial for understanding water uptake and water transport in plants. During the past two decades, many studies have been published regarding this topic in roots, stems and leaves (Tausend et al. 2000, Martinez-Vilalta and Pinol 2002, Domec et al. 2006, Woodruff et al. 2008). Hydraulic conductivity is frequently measured on excised segments from these organs, where K is defined as the volume or mass flow rate of a solution through the segment (F) divided by the applied pressure gradient driving the flow (P/L): K = F/(P/L) (Tyree and Zimmermann 2002). This definition assumes that F and P/L are directly proportional with a flow intercept (F_0) of zero (Fiscus et al. 1983). Although the F by P/L relationship is typically linear for xylem (Kolb et al. 1996), the F_0 is usually non-zero because there is measurable 'passive' flow at P/L = 0. Although $F_0 <> 0$ can arise from leaks in the system or evaporation from the sample or balance reservoir (if F is measured gravimetrically), it typically persists in the absence of these causes, and it may be associated with osmotic uptake by cells (associated with cell wall creep) and capillary uptake by the apoplast, including the refilling of embolized xylem vessels (Taneda and Sperry 2008).

Abbreviations – HPFM, high pressure flow meter; OLS, ordinary least square; PLC, percentage loss of conductivity; SE, standard error.

This paper does not concern the cause of significant F_0, but rather its consequences for the accuracy of K measurements in xylem segments. Some authors have not taken F_0 into account, assuming that $F_0 = 0$ (Cordero and Nilsen 2002, Trifilò et al. 2008, Chen et al. 2010). Others measure F_0 and subtract it from F to obtain the net flow rate ($F_n = F - F_0$), whose value is divided by P/L to derive K (Wheeler et al. 2005, Taneda and Sperry 2008, Hacke et al. 2010). This method assumes a linear F by P/L relationship, and a non-shifting F_0 that is independent of P/L. Often, F_0 stability was confirmed by measuring F_0 before and after the F measurement. Still other authors account for F_0 by measuring the linear F by P/L relationship at multiple P/L values and deriving K directly from the slope. The slope method is most commonly used when entire root or shoot systems are measured using the high pressure flow meter (HPFM; Tyree et al. 1994, Tsuda and Tyree 2000, Bogeat-Triboulot et al. 2002), vacuum chambers (Kolb et al. 1996) or pressure chambers (Fiscus et al. 1983). The importance of taking into account F_0 when measuring K on xylem segments has not been quantified. Errors in segment K could also impact vulnerability curves. These curves describe the loss of segment K as the xylem cavitates from exposure to more negative xylem pressure (Cai and Tyree 2010).

The aim of this study was to evaluate the effect of F_0 on K values of olive root segments, and on the derived vulnerability curves obtained by the centrifuge technique (Pockman et al. 1995, Alder et al. 1997). We used four different methods to estimate K, all of them are based on gravimetric flow measurements as described by Sperry et al. (1988). In this method, a sensitive (0.01 mg resolution) electronic balance allows direct measurement of F_0 as well as the slope of the F vs P/L relationship. The results, however, are equally relevant if F is being measured with a high-resolution flow meter (e.g., Cochard et al. 2000, Espino and Schenk 2011).

Materials and methods

Measurements were made in Olive (*Olea europaea* cv. 'Arbequina') root samples from an experimental orchard near Seville, southwest Spain. Roots of approximately 20 cm in length and 3–4 years in age from fully irrigated trees were sampled on October 4, 2010, wrapped in plastic bags with wet paper towel inside, properly packed and transported to the laboratory of Prof. J. S. Sperry, University of Utah, USA. Once in the lab, six roots were re-cut at approximately 14 cm long and vacuum infiltrated in 20 m*M* KCl degassed solution for 1 h to promote embolism removal. Maximum K

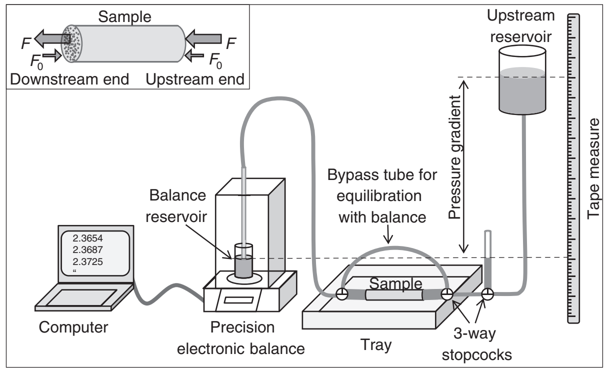

Fig. 1. Tubing apparatus for measuring hydraulic conductivity. Water flows from an upstream reservoir through the xylem sample and into a downstream reservoir on an electronic balance interfaced with a computer. To zero the hydraulic head, the upstream reservoir is switched to the short standpipe, and the bypass is opened to equilibrate the balance reservoir and standpipe. After closing the bypass again, the passive flow rate of water (F_0) was measured. Insert at upper left shows the uni-directional flow under pressure (F) and the bi-directional passive flow that occurs in the absence of any pressure difference.

(K_{max}) was measured after Sperry et al. (1988) by connecting the samples to a tubing system (illustrated in Fig. 1) and measuring the flow rate of filtered (0.2 µm) 20 m*M* KCl solution into a covered reservoir placed on a precision electronic balance (Sartorius, Göttingen, Germany; 0.01 mg resolution). Samples were covered with damp paper towels during the F measurement to prevent water loss from their surface. The hydraulic head was manipulated by changing the height of an upstream reservoir relative to the downstream balance reservoir, with the sample itself typically being 2–4 cm below the level of the downstream balance reservoir (Fig. 1).

The measurement protocol started with measuring the initial F_0 at no hydraulic head (P/L = 0). To insure P/L = 0, the heights of the upstream and downstream reservoirs were equalized by opening a tubing bypass (Fig. 1, upstream reservoir turned to short standpipe). After pressure equilibration, the bypass was shut off and F_0 was measured with the solution being free to flow between the sample and the equilibrated reservoirs (Fig. 1, insert). Flow rate at the downstream balance reservoir was measured at 7 s intervals, with a running mean calculated for the five most recent intervals. Typically, the F_0 was negative, indicating passive uptake of water by the sample. The running means generally showed an initial trend towards becoming less negative, and we waited until the values achieved stability before selecting a value. After this initial F_0 measurement, we switched the upstream reservoir to a hydraulic head of approximately 4 kPa and measured F. Then, the upstream reservoir was raised to a higher hydraulic head of approximately 5.5 kPa and F measured again. The entire time for one point depended on how long the running mean took to achieve a stable value. These pressures were small enough to avoid displacing air from conduits that were cut open at both ends. A certain proportion of such conduits is expected since xylem vessels in olive roots can be longer than 14 cm (Trifilò et al. 2007). After the two pressurized F readings, the head was re-zeroed and the F_0 determination repeated. The second F_0 measurement generally reached stability more quickly than the initial one, particularly if the segment had been centrifuged. If the second F_0 had shifted with regard to the first one, the sequence was repeated until similar (deviation <10%) F_0 was obtained. The end result was four data points: an initial F_0, two F values at a low and a high delivery pressure and a final F_0. The reference K (K_{ref}) was calculated as the slope of the F by P/L linear regression for the four data points (Fig. 2A, line a). The K_{ref} was assumed to be the most accurate value of K because it was based on the greatest number of F by P/L data points. Although accurate, obtaining K from a four-point slope determination is time consuming, and more rapid estimates are generally used. The accuracy of three K estimates was assessed by a comparison with K_{ref}: (1) $K_{1pt} = F/(P/L)$, which assumes that $F_0 = 0$ (Fig. 2B), (2) $K_{2pt} = \Delta F/\Delta(P/L)$ using a two-point slope estimate (Fig. 2C) and (3) $K_{subtr} = F_n/(P/L)$, where $F_n = F - F_0$ (Fig. 2D) and F_0 was the average of the initial and final F_0 measurements.

After the K_{max} measurements, samples were placed in a custom built rotor that allowed them to be spun on a centrifuge (Sorvall RC-5C; Thermo Fisher Scientific, Waltham, MA). Sample ends were immersed in a water-filled Plexiglas reservoir, while the rotor was spinning to induce a maximum xylem tension at the segment center (Alder et al. 1997). Samples were spun for 10 min at successively higher velocities to achieve tensions of 0.5, 1.0, 1.5, 2.0, 3.0 and 4.0 MPa. After each tension, samples were removed from the rotor and their K value after spinning determined from the same four-point measurement sequence described above to produce vulnerability curves. The vulnerability curve was plotted as percentage loss of conductivity (PLC) relative to K_{max}. Four vulnerability curves were generated: one from K_{ref} (VC_{ref}) and one from each of the three K estimation methods.

Statistical analysis

Flow vs pressure regressions were calculated using ordinary least squares (OLS) because error in the x-axis variable (pressure) was considered to be less than in the y-axis variable (flow; McArdle 1988). Although any particular flow rate was measured quite precisely at 0.01 mg resolution, there were more additional sources of variation (e.g. drift in F_0, rate of stabilization, embolism displacement, etc.) than the straightforward height measurements used to calculate pressure. The lower and upper confidence intervals of the slope were used to determinate whether the OLS slopes were significantly greater than zero and whether the K values resulting from estimates 1 to 3 were different than K_{ref}. Percentage deviation from K_{ref} was calculated as 100 [1 − (K_{ref}/K)]. Differences in PLC values between the different methods at each target pressure were determined by a one-way analysis of variance (ANOVA). When the differences were significant, a multiple comparison of means (post hoc Tukey honest significant difference test) was carried out. All analyses were performed by using STATISTICA software (StatSoft Inc.,Tulsa, OK, USA).

Results

Percentage of deviation from K_{ref} is shown for the three K estimates in Fig. 3, with statistically significant deviations indicated by filled symbols. Estimate K_{1pt} (Fig. 3, circles), which ignored F_0, was by far the worst of the three estimates, under-estimating K_{ref} significantly by an average of −81.4 ± 4.7%. The magnitude of the error increased as K_{ref} of the sample decreased because of cavitation (Fig. 3A, post-centrifuged roots only; Table 1) or because of variation in K_{max} (Fig. 3B, K_{max} values only). Only when K_{ref} was relatively high were K_{1pt} and K_{ref} statistically similar. When cavitation was extreme and/or F_0 was very negative, there were cases where $F < 0$ (i.e., negative flow even when $P/L > 0$) in which case we considered $K_{1pt} = 0$. However, K_{ref} could still be positive as long as the slope of the regression was significantly greater than zero (Fig. 2A, regression b). These cases were scored as −100% deviations in Fig. 3.

The two-point slope estimate (K_{2pt}) showed both positive and negative significant deviations from K_{ref} (Fig. 3, squares). The magnitude of K_{2pt} deviations was much less than for K_{1pt} for all the tested xylem tensions (Table 1). The magnitude of the error in K_{2pt} increased as K_{ref} decreased. Unlike K_{1pt}, differences between K_{2pt} and K_{ref} were significant both at low and high K_{ref}.

The F_0 subtraction estimate (K_{subtr}) showed the lowest errors relative to K_{ref} of all three estimates (Fig. 3, triangles). No significant differences were recorded between K_{subtr} and K_{ref} across all measurements. The average deviations were always lower than those for estimates 1 and 2 (Table 1), although they also tended to increase as K_{ref} decreased (Fig. 3).

The vulnerability curve resulting from K_{1pt} values (VC_{1pt}) (Fig. 4) showed a more vulnerable xylem than

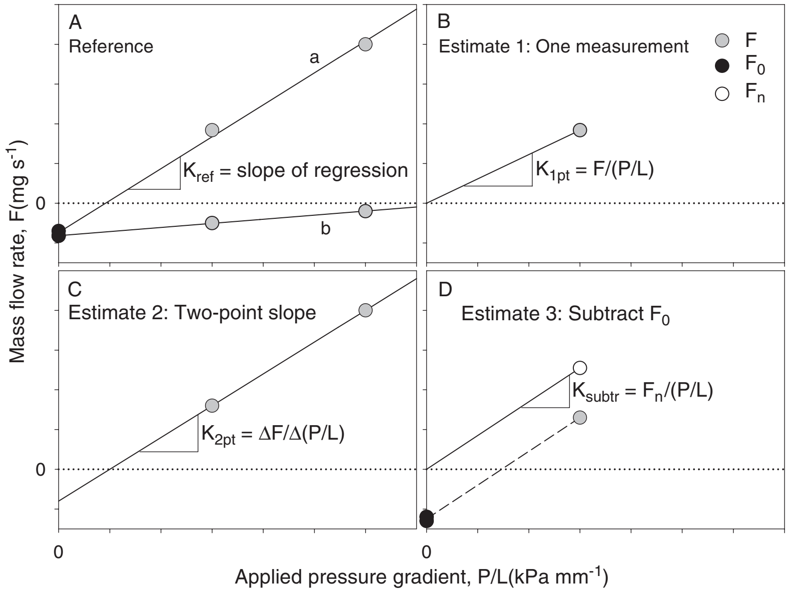

Fig. 2. Each plot corresponds to one of the four methods used to estimate the hydraulic conductivity (K) of olive root segments. Plots show mass flow rate of solution (F) through the sample at a controlled pressure gradient (P/L). F_0 is the F intercept at P/L = 0. (A) The reference method, where K_{ref} = the slope of the regression through all four F_0 and F data points. Two K_{refs} are shown, a where F is positive, and b where F is negative but there is still a significant K_{ref}. (B) The F_0 = 0 estimate where K_{1pt} = F/(P/L). (C) The two-point slope estimate where K_{2pt} = ΔF/Δ(P/L), determined at two pressure gradients. (D) The F_0 subtraction estimate where K_{subtr} = F_n/(P/L), and the net mass flow rate, F_n, was calculated as F−F_0.

Table 1. Mean percentage ± standard error (SE) (n = 6) of the deviation from the reference hydraulic conductivity (K_{ref}) of the three K estimates from Fig. 2: K_{1pt}, K_{2pt} and K_{subtr}. The maximum hydraulic conductivity (K_{max}) corresponds to the xylem tension of 0 MPa. Deviations of −100% indicate K_{1pt} = 0 while K_{ref} > 0.

Xylem tension	K_{1pt} estimate		K_{2pt} estimate		K_{subtr} estimate	
(MPa)	Mean %	±SE	Mean %	±SE	Mean %	±SE
0.0	−33.53	11.74	−1.60	5.07	−0.15	0.47
0.5	−53.69	16.76	−4.42	5.99	−0.42	0.59
1.0	−72.63	14.33	−13.60	5.32	−0.57	0.97
1.5	−82.49	12.56	−3.04	5.19	−0.34	0.43
2.0	−89.47	10.53	−15.21	15.41	−1.49	1.51
3.0	−100.00	0.00	0.17	35.30	−0.03	3.45
4.0	−100.00	0.00	56.28	28.15	10.11	5.14

the vulnerability curve obtained from K_{ref} (VC_{ref}). Thus, VC_{1pt} showed significantly higher PLC values than VC_{ref}, both at target pressures of −1.0 and −1.5 MPa. The vulnerability curves resulting from K_{2pt} (VC_{2pt}) or K_{subtr} (VC_{subtr}) estimates did not show significant differences with regard to VC_{ref} (Fig. 4) at any target pressure.

Discussion

Our results indicate that the non-zero flow intercept, F_0, needs to be accounted for measuring K of xylem segments. The K_{1pt} estimates, i.e. those which do not take F_0 into account, were usually below the actual values, especially in samples with low conductivities. The K_{1pt} estimate is based on a single flow measurement, and because it is the most rapid estimate it is widely used. Assuming a linear flow–pressure relationship, the deviation of K_{1pt} ($1 - K_{ref}/K_{1pt}$) equals the F_0/F ratio, so higher F reduces the error. This is why the K_{1pt} deviation decreased with higher K_{ref} in our experiments (Fig. 3) because higher K_{ref} resulted in greater F for the same P/L. In theory, the error at lower K_{ref} could be reduced by measuring K_{1pt} at a higher P/L (driving down the F_0/F ratio). The P/L required to achieve a desired F_0/F threshold (ERR) is given by:

$$P/L = F_0/K_{ref}\,(1/\mathrm{ERR}) - 1 \qquad (1)$$

For example, if the most negative F_0 is known to reach -0.02 mg s^{-1}, and the minimal K_{ref} of the material is estimated to be 2 mg mm s^{-1} kPa^{-1} (values in our experimental range), the P/L must be at least 0.21 kPa mm^{-1} to keep errors within −5% (ERR = −0.05) using the K_{1pt} method. This P/L is over five times higher than the maximum we could use (approximately 0.04 kPa mm^{-1}) without causing embolism reversal. Using a higher P/L could be an effective way to minimize K_{1pt} error for K_{max} measurements where reversible embolism is not present. It should be noted, however, that excessive P/L can deflect pit membranes and perhaps

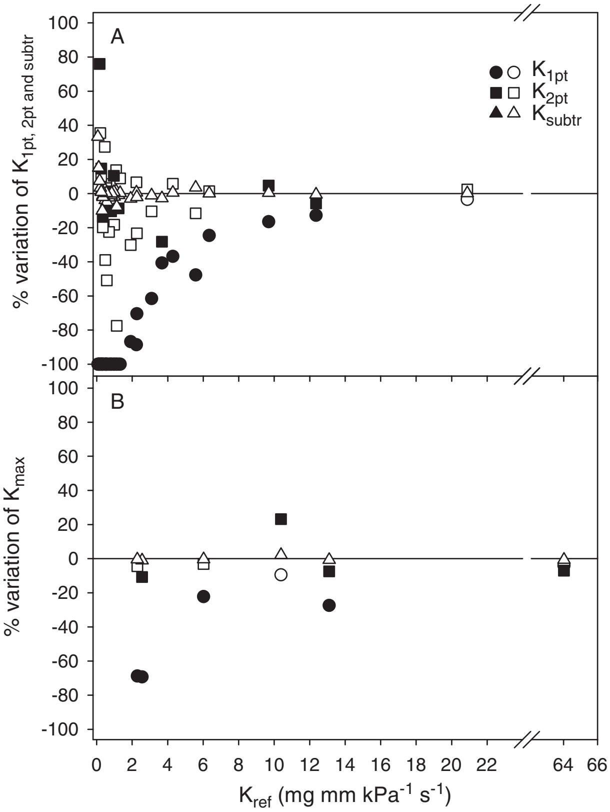

Fig. 3. Agreement between the reference hydraulic conductivity (K_{ref}) and the three hydraulic conductivity (K) estimates from Fig. 2: K_{1pt}, K_{2pt} and K_{subtr} for (A) centrifuged and partially embolized samples and (B) vacuum infiltrated, non-embolized samples (K_{max}). Measurements were made in olive root segments. Closed symbols indicates significant differences ($P < 0.05$) and open symbols indicates non significant differences.

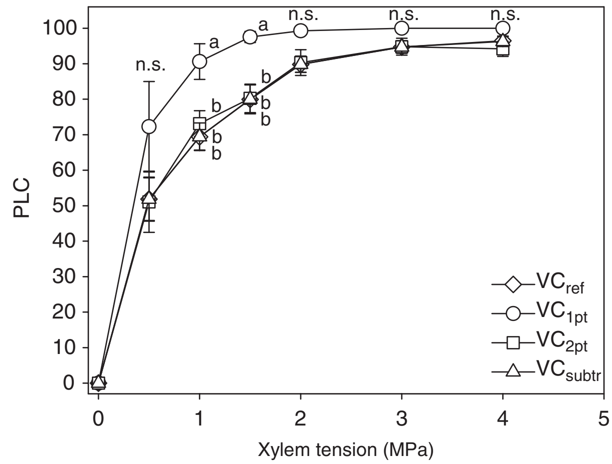

Fig. 4. Xylem vulnerability curves of olive roots obtained from the reference hydraulic conductivity (VC_{ref}) and from the three hydraulic conductivity (K) estimates: VC_{1pt}, VC_{2pt} and VC_{subtr}. Each data represents the mean percentage loss of conductivity [PLC ± standard error (SE), vertical bars] of six samples. Different letters indicate statistically significant differences ($P < 0.05$); n.s. = no significant difference.

cause other problems that alter K (Sperry and Tyree 1990, Domec et al. 2007).

If K is to be measured in the presence of embolism, increasing P/L to minimize K_{1pt} error is not an option because of the possibility for embolism reversal. Erroneous K_{1pt} values can lead to incorrect vulnerability curves that misrepresent the cavitation resistance. In our case, where F was measured from the downstream end, the F_0 was negative, causing K_{1pt} to be an under-estimate (Fig. 1, insert). Vulnerability to cavitation, therefore, was overestimated. However, in cases where F is determined at the upstream end of the sample as in many studies that use an electronic flow meter, F_0 is likely to be positive and would cause K_{1pt} to be an over-estimate. Curves then would be overly resistant. Either way, our results suggest that F_0 needs to be accounted for in K measurements of samples where embolism is present. Presumably, the best estimate of K is obtained by a multi-point slope determination, under conditions where F_0 is not systematically shifting. This method is often used when measuring entire shoot or root systems with the HPFM or other devices (Fiscus et al. 1983, Tyree et al. 1994, 1995, Kolb et al. 1996, Bogeat-Triboulot et al. 2002, Nardini et al. 2006, Gascó et al. 2007, Trifilò et al. 2010). This K_{ref} technique also has the advantage of confirming linearity in the pressure–flow relation. Our results indicate that for excised segments, the more rapid two-point slope (K_{2pt}) and F_0 subtraction (K_{subtr}) estimates provided reasonably accurate alternatives to the time consuming full slope measurement. As reflected by the VC_{2pt} (Fig. 4), the agreement between the K_{2pt} values and the K_{ref} values was enough to obtain reliable values of the vulnerability to cavitation. Still, our results show that K_{2pt} may differ significantly from K_{ref}, which must be taken into account when a reliable K determination needs to be made. Both Figs 3 and 4 show that, when F_0 cannot be directly measured (e.g. systems with one-way flowmeters or with flowmeters with an insufficient resolution to measure F_0), errors can be reduced when determining K by at least two different pressure heads. We have not tested whether a greater number of pressured heads increases the accuracy of the K_{2pt} values, but it seems likely. For testing whether F_0 has shifted during a two-point slope (K_{2pt}) estimate, the first of the two F measurements could be repeated.

When F_0 can be measured directly, the F_0 subtraction estimate (K_{subtr}) is a relatively low time- and labor-consuming method that is enough for an accurate estimation of K. This method has the advantage of confirming constancy in F_0. It was not uncommon for F_0 to become substantially less negative after the F measurement. This was particularly the case after samples had been spun to high tensions in the centrifuge, inducing an initially more rapid rate of passive water uptake that eventually stabilized. Interestingly, however, there was nearly always significant passive uptake, even when samples were fully hydrated and care was taken to minimize evaporation from sample and reservoir.

Negative F_0 was also seen in samples that were flushed with positive pressure to K_{max} (data not shown) indicating that the phenomenon was not unique to the vacuum infiltration treatment described in the Materials and methods. The measurement of xylem K by any of the four methods assumed that F_0 is a constant that is independent of the applied pressure gradient P/L. While we insured that F_0 at $P/L = 0$ was approximately constant during a measurement, we did not determine if the rate of passive uptake changed with P/L. Further refinements in methodology would be required if the uptake varied significantly with pressure.

Our results show the importance of taking into account F_0 values for accurate determinations of K with any method that assumes a linear and steady-state pressure–flow relationship through the sample. The problem applies not only to root samples, but excised segments from any organ or organ system, including leaves. Although the K_{1pt} approach is often used to estimate K on detached transpiring leaves, a recent test suggests the error of not accounting for F_0 can be relatively small in this method (Guyot et al. 2012). In the transpiring leaf, xylem pressures are strongly sub-atmospheric, and P/L can be substantial without reversing embolism. High transpiration rates would keep the F_0/F ratio small enough to trivialize the error. Even so, investigators should confirm that the error is small for their experimental conditions and material.

Conclusions

Methods in which F_0 is not considered and K is determined at a single pressure gradient (K_{1pt}) can lead to significant errors that can also influence vulnerability curves. The error of not accounting for F_0 increases with the F_0/F ratio. When F_0/F is significant, the F_0 value should be measured and subtracted from F for an accurate estimation of K and, therefore, of PLC and vulnerability to cavitation (K_{subtr}). If F_0 cannot be measured, it may be advisable to use a method in which the value of F_0 is implicit, e.g. the two-point slope estimate (K_{2pt}) or a multi-point slope determination (K_{ref}).

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