Predicting Stomatal Closure and Turgor Loss in Woody Plants Using Predawn and Midday Water Potential¹[OPEN]

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Knowledge about physiological stress thresholds provides crucial information about plant performance and survival under drought. In this study, we report on the triphasic nature of the relationship between plant water potential ($\Psi$) at predawn and midday and describe a method that predicts $\Psi$ at stomatal closure and turgor loss exclusively from this water potential curve (WP curve). The method is based on a piecewise linear regression model that was developed to predict the boundaries (termed $\Omega_1$ and $\Omega_2$) separating the three phases of the curve and corresponding slope values. The method was tested for three economically important woody species. For all species, midday $\Psi$ was much more negative than predawn $\Psi$ during phase I (mild drought), reductions in midday $\Psi$ were minor while predawn $\Psi$ continued to decline during phase II (moderate drought), and midday and predawn $\Psi$ reached similar values during phase III (severe drought). Corresponding measurement of leaf gas exchange indicated that boundary $\Omega_1$ between phases I and II coincided with $\Psi$ at stomatal closure. Data from pressure-volume curves demonstrated that boundary $\Omega_2$ between phases II and III predicted $\Psi$ at leaf turgor loss. The WP curve method described here is an advanced application of the Scholander-type pressure chamber to categorize plant dehydration under drought into three distinct phases and to predict $\Psi$ thresholds of stomatal closure and turgor loss.

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T.K. designed and performed most of the experiments, analyzed the data, and wrote the article together with A.J.M.; N.B. developed the piecewise linear regression model, performed measurements on almond trees, and revised the article; M.I.H. helped with measurements of water potential, collected leaf pressure volume curves on walnuts, and revised the article; F.D. helped with measurement of water potential and leaf gas exchange on walnuts; M.K.B. acquired plant material, obtained funding for grapevine research, designed the grapevine experiment, and revised the article; G.S. performed measurements of water potential and leaf gas exchange on grapevines; D.A.K. obtained funding for walnut research, acquired the plant material, helped in experimental design, and revised the article; A.J.M. obtained funding, helped in experimental design, and wrote the article together with T.K.

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Water stress by drought leads to plant mortality linked to carbon starvation and xylem hydraulic failure (Choat et al., 2018; McDowell et al., 2018). Plant survival under drought is dependent on a successful coordination of physiological responses across multiple organizational levels. This includes efficient stomatal regulation to limit excessive water loss to the atmosphere (Brodribb and Holbrook, 2003; Brodribb and McAdam, 2017), changes in root hydraulic and anatomical properties to minimize water loss to a drying soil and reduce the metabolic cost of soil exploration (Zhu et al., 2010; Barrios-Masias et al., 2015; Cuneo et al., 2016), and osmotic regulation and turgor maintenance on a cellular level to avoid negative effects on growth (Blum, 2017). The exact sequence of these physiological events needs to be elucidated across many species, but data indicate that drought-induced stomatal closure is well correlated with leaf turgor loss and the decline in leaf xylem hydraulic conductance (Brodribb and Holbrook, 2003). Scoffoni et al. (2018) showed that drought-induced changes in cell membrane permeability are linked to stomatal closure and that leaf xylem cavitation is negligible above the turgor loss point ($\Psi_{TLP}$). Recent reports suggest that stomatal closure is not triggered by xylem cavitation and primarily driven by a decline in root hydraulic conductance and changes within the root-to-soil hydraulic continuum (Carminati and Javaux, 2020; Rodriguez-Dominguez and Brodribb, 2020). For intact plants subjected to progressive drought stress, in vivo studies indicate that stomatal closure and root cortical cell...
damage precede xylem cavitation in the stem, which precedes the discharge of stored water from xylem fibers surrounding vessels (Coneo et al., 2016; Knipfer et al., 2019).

Drought-induced stomatal closure plays an important role in minimizing excessive negative pressure in xylem sap \((P_x)\), which contributes to increased drought resistance by reducing the risk of xylem cavitation (Martin-StPaul et al., 2017). Although a matter of debate, the isohydry/anisohydry concept provides a framework to describe the efficiency of a plant to control \(P_x\) and in turn plant water potential \((\Psi)\) through stomatal opening/closing under drought (Tardieu and Simonneau, 1998; Martínez-Vilalta et al., 2014; Martínez-Vilalta and García-Forner, 2017; Ratzmann et al., 2019). As suggested by Meinzer et al. (2016), maintenance of plant \(\Psi\) at midday \((\Psi_{md})\) while \(\Psi\) at predawn \((\Psi_{pd})\) declines is indicative for an isohydric behavior, while a decline in \(\Psi_{md}\) together with \(\Psi_{pd}\) points to an anisohydric behavior due to inefficient and/or uncoordinated stomatal closure. However, it remains elusive if \(\Psi\) thresholds corresponding to stomatal closure and leaf turgor \((P)\) loss can be predicted directly from measurements of plant \(\Psi\), but if so, this would certainly allow for a more time-effective and less labor-intensive assessment of both physiological responses.

The cohesion-tension theory predicts that transpirational pull drives the movement of xylem sap from roots to leaves (Dixon and Joly, 1895). The pressure chamber technique allows measuring plant \(\Psi\) in a relatively simple way and provides a good estimate of \(P_x\) when leaf apoplastic is in equilibrium with symplast prior to leaf pressurization (Scholander et al., 1965; Turner, 1981). Commonly, the difference between measured plant \(\Psi\) and actual \(P_x\) is small, since the osmotic potential of the xylem sap is less than 0.1 MPa and the matric potential of the apoplastic is close to \(\Psi\) of the symplast (Turner, 1981). For walnut \((Juglans spp.)\) trees, Cochard et al. (2001) confirmed that the pressure chamber technique accurately predicts the existence of large negative \(P_x\). Since plants function within a hydraulic continuum between soil and atmosphere, plant \(\Psi\) and \(P_x\) are dependent on the rate of water loss by transpiration and water uptake by roots. Because evaporative demand and light intensity change diurnally and impact transpiration via stomatal regulation, plant \(\Psi\) is most negative at midday (i.e. maximum transpiration) and least negative at predawn (i.e. negligible transpiration), especially under well-watered conditions (Klepper, 1968). Plant \(\Psi_{pd}\) provides an indicator for soil \(\Psi\) when nighttime transpiration is minimal and plants can reach an equilibrium with the wettest portion of the soil (Turner, 1981; Donovan et al., 2001). On the other hand, progressive drought stress results in a general decline in plant \(\Psi\) due to limitations in root water uptake and the fact that evaporative demand is not matched by water supply from the soil. In summary, plant \(\Psi\) measurements can provide an integrative measure of plant physiological responses to water stress by drought, but the relationship of \(\Psi_{pd}\) and \(\Psi_{md}\) under these conditions is surprisingly understudied.

About 40 years ago, Turner and Long (1980) showed that the relationship of \(\Psi\) measured on a covered (nontranspiring) and uncovered (transpiring) leaf is nonlinear for plants subjected to progressive drought stress, which highlighted the variable effect of transpiration on measurements of leaf \(\Psi\). Martínez-Vilalta et al. (2014) presented a first theoretical framework regarding the relationship of leaf \(\Psi\) measured at midday and predawn, and the authors interpreted the slope of the relationship as the relative sensitivity of transpiration rate to increasing water stress by drought and the intercept as the maximum transpiration rate per unit of hydraulic transport capacity. Similarly, Meinzer et al. (2016) considered the impact of stomatal regulation on plant \(\Psi\) and found that the relationship of \(\Psi_{pd}\) and \(\Psi_{md}\) (i.e. measured following leaf covering and equilibration) is correlated with a species’ \(\Psi_{TP}\) and degree of isohydricity/anisohydricity (i.e. slope of curve and hysrocape). In contrast, Williams and Araujo (2002) reported that the relationship of \(\Psi_{pd}\) and \(\Psi_{md}\) is linear, but this finding may not hold true under more severe drought and stomatal closure. We revisited these findings for walnut, grapevine \((Vitis spp.)\), and almond \((Prunus dulcis)\) and performed an in-depth analysis of the water potential curve \((WP\) curve\) between \(\Psi_{pd}\) and \(\Psi_{md}\). Measurements of \(\Psi_{pd}\) and \(\Psi_{md}\) were complemented with measurements of leaf gas exchange \((\text{stomatal conductance } [g_s] \text{ and } \text{CO}_2\text{ assimilation rate } [A])\) for walnut and grapevine. In addition, for walnut, leaf sap osmotic pressure \((\pi)\) and pressure-volume curves were collected to obtain an estimate of leaf \(P\) and \(\Psi\) at \(P\) loss. To test if characteristics of the WP curve are dependent on the type of drought experiment (Gilbert and Medina, 2016), walnut trees were subjected to a slow (weeks) and fast (days) drydown (not irrigated). For analysis of the WP curve, a mathematical approach was developed to determine the phases of plant dehydration and to calculate boundaries \(\Theta_1\) and \(\Theta_2\) separating phases I and II and phases II and III, respectively. Together, this allowed us to test the hypothesis that calculated \(\Theta\) values from the WP curve predict \(\Psi\) at stomatal closure and leaf \(P\) loss. To avoid confusion, we will not use the existing terminology of \(\Psi_{leaf}\) and \(\Psi_{stem}\), since in both cases \(\Psi\) is measured on an excised leaf that is either bagged for a relatively short (less than 10 s and nonequilibrated) or long (more than 15 min and equilibrated) time period, respectively.

**RESULTS**

**Walnut**

The relationship between \(\Psi_{pd}\) and \(\Psi_{md}\) exhibited a nonlinear behavior for walnut trees subjected to the slow drydown (Fig. 1A) and the fast drydown (Fig. 1C). Reductions in \(\Psi_{pd}\) and \(\Psi_{md}\) during the slow and fast
Drydown were associated with a decline in soil moisture from around 75% to 45% (w/w) and 80% to 50% (w/w), respectively (Supplemental Fig. S1). In general, the relationship between \( C_{pd} \) and \( C_{md} \) can be described as follows: early during drought stress, \( C_{md} \) was much more negative than \( C_{pd} \); this was followed by minor reductions in \( C_{md} \) while \( C_{pd} \) continued to decline under moderate drought; \( C_{md} \) and \( C_{pd} \) were most similar under severe drought stress (Fig. 1, A and C).

Based on the smoothed line fit included in Figure 1, A and C, transition points (\( \gamma \)) along the WP curve were calculated from \( dC_{md}/dC_{pd} \) slope values (Fig. 1, B and D). Transition points of \( \gamma_1 \) and \( \gamma_2 \) were necessary to parameterize the piecewise linear regression (PLR) model. Subsequently, statistical estimates for boundary \( Q_1 \) between phases I and II and boundary \( Q_2 \) between phases II and III were obtained. The model predicted a boundary \( Q_1 \) for the slow and fast drydown of \(-0.8\) and \(-0.5\) MPa, respectively (Fig. 2; Table 1). Comparing both drydown experiments, \( \Theta_1 \) was less negative (by around 0.3 MPa) during the fast drydown. Boundary \( \Theta_2 \) was at \(-1.3\) (slow) and \(-2.2\) MPa (fast), and \( \Theta_2 \) was more negative (by around 0.9 MPa) during the fast drydown (Fig. 2). Representative images taken during the slow drydown showed that leaves appeared dark green and turgid for trees in phase I, leaves lost turgidity and appeared droopy at boundary \( \Theta_2 \) in phase II, and leaves decolored to light green and started to desiccate and senesce at the bottom of the canopy in phase III (Supplemental Fig. S2).

The boundary \( \Theta_1 \) between phases I and II corresponded to the \( \Psi \) threshold at which leaf gas exchange was substantially reduced in walnut trees (Fig. 3). The different values of \( \Theta_1 \) as determined during the slow and fast drydown with the PLR model reflected the shift in \( \Psi \) threshold at which \( g_s \) and \( A \) reached a minimum. For the slow drydown, \( g_s \) and \( A \) of a mature leaf were reduced by 89% and 86%, respectively, when reaching boundary \( \Theta_1 \) (Fig. 3, A and B). These reductions were similar for the fast drydown (Fig. 3, C and D).

The boundary \( \Theta_2 \) between phases II and III corresponded to the \( \Psi \) threshold that marked the end of the leaf \( P \) maintenance phase and was indicative of the \( \Psi_{TLP} \) (\( = -1.4 \pm 0.14 \) MPa; Fig. 4; Supplemental Table S1). For the slow drydown, leaf \( P \) initially declined by around 60% during phase I, \( P \) was maintained during phase II, and subsequently \( P \) declined again during phase III (Fig. 4A). These drought-induced changes in \( P \) were associated with an increase in leaf sap \( \pi \) (Fig. 4B). For the fast drydown, \( P \) declined predominantly during phase I (Fig. 4C), similar to the slow drydown. However, given the drydown speed, we were not able to collect enough data points of \( P \) and \( \pi \) to conclusively report on the pattern of data points in phases II and III (Fig. 4, C and D).

**Figure 1.** Relationship between plant \( \Psi_{pd} \) and \( \Psi_{md} \) for walnut trees (‘Cisco’). Trees were subjected to a slow drydown (A and B; controlled irrigation over weeks) or a fast drydown (C and D; no irrigation over days). Data are summarized for trees grafted on rootstocks RX1, VX211, and Vlach. A and C, The dashed line is a smoothed line (smoothing factor = 60) that best followed the pattern of data points. The solid line is a linear regression fitted across data points (in A, \( R^2 = 0.87, m = 0.75, P < 0.0001 \); in B, \( R^2 = 0.85, m = 0.89, P < 0.0001 \)). For C, during the fast drydown, the same symbols indicate data collected for the same tree. B and D, Relationship of \( \Psi_{pd} \) and slope values derived for the smoothed lines in A and C. Parameters \( a \) and \( c \) are the maximum slope values, parameter \( b \) is the minimum slope value, and \( \gamma_1 \) and \( \gamma_2 \) are the calculated transition points.
The relationship between $C_{pd}$ and $C_{md}$ was non-linear (from 0 to $2.5 \text{ MPa}$) for grapevine plants subjected to a slow drydown (Fig. 5). Following the initiation of water stress by drought, $C_{md}$ was more negative than $C_{pd}$ (see deviation from the 1:1 line). Subsequently, reductions in $C_{md}$ were minor while $C_{pd}$ continued to decline under moderate drought; values of $C_{md}$ and $C_{pd}$ were most comparable under severe drought (Fig. 5A). Following the smoothed line fit, the transition points along the WP curve were located at $\gamma_1$ of $-0.58 \text{ MPa}$ and $\gamma_2$ of $-1.57 \text{ MPa}$ (Fig. 5B). The PLR model predicted for grapevine that boundary $\Theta_1$ between phases I and II was at $-0.68 \text{ MPa}$ and boundary $\Theta_2$ between phases II and III was at $-1.33 \text{ MPa}$ (Fig. 6; Table 1).

Leaf gas exchange was measured for grapevine (Fig. 7). This showed that boundary $\Theta_1$ between phases I and II matched the $C$ threshold at which leaf gas exchange was substantially reduced (Fig. 7). Following an initial increase of $g_s$ (Fig. 7A) and $A$ (Fig. 7B), that reached a maximum at $\Psi_{pd}$ of around $-0.4 \text{ MPa}, g_s$ and $A$ declined by 93% and 67%, respectively, thereafter reaching values of close to zero at boundary $\Theta_1$.

**Almond**

Similar to data collected for potted walnut (Fig. 2) and grapevine (Fig. 6) plants, data collected for almond trees showed that the relationship of $C_{pd}$ and $C_{md}$ exhibited a triphasic curve shape (Fig. 8). The calculated boundary values using our PLR model were $\Theta_1 = -1.37 \text{ MPa}$ and $\Theta_2 = -1.94 \text{ MPa}$ (Fig. 9; Table 1). See Supplemental Table S2 for statistical comparison of all output parameters obtained with the PLR model (Table 1).

**DISCUSSION**

In this study, we describe a method that allows predicting stress thresholds associated with drought-induced stomatal closure and turgor loss exclusively from measurements of plant $C$ using a Scholander-type pressure chamber. For woody species (walnut, grapevine, and almond), we found that the WP curve between plant $C$ at midday and predawn exhibits a unique triphasic curve shape. To quantify the underlying curve parameters, we developed a PLR model for statistical analysis of the WP curve. For walnut and grapevine, modeling data together with leaf gas-exchange data indicated that boundary $\Theta_1$ between phases I and II marked the $C$ threshold at which $g_s$ was reduced by around 90%. For almond, leaf gas exchange was reduced by approximately 70% at $\Psi_{pd}$ of $-1.4 \text{ MPa}$ (Marsal et al., 1997), and this suggests that our calculated boundary $\Theta_1$ at $-1.37 \text{ MPa}$ for cv Nonpareil most likely predicts stomatal closure as well. Our data of leaf $P$ and $C_{TLP}$ from pressure-volume curves indicated that the boundary $\Theta_2$ between phases II and III marked the end of the leaf $P$ maintenance phase and $C$ at $P$ loss for walnut. For grapevine and almond, literature data of $C_{TLP}$ ($-1.4 \text{ MPa}$ for grapevine ‘Chardonnay’ [Alsina et al., 2007] and $-2.1 \text{ MPa}$ for almond ‘Garrigues’ [Torrecillas et al., 1996]) closely matched our calculated $\Theta_2$. The robustness of our WP curve method was tested for walnut by analyzing two types of drydown experiments (slow over weeks versus fast over days and no irrigation). This showed that the WP curve method was successful in identifying the shift in $C$ at stomatal closure depending on the type of drought experiment. Moreover, preliminary data collected for commercially
available walnut rootstocks RX1 (Juglans microcarpa × Juglans regia) and VX211 (Juglans hindsii × J. regia) indicated that WP curves are genotype specific (Supplemental Fig. S3). In summary, the WP curve method provides for a new approach to evaluate plant drought responses (stomatal closure and turgor loss) in a cost-effective and relatively simple way that only requires access to a Scholander-type pressure chamber.

Drought-Induced Stomatal Closure

In the past, Turner and Long (1980) reported on the nonlinear behavior of the relationship of \( C_{pd} \) measured simultaneously on a covered leaf prior to excision and an uncovered leaf. Their data show a triphasic curve shape with values approaching 1:1 due to stomatal closure when plants experience severe drought. Similarly, our data demonstrate that the relationship of \( C_{pd} \) and \( C_{md} \) is nonlinear and triphasic. Our leaf gas-exchange measurements indicated that \( g_s \) started to reach a minimum at the transition point between phases I and II (i.e. boundary \( Q_1 \)) of the WP curve, which confirmed that stomatal closure was the main driver that caused the initial change in shape of the WP curve. We speculate that the shape of the curve between \( g_s \) and \( C_{pd} \) until reaching boundary \( Q_1 \) is dependent on how

### Table 1. Summary of output parameters from the PLR model as used for analysis of the relationship between \( \Psi_{pd} \) and \( \Psi_{md} \) for woody species (walnut ‘Cisco’, grapevine ‘Chardonnay’, and almond ‘Nonpareil’)

| Parameter | Walnut | Grapevine | Almond |
|-----------|--------|-----------|--------|
| \( N \) | 92     | n/a       | 24     |
| \( \Theta_1 \) (MPa) | -0.84 (0.08, 0.01) | 2.40 (0.10, 0.01) | 80 (n/a, n/a) |
| \( \Theta_2 \) (MPa) | -1.31 (0.16, 0.01) | 2.22 (0.26, 0.01) | -0.68 (0.14, 0.01) |
| \( \beta_1 \) | 1.33 (0.23, 0.01) | 3.64 (0.57, 0.01) | 1.23 (0.63, 0.05) |
| \( \beta_2 \) | 0.09 (0.20, 0.01) | 0.49 (0.63, 0.05) | -0.03 (1.02, 0.17) |
| \( \beta_3 \) | 0.73 (0.07, 0.01) | 1.80 (0.95, 0.01) | 1.39 (0.86, 0.11) |
| \( \alpha \) (MPa) | -0.62 (0.08, 0.01) | 0.32 (0.28, 0.01) | 0.60 (0.24, 0.01) |
| \( R^2 \) | 0.91 (n/a, n/a) | 0.90 (n/a, n/a) | 0.62 (n/a, n/a) |

Simultaneously, on a covered leaf prior to excision and an uncovered leaf. Their data show a triphasic curve shape with values approaching 1:1 due to stomatal closure when plants experience severe drought. Similarly, our data demonstrate that the relationship of \( \Psi_{pd} \) and \( \Psi_{md} \) is nonlinear and triphasic. Our leaf gas-exchange measurements indicated that \( g_s \) started to reach a minimum at the transition point between phases I and II (i.e. boundary \( \Theta_1 \)) of the WP curve, which confirmed that stomatal closure was the main driver that caused the initial change in shape of the WP curve. We speculate that the shape of the curve between \( g_s \) and \( \Psi_{pd} \) until reaching boundary \( \Theta_1 \) is dependent on how...
well drought-induced closure between individual stomates of a leaf is coordinated. For example, a distinctive drop-off in measured $g_s$ should only be observed at boundary $Q_1$ when all stomates transition instantaneously from an open to a closed state (Gollan et al., 1985). Following this logic, we hypothesize that the shape of the WP curve during phase I is predominantly affected by the ability of a plant to efficiently coordinate stomatal closure under increasing water stress by drought.

Stomatal closure/opening can be triggered by several factors, including light intensity, vapor pressure deficit (VPD), abscisic acid (ABA) concentration, and/or hydrostatic pressure (Farquhar and Sharkey, 1982; Franks, 2013; Tombesi et al., 2015). We did not measure ABA concentrations, but the role of ABA in stomatal closure can be summarized as follows. A root tip that is exposed to dry soil conditions commonly synthesizes additional amounts of ABA, which ultimately arrives in leaves depending on the transport efficiency of the transpiration stream (Zhang et al., 1987). Gollan et al. (1986) showed that when leaves are kept turgid by pressurizing roots while the soil is drying, stomates still closed, which was interpreted as further evidence for...
the importance of root-to-shoot signaling via ABA translocation. However, McAdam and Brodribb (2018) found that leaf mesophyll cells are the main location for ABA biosynthesis under drought stress, which deemphasizes the importance of ABA delivery from roots to leaves via the xylem to initiate stomatal closure. Drought-induced increases in root ABA concentrations may also trigger aquaporin-mediated changes in root hydraulic conductivity and/or result in modifications of root architecture (Aroca et al., 2012). Recent studies suggest that drought-induced changes in root and soil hydraulic properties drive stomatal closure (Carminati and Javaux, 2020; Rodriguez-Dominguez and Brodribb, 2020). Besides ABA, drought-induced stomatal closure can be mediated by a pressure-induced passive mechanism depending on $P_s$ (Brodribb and McAdam, 2011; Franks, 2013). Tombesi et al. (2015) reported that $g_s$ in grapevine subjected to drought stress is predominantly regulated by such a passive hydraulic signal. The authors came to this conclusion because leaf ABA increased only after complete stomatal closure, and it was hypothesized that this is of importance for long-term drought recovery to facilitate xylem embolism repair by forcing stomatal closure when the soil is rehydrated. In summary, and in the context of collecting WP curves for various woody plants, we speculate that if leaf ABA accumulation is observed in phase I, this points to a predominant ABA-mediated mechanism driving stomatal closure, whereas if leaf ABA accumulation is observed in phase II, this points to a predominant pressure-driven (passive) mechanism driving stomatal closure.

Atmospheric evaporative demand affects the rate of transpiration (see introduction; Klepper, 1968). Gollan et al. (1985) performed an extensive study on the relationship of VPD, $\Psi$ determined on leaves with in situ psychrometers, and soil water content on leaf gas exchange. The authors showed that the relationship of $\Psi$ and leaf gas exchange is dependent on VPD. During increasing drought, their data point to a sharp drop-off in $g_s$ at high VPD (25 Pa kPa$^{-1}$), whereas the reduction in $g_s$ was gradual until reaching a minimum (as observed in this study) at low VPD (10 Pa kPa$^{-1}$). Due to natural fluctuations in VPD and its effect on transpiration, plant $\Psi$ can vary between days and over the course of a day for the same soil water status. For this reason, $\Psi_{md}$ (as measured on an equilibrated leaf using a pressure chamber [i.e. $\Psi_{stem}$]) is most meaningful as a water stress indicator over the growing season when compared with baseline values under well-irrigated conditions (Turner, 1990; Shackel et al., 1997). Based on our findings, we hypothesize that the effects of VPD on the shape of the WP curve are most pronounced during phase I prior to stomatal closure, and high versus low VPD conditions result in either a steeper (higher transpiration) or shallower (lower transpiration) slope $\beta_1$. On the other hand, and for a given plant species, we speculate that the effect of VPD on boundary $\Theta_1$ is negligible if stomatal closure is
Figure 8. Relationship between $\Psi_{pd}$ and $\Psi_{md}$ for almond (‘Nonpareil’). A, Data were obtained for trees grafted onto rootstock Nemaguard. The dashed line is a smoothed line (smoothing factor = 60) that best followed the pattern of data points. The solid line is a linear regression fitted across data points ($R^2 = 0.94$, $m = 0.79$, $P < 0.0001$). B, Relationship of $\Psi_{pd}$ and slope values derived for the smoothed line in A. Parameters $a$ and $c$ are the maximum slope values, parameter $b$ is the minimum slope value, and $\gamma_1$ and $\gamma_2$ are the calculated transition points.

Figure 9. PLR model describing the triphasic relationship between $\Psi_{pd}$ and $\Psi_{md}$ for almond trees (corresponding to Fig. 8). The model is shown as a thick solid line. Roman numerals I to III designate the three phases of the WP curve. Vertically solid lines are the boundaries between phases I and II ($\Theta_1$) and phases II and III ($\Theta_2$), and corresponding $n$ values are indicated in gray color. Model output parameters are summarized in Table 1.

Leaf Turgor Loss

Our data provide evidence that boundary $\Theta_2$ separating phases II and III of the WP curve predicts $\Psi_{TLP}$. For walnut (‘Cisco’), average $\Psi_{TLP}$ determined from pressure-volume curves of $-1.39$ MPa (Supplemental Table S1) was only slightly more negative as compared with $\Theta_2$ of $-1.31$ MPa (Table 1). For grapevine (‘Chardonnay’), Alsina et al. (2007) reported an average $\Psi_{TLP}$ of $-1.36$ MPa at veraison and prior to berry maturation, which was similar to our $\Theta_2$ of $-1.33$ MPa (Table 1). For almond, Torrecillas et al. (1996) determined a $\Psi_{TLP}$ of around $-2.2$ MPa (cv Garrigues) and $-2.3$ MPa (cv Ramilete) for well-watered trees as compared with our $\Theta_2$ of $-1.9$ MPa (cv Nonpareil; Table 1). Together, these findings suggest that $\Psi_{TLP}$ can be predicted from the triphasic WP curve using our PLR model for a variety of woody species.

For walnut trees subjected to the slow drydown, we were able to collect sufficient data points to elucidate the pattern of drought-induced variations in $\Delta P$ ($\Delta P = \Psi - \pi \sigma$). To calculate $\Delta P$, we assumed that $\pi \approx \pi_{symplast}$ and that the reflection coefficient for solutes ($\sigma$) was unity. If we consider that the remaining apoplastic sap in the centrifuged leaf tissue resulted in a dilution effect (Wardlaw, 2005), then our measured $\pi$ underestimated true $\pi_{symplast}$. Hence, true $\Delta P$ should be slightly lower than our calculated $\Delta P$, which would explain why our calculated $\Delta P$ was $0.3$ MPa and not $0$ MPa at $\Theta_2$ of $-1.31$ MPa (Fig. 4A). On the other hand, $\pi_o$ at full hydration obtained from pressure-volume curves was $1.1$ MPa (Supplemental Table S1) but $\pi$ at $\Psi_{pd}$ of $0$ MPa was around $1.7$ MPa (Fig. 4A), which points to a possible overestimation of $\pi_{symplast}$. The indirect approach used here provides a relatively easy way to obtain information on drought-induced $\Delta P$ changes, but if experimentally feasible, $\Delta P$ and $\pi_{symplast}$ are best determined directly using a combination of cell pressure probing and picoliter osmometry (Tomas and Leigh, 1999; Fricke and Peters, 2002; Knipfer et al., 2014).

Although controversial, it has been reported that negative $\Delta P$ exists in plant cells according to indirect measurements from pressure-volume curves (Tyree, 1976; Rhizopoulou, 1997; Ding et al., 2014). Our $\Delta P$ data obtained indirectly by $\Psi - \pi \sigma$ also point to the existence of negative $\Delta P$, which occurred in phase III following the $\Psi_{TLP}$. However, one factor that may explain the measurement of negative $\Delta P$ when determined indirectly is $\sigma$, which provides a measure of solute permeability/leakage of the cell membrane (Staverman, 1951; Knipfer et al., 2014). Drought stress results in increased solute leakage and modulation of the physical state of the membrane (Blum and Ebercon, 1981; Premachandra and Shimada, 1987; Couchoud et al., 2019). If solute leakage plays a role during

predominantly driven by soil-to-root interactions (see previous paragraph).
drought stress, then $P$ is best determined by $\Psi - \pi\sigma_i$ where $i = \text{level of water stress}$. Hence, if we imagine that solute leakage becomes more and more severe under increasing drought stress, $\sigma$ would become smaller and smaller and the term $\pi\sigma$ goes toward zero, which in turn would result in a calculated $P$ that may not reach negative values. Comparing the slow versus fast drydown, our data suggest that $P$ reaches values closer to zero during the fast drydown at boundary $\Theta_1$. This may be due to generally higher $\pi$ values in mature leaves of these trees because of seasonal effects (i.e. this drydown was performed in September versus June/July) or less severe cell membrane damage and electrolyte leakage during phase I when the drydown is fast (i.e. this drydown was performed over a couple of days versus weeks). However, the biophysical properties of the cell membrane under various levels of drought stress and in response to the type of the drought experiment remain unknown, and only direct $P$ measurements using a cell pressure probe would allow us to resolve these open questions (see previous paragraph).

Pressure-Volume Curve

The Scholander-type pressure chamber has been used successfully to generate pressure-volume curves for the determination of tissue properties such as $\Psi$ (Tyree and Hammel, 1972; Ding et al., 2014). The advantage of the pressure-volume curve is that $\Psi_{\text{TLP}}$ can be determined from a single leaf measurement. The disadvantage is that generating a pressure-volume curve can be time consuming (more than 10 h) depending on the speed of leaf dehydration and requires accessibility to an analytical digital balance to determine relative water content; one technical difficulty is finding the right time interval for progressive leaf dehydration and data collection.

WP Curve

The WP curve method allows predicting $\Psi$ at stomatal closure and $\Psi_{\text{TLP}}$ exclusively from measurements of $\Psi_{\text{pd}}$ and $\Psi_{\text{md}}$ using a Scholander-type pressure chamber. This can be especially useful under remote field conditions or during research operations with limited access to a leaf gas-exchange system, analytical balance, and laboratory space. Moreover, the WP curve method can provide for a time-integrative measurement of $\Psi$ at stomatal closure and $\Psi_{\text{TLP}}$ when collected over the growing season. In this case, it is recommended that plant $\Psi$ be measured during the phenological time frame following leaf maturation and prior to senescence to ensure that leaf cells, xylem, and cuticle are fully developed. For establishing the WP curve, we advocate the following stepwise procedure for the measurement of plant $\Psi$ (Fig. 10).

Step 1: covering of leaf to minimize transpiration. Identify a representative mature leaf in the canopy and cover the leaf with a plastic bag. Seal the plastic bag to allow for a humid environment that aids stomatal closure and minimizes transpiration (Turner and Long, 1980).

Step 2: equilibration of leaf apoplast and symplast. Wrap the sealed plastic bag with aluminum foil to exclude ambient light from the leaf surface. This will further aid stomatal closure. Wait for more than 30 min to allow for equilibration of leaf internal $\Psi$ of apoplast (liquid to the outside of the cellular membrane including xylem liquid) and symplast (liquid to the inside of the cellular membrane). Including the equilibration will ultimately provide a measure of plant $\Psi$ that closely reflects $P_x$ (see introduction).

Step 3: excision of the leaf. Cut the leaf at the petiole (or petiolule) end using scissors or a razor blade.

Step 4: measurement of plant $\Psi$. Insert the covered leaf into the pressure chamber with the cut surface protruding through the seal of the chamber lid. Slowly raise the pressure in the chamber while monitoring the cut surface; use a magnifying glass and a light source if it is difficult to identify xylem vessels. Record the pressure when liquid starts to emerge from open xylem vessels and a meniscus forms on the cut surface.

One alternative procedure to save time is to skip the equilibration step and immediately proceed from step 1 to step 3 (Fig. 10). However, excluding the equilibration step 2 provides a less accurate estimate of $P_x$, since leaf internal $\Psi$ gradients between apoplast and symplast are not minimized prior to leaf excision (Shackel et al., 1997). Another alternative procedure is to seal the excised leaf in a second plastic bag (i.e. to minimize leaf water loss as much as possible) and store the sample at around 4°C for up to 24 h prior to measurement of plant $\Psi$ (Fig. 10); the assumption is that leaf internal $\Psi$ is maintained constant during the storage period, but this should ideally be tested first on a subset of leaves through frequent measurements of $\Psi_{\text{pd}}$ and $\Psi_{\text{md}}$ during the storage period.

Our data show that, opposite to a fast drydown, a controlled and slow drydown (weeks) has the advantage of collecting a higher number of data points during all three phases of the WP curve because $\Psi_{\text{pd}}$ can be determined for a wide range of soil moisture contents. When establishing the WP curve, $\Psi_{\text{pd}}$ can be interpreted as a measurement of soil $\Psi$ when nighttime water loss by transpiration is negligible for the plants analyzed. For plant species that exhibit relatively high rates of nighttime transpiration, the required equilibrium between $\Psi_{\text{pd}}$ and soil $\Psi$ cannot be reached (Donovan et al., 2001). Donovan et al. (2001) came to this conclusion by comparing measurements of soil $\Psi$ and $\Psi_{\text{pd}}$ as obtained from plants with completely bagged and nonbagged canopies during the night period. For walnut trees subjected to the slow drydown, nighttime canopy conductance was more than 100-fold smaller compared with daytime values (Supplemental Fig. S4, inset), and we can conclude that our measurements of

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Ψpd closely reflected soil Ψ for this type of experiment. For every soil type, soil Ψ can be interpreted as the ability of a plant to extract water from the soil (Jones, 2007). However, the breakdown of the soil-to-root hydraulic continuum is suspected to be the trigger for stomatal closure (Carminati and Javaux, 2020). In turn, it can be speculated that boundary Θ1 also provides an estimate when the hydraulic continuum between root and soil starts to break down under increasing water stress by drought independent of the soil type.

Type of Drought Experiment

Limited soil water availability is the factor that causes drought stress. Plant stress responses to drought depend on drought severity and a plant’s ability to adapt to the stress over time (Gilbert and Medina, 2016). Therefore, the experimental procedure of how drought is induced over time (i.e., days versus weeks) can result in a more or less severe stress response for a given level of soil moisture. A slow drydown (weeks) would provide time for stress adaptations linked to plant anatomical changes, for example, root suberization to minimize water loss back to the soil and reductions in vessel diameters to secure long-distance transport capacity (Barrios-Masías et al., 2015; Knipfer et al., 2015, 2018, 2020). Here, we conducted slow and fast drydown experiments for walnut trees to test if the WP curve (1) remains triphasic and (2) predicts stomatal closure in both types of experiments. Our data show that the WP curve was triphasic in both drydown experiments, but the character of the WP curve differed between the slow and fast drydown and boundary values Θ1 and Θ2 were specific to the drydown experiment (Table 1). Boundary Θ1 predicted the shift in the Ψ threshold of stomatal closure in both types of drydown experiment. We speculate that boundary Θ1 was less negative during the fast drydown (i.e., earlier stomatal closure) because the time period for anatomical adaptations, which would aid in maintaining plant performance for the imposed level of stress, was too short. Data by Knipfer et al. (2020) show that walnut fine roots develop a multiseriate endodermis in response to a slow drydown, and data by Meyer et al. (2009) indicate that the development of a multiseriate exodermis in Iris germanica requires ~12 d. However, and especially for the purpose of using the WP curve method to select for genotypes with improved drought resistance, future work is required to exactly determine the effects of experimental time period, soil medium, irrigation frequency, and pot characteristics on the shape of the WP curve (Turner, 2019).

CONCLUSION

The WP curve method represents a methodological advancement of the Scholander-type pressure chamber to predict Ψ at stomatal closure and turgor loss. Based exclusively on measurements of plant Ψ at predawn and midday, the method presented here provides an alternative tool for the study of plant stress physiology in natural and agricultural ecosystems. According to published data for grapevine and under the assumption that plant Ψ reported by these authors (i.e., measured under laboratory low-light conditions) is most representative of Ψpd (Choat et al., 2010; Brodersen et al., 2013; Cuneo et al., 2016), the following can be concluded: (1) root cell damage is initiated at boundary Θ1 at a Ψ threshold that corresponds to stomatal closure; (2) root hydraulic conductivity declines progressively during phases I and II and reaches a minimum at boundary Θ2 at a Ψ threshold that corresponds to leaf turgor loss; and (3) vessel cavitation is initiated at boundary Θ2 and cavitated vessels accumulate during phase III. This example demonstrates that our WP curve method can be used to categorize the sequence of physiological and anatomical events that occur under progressive drought stress into three distinct phases. Moreover, we propose that the WP curve method can assist in the determination of Ψ thresholds that mark the breakdown of the soil-to-root hydraulic continuum (Carminati and Javaux, 2020; Rodriguez-Dominguez and Brodribb, 2020) and xylem hydraulic failure by embolism (Cuneo et al., 2016; Knipfer et al., 2019) and facilitate the selection for woody perennial genotypes with improved drought resistance (Knipfer et al., 2020).
MATERIALS AND METHODS

PLR Model

Initial data inspection showed that a simple linear model did not appropriately describe the relationship of plant $\Psi$ and $\Psi_{sm}$ for the three woody species, walnut (Juglans spp.), grapevine (Vitis spp.), and almond (Prunus dulcis). For this reason, the PLR model was developed for analysis of the WP curve:

$$
\Psi_{sm}(\Psi) = \left\{ \begin{array}{ll}
\alpha + \beta_1 \Psi & , \quad \Psi \leq \Psi_1 \\
\alpha + \beta_2 (\Psi - \beta_1) + \beta_3 \Psi & , \quad \Psi_1 < \Psi \leq \Psi_2 \\
\alpha + \beta_3 (\Psi - \beta_2) + \beta_4 (\Psi - \beta_3) + \beta_5 \Psi & , \quad \Psi > \Psi_2
\end{array} \right.
$$

The PLR model was used to calculate the boundaries between linear phases ($\Psi_1$ to $\Psi_2$) and phases II and III and corresponding slope values ($\beta_1$, $\beta_2$, $\beta_3$, $\beta_4$, $\beta_5$, $\Psi_1$, $\Psi_2$). To parameterize the PLR model, an estimate of the transition points between phases I and II ($\Psi_1$) and phases II and III ($\Psi_2$) was obtained mathematically as follows. First, a smoothed line was fitted to the relationship of $\Psi_{sm}$ and $\Psi_{sm}$ that best described the data pattern. Subsequently, slope values ($m$) of the smoothed line were determined by $\partial \Psi_{std}/\partial \Psi_{st}$ for $d = 0.01$, and maximum $m$ in phase I ($-\infty$) and phase III ($+\infty$) and minimum $m$ in phase II ($-b$) were identified. Parameters $a$, $b$, and $c$ were used to determine $\gamma = [-b - a] / 2 + a$ and $\gamma_1 = [-b - a] / 2 + b$. Based on the information of $\gamma_1$ and $\gamma_2$, all parameters for the PLR model were fitted using a least-squares solver implemented in Python (pwl package version 1.1.6; Jekel and Venter, 2019). This allowed for fitting a continuous piecewise linear function to corresponding data of $\Psi_{sm}$ and $\Psi_{std}$ for a specified number of three line segments. Our method used a limited memory Broyden-Fletcher-Goldfarb-Shanno algorithm for bound constrained optimization to obtain a statistical solution of boundary values $\Psi_1$ and $\Psi_2$ from the initial estimates of $\gamma_1$ and $\gamma_2$ and $P$ values corresponding to output parameters of the PLR model were the result of using this optimization procedure to find boundaries $\Psi_1$ and $\Psi_2$ that best satisfied the specified number of linear segments; $s$ values were obtained following the derivation of Coppe et al. (2011) for linear regression problems.

Plant Materials

The WP curve method was tested initially for data collected during a drought screening trial on potted walnut trees (lathouse trial). Subsequently, the method was further evaluated by reanalyzing data that were collected during drought screening trials of potted grapevines (greenhouse trial) and potted almond trees (open field trial) at the University of California, Davis. Therefore, growing conditions and experimental procedures for data collection for the three woody species were not identical and are best summarized as follows based on the information available.

Walnut Experiments

Experiments were performed in 2019 on $n = 71$ walnut trees (’Cisso’). Trees (stem diameter and height of around 2.5 cm and 1 m, respectively) were obtained from Sierra Gold Nursery and transplanted into 15-L plastic pots on February 17, 2019. Pots were filled with a similar amount of soil mix (~30% washed sand and 50% sphagnum peat moss) by leaving a gap of around 2 cm to the upper edge of the pot. A slow-release fertilizer (Osmocote Smart-Release Plus) was added to the topsoil layer. Growth was maintained under ambient atmospheric conditions (day/night length was on average 14/10 h, and temperature was ~13°C/32°C) in a lathouse at the University of California, Davis. Trees were irrigated by supplying water to the top of the soil every 2 d and maintained well-watered for 3 months after transplanting to ensure sufficient time for tree establishment. All physiological measurements were performed on mature leaves of current-year shoots. A temperature and relative humidity sensor (HMP50; Vaisala) was installed at the plot site to monitor VPD (Supplemental Fig. SS). To investigate possible effects of the type of drought experiment, trees were subjected to a slow drydown (i.e. weeks, adjustment of irrigation) or a fast drydown (i.e. days, no supplemental irrigation). The slow drydown experiment was performed on $n = 65$ trees. Irrigation was adjusted based on estimates of bulk soil moisture ($\% = \text{weight change/weight dry (as-} \text{wet)}$) as calculated from pot weights (for details, see Knipfer et al., 2020). A subset of $n = 27$ plants was located on mini-weighing lysimeters to continuously monitor pot weight and SM (see Supplemental Fig. S6 for representative data of two individuals); to account for temporal effects, trees were either maintained well-watered or subjected to a drydown. At 103 d after transplanting, measurements were performed on $n = 27$ trees with SM of individuals ranging from 40% to 90% (w/w); VPD ranged from 2.1 to 2.9 kPa between 11 AM and 1 PM. At 150 d after transplanting, measurements were carried out on $n = 36$ trees with SM ranging from 58% to 87% (w/w); VPD ranged from 1.9 to 2.5 kPa). At 162 d after transplanting, measurements were performed on $n = 30$ trees with SM ranging from 44% to 75% (w/w); VPD ranged from 1.7 to 2.2 kPa). The fast drydown experiment was performed on $n = 6$ trees that were maintained well-watered until the start of the drydown. Supplemental irrigation was stopped at 210 d after transplanting. Trees were analyzed at 210 d (SM ranging from 73% to 100% [w/w]), 213 d (69% to 96% [w/w]), 216 d (54% to 86% [w/w]), 218 d (49% to 77% [w/w]), and 220 (47% to 72% [w/w]) after transplanting.

Ψ

A pressure chamber (PMS Instrument; model 1305D) was used to measure plant $\Psi$ following leaf covering and equilibration (Fig. 10). Measurements were performed on a leaflet of a mature leaf that was covered with aluminum foil and equilibrated for more than 1 h using a sealed plastic bag. Following excision of the leaflet at the petiole, the plastic bag was removed and leaflets still covered with foil (i.e. to exclude effects of transpiration; Turner and Long 1980) were inserted into the pressure chamber. The pressure in the chamber was raised slowly at a constant rate (about 0.01 MPa s$^{-1}$), and pressure was recorded when a water meniscus started to form on the cut petiole (midvein of leaflet) surface. For the same plant, $\Psi_{sm}$ was measured prior to sunrise between 4 and 6 AM. $\Psi_{sm}$ was measured between 11 AM and 1 PM Pacific Daylight Time. Watering was always completed the day before measurement of plant $\Psi$ to allow for soil water distribution.

Leaf Gas Exchange

$g$, and $A$ were measured between 11 AM and 1 PM using a LICOR-6800 gas-exchange system (fan speed at 10,000 rpm, leaf temperature at 24.5°C, CO$_2$ sample at 400 µL L$^{-1}$, and 1,500 µmol m$^{-2}$ s$^{-1}$ light intensity). One nonsheathed leaflet of a mature leaf was measured on each sapling that was in proximity to the leaflet used for measurements of plant $\Psi$ (as described above). Since the leaf area inserted in the cuvette of the gas-exchange system during measurement occupies many stomata and the response of individual stomates within this area can be more or less coordinated, the point of stomatal closure was defined as the point where $g$, was reduced by around 90%.

P and π

From the same leaf used for plant $\Psi$ and leaf gas exchange, a leaflet was excised with scissors. Immediately after the leaf lamina was separated from the midvein using a razor blade (i.e. to minimize the contribution of apoplastic xylem sap), the leaf lamina (2 cm-long portion located halfway along the leaflet) was placed in a 2-mL Eppendorf tube containing a mesh filter and stored on ice. Great care was taken to ensure that the procedure, from initial leaflet excision to storage of the Eppendorf tube on ice, was completed within 30 s to minimize drying artifacts. All samples were kept on ice, transported to the laboratory within 1 h following sampling, and stored at ~8°C until further analysis. For analysis of leaf sap $p$, samples were thawed at room temperature for 15 to 20 min, centrifuged at 8,000 rpm for 15 min to extract leaf sap, and the osmolality of leaf sap was measured using a vapor pressure osmometer (VAPRO 5600; Wescor; Barrios-Masatas et al., 2019; Knipfer et al., 2020). Measured values of sap osmolality (in mOsmol kg$^{-1}$) were converted to units of $\pi$ (0.1 MPa = 40.75 mOsmol kg$^{-1}$). $\Psi_{tLP}$ was determined from pressure-volume curves measured on a leaflet. Prior to measurements, one mature leaf per plant was excised, the cut end of the petiole was submerged in water, and the leaf was covered with a plastic bag and transported to the lab within 30 min. Subsequently, the leaf was allowed to rehydrate in the darkness for 24 h. A leaflet was excised from the compound leaf, and $\Psi_{sm}$ was measured using the benchtop drydown method as described by Sack and Pasquet-Kok (2010). Leaflet fresh weight was measured with a digital balance before and after each $\Psi$ step measurement (PMS Instrument; model 1305D). Measurements were repeated until five measurements after the $\Psi_{tLP}$.

Grapevine Experiments

Experiments were performed in 2019 on 48 grafted vines (cv Chardonnay on rootstock SC, 420A, Riparia Gloire, 101-14, Ramsey, 140Ru, 1103P, and 110R).
Vines were planted in 6-L pots. Pots were filled with similar amounts of soil mix (approximately 75% coconut coir and 25% perlite) by leaving a gap of around 2 cm to the upper edge of the pot. Growth was maintained in a greenhouse on the University of California, Davis, campus. Vines were allowed to establish for 3 months prior to data collection. During the establishment period, the vines were pruned to a single shoot, which was staked and tied after reaching approximately 0.5 m in length. The pots were weighed and irrigated by supplying water to the top of the soil three times per week to a target weight (pot weight at saturation + half of pot evapotranspiration; evapotranspiration was the difference in pot weights). On September 9, half of the vines were randomly assigned to each of two watering treatments. The well-watered vines continued to receive the same watering regime as during the establishment period, while the water-stressed vines were watered to 40% of the saturated pot weight plus half of pot evapotranspiration. Water was withheld from water-stressed vines until the pots reached the target weight. Three vines per rootstock × treatment combination (n = 48) were assessed for gas exchange and \( \Psi_{pd} \) and \( \Psi_{sat} \) on November 4 and 12. On December 9, supplemental watering was stopped for one vine per rootstock from the water-stressed treatment to allow for severe drought conditions.

**Leaf Gas Exchange**

Measurements were performed for one mature, fully expanded leaf per vine that was in proximity to the leaf used for \( \Psi \) measurements. The sampled leaves were selected from exterior, sunlit canopy positions. The gas-exchange measurements were conducted at the same time as plant \( \Psi \) measurements using a LiCor-6800 gas-exchange system at a constant fan speed (10,000 rpm), sample chamber VPD (1.5 kPa), CO\(_2\) concentration (400 \( \mu \)mol mol\(^{-1} \)), and light intensity (1,000 \( \mu \)mol m\(^{-2} \) s\(^{-1} \)). Leaf temperature ranged from 26.9°C to 27.5°C.

**Almond Experiments**

Experiments were performed in 2014 on \( n = 16 \) potted 4-year-old almond trees (‘Nonpareil’ grafted on rootstock Nemaguard). Pots (56 L) were filled with similar amounts of soil mix (approximately 60% plaster sand and 40% pea moss). Slow-release fertilizer spikes (Miracle-Gro Fruit Fertilizer) were inserted in each pot. Potted vines were maintained in an open field at the Orchard Park Greenhouse Complex at the University of California, Davis, campus. Throughout the experimental period, the daily maximum temperature ranged from 19.4°C to 50.6°C. Over the experimental period, trees were watered by supplying water to the top of the soil either once every other day (approximately 0.5 m in length). The pots were weighed and irrigated by supplying water to the top of the soil three times per week to a target weight (pot weight at saturation + half of pot evapotranspiration; evapotranspiration was the difference in pot weights). On September 9, half of the vines were randomly assigned to each of two watering treatments. The well-watered vines continued to receive the same watering regime as during the establishment period, while the water-stressed vines were watered to 40% of the saturated pot weight plus half of pot evapotranspiration. Water was withheld from water-stressed vines until the pots reached the target weight. Three vines per rootstock × treatment combination (n = 48) were assessed for gas exchange and \( \Psi_{pd} \) and \( \Psi_{sat} \) on November 4 and 12. On December 9, supplemental watering was stopped for one vine per rootstock from the water-stressed treatment to allow for severe drought conditions.

**Supplemental Data**

The following supplemental materials are available.

**Supplemental Figure S1.** Recordings of pot weight and bulk soil moisture for walnut trees.

**Supplemental Figure S2.** Representative images of the canopy of walnut trees.

**Supplemental Figure S3.** \( \Psi \) curve collected for walnut genotypes RX1 and VX211.

**Supplemental Figure S4.** Relationship between \( \Psi \) and canopy conductance of walnut trees.

**Supplemental Figure S5.** Recordings of VPD for walnut trees.

**Supplemental Figure S6.** Relationship of \( \Psi_{pd} \) and bulk soil moisture of walnut trees.

**Supplemental Table S1.** \( \Psi_{FLP} \) determined from pressure-volume curves for walnut trees.

**Supplemental Table S2.** Z test used for statistical comparison of output parameters shown in Table 1.

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