Role of leaf hydraulic conductance in the regulation of stomatal conductance in almond and olive in response to water stress

RUNNING TITLE: LEAF HYDRAULIC FUNCTION IN ALMOND AND OLIVE

VIRGINIA HERNANDEZ-SANTANA*, CELIA M. RODRIGUEZ-DOMINGUEZ, J. ENRIQUE FERNÁNDEZ, ANTONIO DIAZ-ESPEJO

Irrigation and Crop Ecophysiology Group, Instituto de Recursos Naturales y Agrobiología de Sevilla (IRNAS, CSIC). Avenida Reina Mercedes, nº 10, 41012-Sevilla, Spain. +34 954624711.

* Corresponding author: virginiahsa@gmail.com

Key words: drought, Dynamic rehydration kinetics method, Evaporative flux method, fruit crops, deficit irrigation, Olea europaea, Prunus dulcis
ABSTRACT

The decrease of stomatal conductance ($g_s$) is one of the prime responses to water shortage and the main determinant of yield limitation. Understanding the mechanisms related to stomatal closure in response to imposed water stress is crucial for a correct irrigation management. The loss of leaf hydraulic functioning is considered one of the major factors triggering stomatal closure. Thus, we conducted an experiment to quantify the dehydration response of leaf hydraulic conductance ($K_{leaf}$) and its impact on $g_s$ in two fruit tree Mediterranean species, one deciduous (almond) and one evergreen (olive).

Our hypothesis was that a higher $K_{leaf}$ would be associated with a higher $g_s$ and the reduction in $K_{leaf}$ would predict the reduction in $g_s$ in both species. We measured $K_{leaf}$ in olive and almond during a cycle of irrigation withholding. We also compared the results of two methods to measure $K_{leaf}$: dynamic rehydration kinetics and evaporative flux methods. We also determined $g_s$, leaf water potential ($\Psi_{leaf}$), vein density, photosynthetic capacity and turgor loss point. Results showed that $g_s$ was higher in almond than in olive and so was $K_{leaf}$ ($K_{max} = 4.70$ and $3.42$ mmol s$^{-1}$ MPa$^{-1}$ m$^{-2}$, in almond and olive, respectively) for $\Psi_{leaf}>-1.2$ MPa. At greater water stress levels than -1.2 MPa, however, $K_{leaf}$ decreased exponentially being similar for both species while $g_s$ was still higher in almond than in olive. We conclude that although the $K_{leaf}$ decrease with increasing water stress does not drive unequivocally the $g_s$ response to water stress, $K_{leaf}$ is the variable most strongly related to the $g_s$ response to water stress, especially in olive. Other variables such as the increase in ABA may be playing an important role in the $g_s$ regulation although in our study, the $g_s$ -ABA relationship did not show a clear pattern.
**Introduction**

Fruit tree orchards are among the agronomical systems that are most threatened by reduced water availability and climate change. Despite the high water demand of these orchards, they respond markedly to deficit irrigation practices (Fereres and Soriano 2007, Ruiz-Sanchez et al. 2010). This explains the increasing demand to understand the physiological mechanisms involved in their response to imposed water stress (Rinaldi and He 2014). This is particularly important when plant-based sensors are used for water stress assessment. A correct interpretation of the collected outputs is then required for accurate irrigation scheduling. Stomatal conductance regulation is considered a major mechanism responsible for regulating the plant response to water stress, since stomatal closure is one of the earliest responses to water shortage and the main determinant of limitation to photosynthesis (Flexas et al. 2014), and hence, yield.

Stomatal control is regulated to optimize both the outward diffusion of water vapor and the diffusion of CO₂ into the leaf during photosynthesis (Hetherington and Woodward 2003). However, the mechanisms producing stomatal closure under water stress conditions still remain a matter of debate (Buckley 2005, Brodribb 2009, Brodribb and MacAdam 2011, Pantin et al. 2013). The loss of plant hydraulic functioning is considered one of the main driving factors of stomatal closure (Brodribb and Holbrook 2003, Brodribb and Jordan 2008, Brodribb and Cochard 2009, Torres-Ruiz et al. 2014), since the same volume of water leaving the stomata as vapor must be replaced by liquid water flowing through the vascular system (Brodribb 2009). The other major mechanism considered to trigger stomatal closure is the increase of chemical signals such as ABA (Abscisic acid) in the leaf (Tardieu and Simonneau 1998, Dodd 2005).
Among all the resistances that water has to overcome through the plant, the leaf has been demonstrated to be a major one (Nardini and Salleo 2000, Nardini et al. 2001, Sack et al. 2003, Brodribb and Holbrook 2003) and, thus, it may play an important role in the regulation of stomata (Guyot et al. 2012). Consequently, leaf hydraulic conductance ($K_{\text{leaf}}$) may be a major determinant of plant productivity and growth (Sack and Holbrook 2006). However, the effect of $K_{\text{leaf}}$ on stomatal conductance ($g_s$) is still poorly understood and few studies have addressed the coordination dynamics between both variables (Brodribb and Holbrook 2004, Lo Gullo et al. 2005, Brodribb and Jordan 2008, Blackman et al. 2009, Gortan et al. 2009, Johnson et al. 2009). Furthermore, the extent to which $K_{\text{leaf}}$ declines with water stress varies from species to species, even within a particular habitat (Sack et al. 2003, Brodribb and Hobrook 2003, Lo Gullo et al. 2005, Johnson et al. 2009, Scoffoni et al. 2012) and the knowledge of its relationship to leaf structure and ecological strategy remains incomplete (Blackman et al. 2010). For these studies, reliable and fast $K_{\text{leaf}}$ measurements would be of great help. Although there are new approaches that can provide $K_{\text{leaf}}$ measurements within minutes, such as the “dynamic rehydration kinetics method” (DRKM, Blackman and Brodribb 2011), in opposition to more traditional and time-consuming options such as the “evaporative flux method” (EFM), some uncertainties are still unresolved. These include the identification of the flow pathways during leaf rehydration, upon which DRKM measurements are based, and how similar these pathways are to those of transpiration (Flexas et al. 2013).

Thus, we conducted a study with the ultimate objective of contributing to the understanding of $g_s$ regulation. We focused on the impact of $K_{\text{leaf}}$ on $g_s$ in an evergreen (olive) and a deciduous (almond) Mediterranean species, i.e. two species occurring in the same area but with different ecological strategies. Specifically, our objectives were: (i) to test the impact of DRKM and EFM as well as tree age on the values of $K_{\text{leaf}}$; (ii) to
determine the $K_{\text{leaf}}$ response to dehydration in both olive and almond, and potentially
related structural and functional leaf traits; and finally (iii) to study the effect of $K_{\text{leaf}}$ on
$g_s$ in response to water deficit. We further compared the effect of $K_{\text{leaf}}$ on $g_s$ with the
impact of ABA on $g_s$. For the first objective, we hypothesized that both methods would
produce similar $K_{\text{leaf}}$ values, because the flow pathways used in leaf rehydration (basis
for DRKM measurements) would be the same as the pathways followed by the water on
its way to the leaf evaporation sites (basis for EFM measurements). For the second
objective, we hypothesized that we would find differences by comparison between the
species in maximum $K_{\text{leaf}}$ and leaf traits such as vein density, photosynthetic capacity,
and turgor loss point. For the third objective, we expected that a higher $K_{\text{leaf}}$ would be
associated with a higher $g_s$, and the reduction in $K_{\text{leaf}}$ would predict the reduction in $g_s$,
to a greater extent than ABA level.

Materials and methods

The olive (Olea europaea L., cv Arbequina) and almond (Prunus dulcis (Mill.) D.A.
Webb, cv. Guara) trees used in this study were located in Seville (Spain) (37º 15’ N, -5º
48’ 102 W). The area has a Mediterranean climate with hot and dry weather from May
to September, being mild and wet for the rest of the year. Measurements were taken
from May to October, both in 2013 and 2014. The sampled plants were regularly
irrigated before the beginning of the experiments to replace their water needs.

Response of leaf hydraulic conductance to dehydration: methods and tree age effect

We aimed to conduct a study comparing $K_{\text{leaf}}$ values obtained using different methods,
tree age material, and single leaves vs. terminal parts of shoots. The data obtained in
these methodological experiments were independent of the data collected in the water
deficit experiment. Leaf hydraulic conductance was measured in fully developed, current year and sun-exposed leaves in both species. This was the same for the test comparing plant material, but in this test also the terminal parts of olive shoots were used. Before taking the measurements, we cut branches long enough to avoid any xylem embolism and put them in dark plastic bags with wet paper towel inside to equilibrate for at least 30 min. Leaves and terminal parts of shoots were cut from the branches under purified water. They were then rapidly connected under water to a flowmeter consisting of silicon tubing containing purified and degassed water. The tubing connected the leaf to a pressure transducer (PX26-005GV, Omega Engineering Ltd., UK), which was, in turn, connected to a Campbell data logger CR1000 (Campbell, Campbell Scientific Ltd., UK) to register and store readings every 1 s to calculate the flow rate through the leaf (mmol s\(^{-1}\) m\(^{-2}\)). Reference tubing of different resistances was used to minimize measurement errors (Sack et al. 2011, Melcher et al. 2012). Some branches were allowed to dehydrate before measurement for increasing periods to obtain a wide range of leaf water potential values (\(\Psi_{\text{leaf}}\)).

**Comparison of DRKM and EFM methodologies**

To assess the differences between the \(K_{\text{leaf}}\) vulnerability curves potentially produced by the use of the two tested methods, a group of leaves (Table 1) was measured with either DRKM \((K_{\text{IDRKM}})\) or EFM \((K_{\text{EIFM}})\). The examination of leaf hydraulic methods was conducted using different types of plant material. With olive, we used leaves from eight 6-year-old potted trees and three 8-year-old trees and with almond, we used leaves from eight 6-year-old potted trees and three 13-year-old trees. For the DRKM measurements (Brodribb and Holbrook 2003, Blackman and Brodribb 2011) the leaves were connected to the flowmeter, as described in the former section,
until flow rate decayed from its maximum as leaves rehydrated. Initial flow was
determined by fitting an exponential curve through the first 20 s of the rehydration flow
data and extrapolating back to the initial point of leaf excision, considering the time (s)
required to connect the leaf to the flowmeter (Blackman and Brodribb 2011). After
connecting the leaves to the flowmeter, they were covered with moist paper and had no
exposure to light in order to prevent transpiration. Prior to the flow rate determination,
initial leaf water potential ($\Psi_0$) was measured in the neighboring leaf. Final leaf water
potential ($\Psi_{DRKM}$) was also measured in the leaf used to determine the flow after being
allowed to equilibrate for at least 30 min. A test conducted with rehydrated olive leaves
at different leaf water potentials demonstrated that after 30 min the leaf water potential
hardly changed.

For the EFM measurements (Scoffoni et al. 2012), the leaves were connected to the
same flowmeter, but they were allowed to transpire above a large box fan and under a
light source (> 1,000 μmol m$^{-2}$ s$^{-1}$ photosynthetically active radiation) for at least 30 min
until the flow was stable (coefficient variation <5% in the last 5 min). In EFM, it is
essential that the flow rate reaches a steady state, because the method assumes a stable
leaf water potential ($\Psi_{EFM}$) after flow rate reaches the steady state (Scoffoni et al.
2012). $\Psi_0$ was measured as in DRKM.

In addition, and to compare the effect of both methods on $K_{leaf}$ values and not
only on $\Psi_{leaf}$– $K_{leaf}$ curves, direct comparisons were conducted by determining $K_{leaf}$ in 26
leaves of both species using both methods. After connecting the leaves to the flowmeter,
transpiration was prevented, as previously described for DRKM, until the leaves were
rehydrated. Then, initial flow rate was determined following DRKM. Later, and while
the leaves were still connected to the flowmeter, they were allowed to transpire under
the same conditions described before for EFM, and then steady-state flow rate and
Ψ_{EFM} were obtained following EFM.

Thus, for each method, \( K_{\text{leaf}} \) was calculated as:

\[
K_{\text{IEFM}} = \frac{E}{(-\Psi_{\text{EFM}} - 0)A_{\text{leaf}}}, \quad (1)
\]

\[
K_{\text{IDRKM}} = \frac{-I}{\Psi_0 A_{\text{leaf}}}. \quad (2)
\]

In Eq. 1, \( K_{\text{IEFM}} \) is leaf hydraulic conductance measured using EFM, \( E \) is steady-state transpiration determined with the readings of the flowmeter (mmol s\(^{-1}\)), and \( A_{\text{leaf}} \) is leaf area (m\(^2\)). In Eq. 2, \( K_{\text{IDRKM}} \) is leaf hydraulic conductance measured with DRKM, \( I \) is the instantaneous initial maximum flow rate into the leaf (mmol s\(^{-1}\)), and \( \Psi_0 \) is the initial leaf water potential (MPa). To correct for changes in \( K_{\text{leaf}} \) induced by temperature dependence of water viscosity, standardized \( K_{\text{leaf}} \) values at 25°C were calculated (Scoffoni et al. 2012).

To construct the vulnerability curve for each species, \( K_{\text{leaf}} \) was then related to the lowest \( \Psi_{\text{leaf}} \), i.e. \( \Psi_0 \) in DRKM and \( \Psi_0 \text{ or } \Psi_{\text{EFM}} \) in EFM (Scoffoni et al. 2012). All \( \Psi_{\text{leaf}} \) measurements were made with a Scholander-type pressure chamber (PMS Instrument Company, Albany, Oregon, USA).

**Tree age test**

For the tree age test, for olive we compared the \( K_{\text{leaf}} \) data of eight 6-year-old potted trees with \( K_{\text{leaf}} \) data of three 8-year-old trees. With almond, we compared \( K_{\text{leaf}} \) of eight potted 6-year-old trees with \( K_{\text{leaf}} \) of three 13-year-old trees. Only DRKM was used in the \( K_{\text{leaf}} \) determination.
Single leaves—terminal shoots test

An additional experiment was performed, with the olive trees only, to assess the effect on $K_{\text{leaf}}$ of any potential artifact derived from the measurement of the terminal parts of shoots as opposed to the single leaves. For this test, we used plant material of the same eight 6-year-old potted olive trees. $K_{\text{leaf}}$ was measured using DRKM.

Structural and functional leaf traits

The following measurements were conducted to analyze the coordination between leaf traits.

Vein density

Vein density (VD), defined as length of vein per unit leaf area (mm mm$^{-2}$), was sampled using ten leaves per species. They had similar characteristics to the leaves used for the rest of measurements (fully developed, current year and sun-exposed leaves of potted trees of 6- and 8-year-old olive trees and 6- and 13-year-old almond trees). The major first vein was not considered. Leaves were slightly sanded and cleared using 5% NaOH solution, changed every 2 days for a total of 8 days in olive and 5 days in almond. To remove any remaining pigment from the leaves after the chemical clearing, we used a 50% bleach solution on the leaf for 10–20 s. Images of cleared and stained leaves with 1% safranin were captured using a Canon Powershot A620 camera mounted on a stereoscope (Zeiss Stereo Discovery V8, Germany). Images of 11 mm$^2$ were taken centrally in the top, middle, and bottom thirds of the leaves and the ImageJ program 1.48v (Schneider et al. 2012) was used to quantify the vein lengths.
Leaf hydraulic vulnerability parameters

To compare the two species in their hydraulic parameters, $K_{\text{leaf}}$ at full hydration ($K_{\text{Lmax}}$) was determined for both species using the average $K_{\text{leaf}}$ for data above -0.5 MPa (Scoffoni et al. 2012). $\Psi_{\text{leaf}}$ values, for which $K_{\text{Lmax}}$ had declined by 50% and 80% (P50 and P80, respectively), were calculated. P50 and P80 were used as indicators of vulnerability of leaf hydraulic conductance to decreasing water potential.

Pressure-volume curves and turgor loss point

We used five leaves of the 13-year-old almond and 8-year-old olive trees to calculate the pressure-volume curves. Leaves were sampled in the morning of October 7 of 2014 (a few days after the water deficit experiment ended) and were rehydrated for 24 h, then left to desiccate. Leaf weight and leaf water potentials were measured at least nine times during that period until the leaves reached minimum $\Psi_{\text{leaf}}$ values of ca. -5 MPa. The turgor loss point (TLP) was calculated as the intersection point of the two curves represented by the inflection point of the relation $1/\Psi_{\text{leaf}}$ vs. 1-relative leaf water content.

Photosynthetic response curves

Five $A$-$C_i$ response curves (the response of net $\text{CO}_2$ assimilation to varying intercellular $\text{CO}_2$ concentration) per species were measured between 09:00 and 13:00 GMT during the experimental period. Measurements were made using a Li-cor LI-6400 portable photosynthesis system (Li-cor, Lincoln, NE, USA) at ambient temperature, saturating PPFD (photosynthetic photon flux density, 1,600 $\mu$mol m$^{-2}$ s$^{-1}$) and an ambient $\text{CO}_2$ concentration ($C_a$) of between 50 and 1,500 $\mu$mol mol$^{-1}$. After steady-state photosynthesis was achieved, the response of $A$ to varying $C_i$ was measured by lowering
Ca stepwise from 390 to 50 µmol mol\(^{-1}\), returning to 390 µmol mol\(^{-1}\), and then increasing Ca stepwise from 390 to 1,500 µmol mol\(^{-1}\). Each A-C\(_i\) curve comprised 16 measurements, each made after at least 3 min at each Ca. Maximum carboxylation rate (\(V_{\text{cmax}}\)) was estimated by the curve fitting method proposed by Ethier and Livingston (2004). Rubisco kinetic parameters were taken from the literature (Bernacchi et al. 2002).

Response to soil water deficit: the effect of leaf hydraulic conductance on stomatal conductance

This last study consisted of a drydown experiment using potted plants, which aimed at determining whether the decline in \(K_{\text{leaf}}\) with increasing dehydration matched the decline in \(g_s\). Three potted olive and almond trees of 8 and 13 years, respectively, were gradually stressed by withholding irrigation for 13 days with olive and 24 days with almond. No plant deaths were reported. Measurements of \(K_{\text{leaf}}\), \(g_s\), \(\Psi_{\text{leaf}}\), and \(\text{ABA}_{\text{leaf}}\) were conducted on the terminal parts of shoots and leaves of the two species, sampled from the outer part of the canopy facing S-E at ca. 1.5 m above ground. Two samples from three trees per species were used at predawn (05:00) and at 08:30 GMT, when \(g_s\) is at its maximum (\(g_{\text{smax}}\)). We obtained the time for \(g_{\text{smax}}\) through daily \(g_s\) measurement cycles in olive (Fernández et al. 1997) and almond (Rodriguez-Dominguez et al., personal communication). Maximum stomatal conductance was measured in the same leaves throughout the experiment, when possible. \(K_{\text{leaf}}\) and \(\Psi_{\text{leaf}}\) were measured using leaves or terminal parts of shoots from the same branches. The measurements were conducted on four clear and sunny days in September 2014 (September 2, 5, 10, and 15 for olive and 2, 5, 15, and 26 for almond; technical problems impeded same-day measurements in olive and almond). \(K_{\text{leaf}}\) was measured using DRKM as described.
before. The maximum stomatal conductance \( (g_{\text{max}}, \text{mol m}^{-2} \text{s}^{-1}) \) was measured with a Li-cor LI-6400 portable photosynthesis system (Li-cor) with a 2 × 3 cm standard chamber at ambient light and CO₂ conditions.

Leaves collected at the same time intervals as the rest of measurements were used to determine ABA_{leaf} concentration. ABA_{leaf} was measured by the liquid chromatography-electrospray/tandem mass spectrometry method of Gómez-Cadenas et al. (2002). Samples of about 400 mg of frozen leaf tissue, midribs not included, were milled, homogenized and extracted in 5 mL of distilled water using a benchtop homogenizer (Polytron PT 1600 E, Kinematica AG, Switzerland). An aliquot of 50 μL of 2-ppm deuterated abscistic acid (dABA) was previously added as an internal standard. Samples were centrifuged (20,000 rpm, 15 min, 4°C), supernatants were acidified to pH 3.0 (150 μL acetic acid 30% v/v), and leaf extracts were twice partitioned with 3 mL of diethyl ether. Organic phases were collected in Erlenmeyer flasks and evaporated using a vacuum pump. Tube walls were washed with 1 mL diethyl ether and desiccated again. Dry residues were re-suspended in 500 μL methanol, completed to a total volume of 1 mL with Milli-Q quality (reverse osmosis) water and filtered through a 13 mm diameter polypropylene membrane syringe filter (Ø 0.22 μm, RephiQuik PTFE Non-sterile Syringe Filter, RephiLe Bioscience Ltd., China). A calibration line was also prepared with different ABA concentrations (5, 10, 25, 50, and 100 ppb) and the internal standard dABA. Measurements were conducted using an Agilent 1290 Infinity HPLC system (Agilent Technologies Inc., CA, USA) coupled with an electrospray/tandem mass spectrometer (3200 QTRAP® LC/MS/MS System, AB SCIEX, Framingham, MA, USA) and data were analyzed with mass spectrometry software (Analyst® Software, AB SCIEX). Leaf ABA was normalized by fresh weight (g).
The main weather variables in the area were monitored by a Campbell weather station (Campbell Scientific Ltd., Shepshed, UK). The station recorded 30 min average values of air temperature ($T_a$), air humidity ($\text{RH}_a$), global solar radiation ($R_s$), and photosynthetically active radiation (PAR), among other variables.

Leaf hydraulic conductance measured at 8:30 GMT was compared with $K_{\text{plant}}$, calculated according to Ohm’s law analogy assuming steady-state conditions:

$$K_{\text{plant}} = \frac{g_{\text{smax}} D}{\Psi_s - \Psi_{\text{leaf}}}, \quad (3)$$

where $g_{\text{smax}}$ (mol m$^{-2}$ s$^{-1}$) is the stomatal conductance measured at 08:30 GMT, $D_a$ is the air vapor pressure deficit (mmol mol$^{-1}$) determined from measurements by the weather station mentioned above, $\Psi_s$ is the soil water potential that is assumed to be similar to $\Psi_{\text{leaf}}$ at predawn ($\Psi_{\text{pd}}$, MPa), and $\Psi_{\text{leaf}}$ is the leaf water potential measured at 08:30 GMT. All leaf water potentials were measured with the Scholander-type pressure chamber already mentioned.

**Data processing and statistical analysis**

The most-used functions in plant hydraulic studies (linear, sigmoidal, logistic, and exponential) were fitted to our dataset of leaf vulnerability curves ($\Psi_{\text{leaf}} - K_{\text{leaf}}$), using maximum likelihood, as described in Scoffoni et al. (2012). The function with the lowest Akaike information criterion (AIC) and highest $R^2$ was chosen as the best fit function. Outlier tests were conducted for each 0.5 MPa interval using Dixon’s test (Sokal and Rohlf, 1995) for the vulnerability curves.

Statistical analyses were used to compare $g_{\text{smax}}$, $K_{\text{leaf}}$, and ABA_{leaf} between species for two $\Psi_{\text{leaf}}$ ranges: one going from -1.2 to -2.1 MPa ($n = 6$ and 9 for olive and...
almond, respectively) and the other from -2.7 to -3.0 MPa (n = 2 and 3, for olive and almond, respectively). We selected these two groups, considering that there were enough data for the comparison of the two species and avoiding the range of $\Psi_{\text{leaf}}$, where $g_{\text{smax}}$ of both species changed dramatically (between -2.1 and -2.7 MPa). We were also able to compare $K_{\text{leaf}}$ by species for higher values of $\Psi_{\text{leaf}}$ (<-1.1 MPa) from the leaf vulnerability curves. The Mann–Whitney U-test was used instead of Student’s t-test for the comparisons due to the lack of normality in some cases. Significant differences were reported when variations between the groups were $P <0.05$. The same test was used to analyze the differences on $K_{\text{leaf}}$ by method, tree age, and plant material.

Binned values by 0.2 MPa intervals of $K_{\text{leaf}}$ and ABA$_{\text{leaf}}$ were used to analyze their linear effect on binned values of $g_{\text{smax}}$ to make the trends and correlations more robust, given that the variables were not measured in the same leaves. Simple regressions were run to determine whether $K_{\text{leaf}}$ and ABA$_{\text{leaf}}$ were significantly related to $g_s$ ($\alpha = 0.05$).

The points at where the slope of the $\Psi_{\text{leaf}}$ – $K_{\text{leaf}}$ and $\Psi_{\text{leaf}}$ – $g_{\text{smax}}$ curve changes, were determined with a piecewise regression using the R package “segmented.”

R software was used for all the analyses (R version 3.1.1) except for the Mann–Whitney U-test, where SigmaPlot (version 12.0, Systat Software, Inc., San Jose California USA) was used.

Results

Effect of methods and tree age on $K_{\text{leaf}}$ values

The $\Psi_{\text{leaf}}$ – $K_{\text{leaf}}$ relationships determined by the two methods were statistically indistinguishable (Table 1 and Fig. 1) and $K_{\text{leaf}}$ obtained in the same leaves with both methods rendered similar values (Fig. 2), although with a slight tendency for EFM to
return higher values of $K_{\text{leaf}}$ as compared to DRKM (slope $K_{\text{EIFM}} \text{ vs. } K_{\text{DRKM}} = 1.11$). The vulnerability curves obtained using leaves of different ages of tree were also statistically similar and, in the case of olive, data from terminal parts of shoots and single leaf were overlaid (Table 1).

Due to the lack of differences by tree age or part of the plant, all of the data obtained with DRKM were pooled together for each species. Both olive and almond best fitting curves were exponential (Fig. 3).

**Structural and functional leaf traits**

The higher $K_{{\text{max}}}^{{\text{in almond}}} (4.70 \text{ mmol m}^{-2} \text{s}^{-1} \text{ MPa}^{-1})$ compared to olive ($3.42 \text{ mmol m}^{-2} \text{s}^{-1} \text{ MPa}^{-1}$) related well with the leaf $VD$ of each species: $11.33 \pm 0.28 \text{ mm mm}^{-2}$ for almond and $6.74 \pm 0.19 \text{ mm mm}^{-2}$ for olive (Table 2). This higher hydraulic capacity in almond than in olive also correlated with a higher photosynthetic capacity of leaves ($185.7 \mu\text{mol m}^{-2} \text{s}^{-1}$ in almond and $128.0 \mu\text{mol m}^{-2} \text{s}^{-1}$ in olive). The slope of the transition between maximum and minimum $K_{\text{leaf}}$ values was steeper for almond than for olive (Fig. 3). Thus, $P50$ and $P80$ were higher for almond than for olive (Table 2). Accordingly, TLP was also higher for almond than for olive ($-2.26$ for almond and $-3.30$ MPa for olive), corresponding to a $K_{\text{leaf}}$ loss of $65\%$ in almond and $69\%$ in olive (values derived from the vulnerability curves for each species). We also calculated the ratio $g_{\text{smax}}/K_{\text{leaf}}$ as an index of the degree of the stomata’s hydraulic buffering against changes in $Ds$ and drought (Brodribb and Jordan 2008, Scoffoni et al. 2015). We observed that this ratio was lower for olive ($88.02 \pm 12.10$) than for almond ($140.37 \pm 25.7$) and for any $\Psi_{\text{leaf}}$, but differences were not significant in the range of $\Psi_{\text{leaf}}$ for the number of replicates allowed to conduct statistical comparisons ($-1.2/-2.1$ MPa).
Effect of leaf hydraulic conductance on stomatal conductance in response to water stress

During the days of September of 2014 when the irrigation withholding study was conducted, air temperature varied between 16°C and 21°C at dawn to 21°C and 26°C at 8:30 GMT, when measurements of gs\textsubscript{max} were made. Da was 0.04 ± 0.03 kPa at dawn and increased to 0.81 ± 0.07 kPa at 8:30 GMT.

The lack of irrigation reduced Ψ\textsubscript{pd} (taken as proxy of soil water potential) from -0.47 ± 0.05 MPa to -1.20 ± 0.04 MPa in almond, and from -0.76 ± 0.11 MPa to -4.12 ± 0.41 MPa in olive. Minimum values of Ψ\textsubscript{leaf} at 8:30 GMT (Fig. 4a) were reached at the end of the experiment, being of -3.10 ± 0.02 MPa in almond and -6.14 ± 0.01 MPa in olive. Both gs\textsubscript{max} and K\textsubscript{leaf} decreased with lowering Ψ\textsubscript{leaf}, for both species (Fig. 4a and b).

However, K\textsubscript{leaf} started to decrease at higher Ψ\textsubscript{leaf} values than gs\textsubscript{max}. Moreover, the Ψ\textsubscript{leaf} values where gs\textsubscript{max} reached its minimum values, -2.68 MPa in olive and -2.14 MPa in almond, represented a K\textsubscript{leaf} reduction of 65% in olive and 63% in almond. These values were higher but not very different from the TLP values (-3.30 MPa in olive and -2.26 MPa in almond). The other variable analyzed, ABA\textsubscript{leaf} (Fig. 4c), did not follow clearly the gs\textsubscript{max} trend either. Interestingly, in olive, the relationship of Ψ\textsubscript{leaf} with ABA\textsubscript{leaf} was not even statistically significant (P > 0.05). The rest of the relationships shown in Fig. 4, between Ψ\textsubscript{leaf} and gs\textsubscript{max}, and K\textsubscript{leaf} and ABA\textsubscript{leaf}, were statistically significant (P < 0.05).

We further analyzed the linear relationships of K\textsubscript{leaf} and ABA\textsubscript{leaf} with gs\textsubscript{max} and found that K\textsubscript{leaf} was the only variable significantly correlated to gs\textsubscript{max} in olive and almond (r\textsuperscript{2} = 0.79 and 0.47, respectively). Thus, adding ABA\textsubscript{leaf} as a predictor did not significantly improve the prediction of gs from K\textsubscript{leaf}.

The results of comparing gs\textsubscript{max} by species showed that it was significantly lower in olive than in almond (P < 0.05) for the two Ψ\textsubscript{leaf} ranges analyzed in both species (-
This same test showed no differences in $K_{\text{leaf}}$ between the species. However, almond $K_{\text{leaf}}$ was significantly higher than olive $K_{\text{leaf}}$ for the $\Psi_{\text{leaf}}$ range between -0.4 and -1.1 MPa, using the leaf vulnerability curves (Fig. 3). On the contrary, ABA$_{\text{leaf}}$ was significantly higher for olive than for almond in the -1.2/-2.1 MPa $\Psi_{\text{leaf}}$ range (Fig. 4d). Unfortunately, there were not enough replicates of ABA$_{\text{leaf}}$ for the -2.7/-3.0 MPa range comparison.

An independent estimate of $K_{\text{plant}}$ was calculated from leaf gas exchange and leaf water potential measurements (Eq. 3). Despite the shortcomings of this estimate (transpiration was not measured in the same leaves used for $K_{\text{leaf}}$ measurements and root water potential was assumed to be the same at predawn and midday), our data (Fig. 5) confirm that the curve shape found for $K_{\text{leaf}}$ in Fig. 3. $K_{\text{leaf}}$ and $K_{\text{plant}}$ decreased markedly at high leaf water potentials, the decrease for both $K_{\text{leaf}}$ and $K_{\text{plant}}$ being steeper in almond than in olive.

**Discussion**

*Measuring the vulnerability of $K_{\text{leaf}}$ to dehydration: methodological considerations*

Values of $K_{\text{leaf}}$ determined by both DRKM and EFM showed good agreement, both in absolute values (Fig. 2b) and vulnerability to dehydration (Table 1, Fig. 1). This was despite methodological limitations (common and specific to each method, Scoffoni et al. 2012) and different measurement principles (Scoffoni et al. 2012, Blackman and Brodribb 2011). To our knowledge, this is the first time that both methods have been tested on the same plant material. We did not find any difference due to the tree age or the sampled plant material (Table 1). In the first case, this was likely because all leaves were of the current year, exposed to sun and all trees had been well-irrigated until the beginning of the water withholding experiment, and, presumably, no cavitation episodes.
occurred (Hacke et al. 2001). Regarding the sampled plant material experiment, the lack of differences between the measurements made in single leaves and terminal parts of shoots with a few leaves could have been due, as suggested before (Blackman and Brodribb 2011, Nardini and Salleo 2000) and tested here, to the major resistance being located in the leaves and, thus these organs and not the shoots, being responsible for the total conductance (Nardini et al. 2003, Sack and Holbrook 2006).

$K_{\text{leaf}}$ response to water stress and interspecific differences in structural and functional leaf traits

The high hydraulic capacity in almond, likely related to its higher VD, agrees with its high growth and water use rates, when soil water conditions are not limiting. Maximum $K_{\text{leaf}}$ has been found to be related to maximum stomatal conductance and photosynthesis across different species (Sack et al. 2003, Sack and Holbrook 2006). Species with large photosynthetic capacity must show a high leaf hydraulic capacity to cope with the high $g_s$ values required to avoid diffusional limitations to photosynthesis (Flexas et al. 2004).

In agreement with that, our data show that the photosynthetic capacity in almond leaves, estimated as $V_{\text{cmax}}$, was 1.44-fold that of olive (Table 2). These data are in agreement with a potential trade-off between hydraulic safety and efficiency. The steeper slope between $K_{\text{leaf}}$ and $\Psi_{\text{leaf}}$ in almond at high $\Psi_{\text{leaf}}$ values (Fig. 3) shows that almond is more vulnerable to dehydration than olive, as its P50 and P80 values also suggest (Table 2).

According to its higher leaf hydraulic vulnerability, TLP in almond was higher than in olive (Table 2). Indeed, deciduous species have been shown to exhibit far more rapid transitions from high to low $K_{\text{leaf}}$ values than evergreen ones (Brodribb and Holbrook 2003), as well as higher TLP (Corcuera et al. 2002). Moreover, the lower ratio $g_{\text{smax}}/K_{\text{leaf}}$ for olive than almond indicates that olive had a higher degree of
hydraulic buffering of $g_s$ against declines in $\Psi_{\text{leaf}}$ during leaf dehydration. As TLP was measured in leaves following the drought-response experiment, higher TLP values would have been found before due to osmotic adjustment.

High cavitation thresholds in leaves have been reported for species belonging to the same genus as almond (Prunus mahaleb, -0.75 MPa) and the same family as olive (Phillyrea angustifolia, -0.9 MPa) (Kikuta et al. 1997). In general, however, lower P50 values and shallower, more linear in shape $\Psi_{\text{leaf}}$ declines would have been expected for drought-tolerant species (Scoffoni et al. 2012) such as olive and almond. The P80 values of this study (-4.21 MPa in almond and -5.35 MPa in olive) were in the range of previously published P80 values of drought-tolerant species. For example, Scoffoni et al. (2012) reported P80 values for species of dry habitats ranging from -4.12 MPa for Heteromeles arbutifolia to -5.25 MPa for Cercocarpus betuloides. P80 has been reported to be more useful for comparison of species’ vulnerabilities, because P50 values often occur in the middle of the steepest decline (Scoffoni et al. 2012). Indeed, as P50 and P80 values are a function of $K_{\text{max}}$, these values may be artifactually skewed.

Maximum $K_{\text{leaf}}$, contrary to what happens with stems, cannot be measured and it is usually estimated from the highest $K_{\text{leaf}}$ values or extrapolating the $\Psi_{\text{leaf}}$: $K_{\text{leaf}}$ adjusted curve to $\Psi_{\text{leaf}} = 0$ (Scoffoni et al. 2012).

Apparently, the loss of hydraulic conductance at high $\Psi_{\text{leaf}}$ could be surprising since the functional range of $\Psi_{\text{leaf}}$ for both species is usually lower, with minimum $\Psi_{\text{leaf}}$ around -3.5 MPa in almond (Egea et al. 2012) and -4.5 MPa in olive (Torres-Ruiz et al. 2013). Although steep decreases of $K_{\text{leaf}}$ to high $\Psi_{\text{leaf}}$ have been often reported (Blackman et al. 2009, Scoffoni et al. 2012), methodological artifacts in $\Psi_{\text{leaf}}$ measurement should be considered. When leaves are well-hydrated, $\Psi_{\text{leaf}}$ is high and the gradient of water potential during measurements is small. Mistakes made in the correct
determination of high \( \Psi_{\text{leaf}} \) would lead to large errors in the estimation of \( K_{\text{leaf}} \).

However, absolute errors in the measurement of \( \Psi_{\text{leaf}} \) at high water potentials, 0.1 MPa for example, cannot explain the high values of \( K_{\text{leaf}} \) observed and thus, a \( \Psi_{\text{leaf}} \) measurement artifact can be disregarded. Additional evidence suggesting that our results were not affected by a methodological artifact comes from the comparison of the response of \( K_{\text{leaf}} \) and \( K_{\text{plant}} \) to \( \Psi_{\text{leaf}} \) (Fig. 4), since both curves show a similar shape.

The early loss of \( K_{\text{leaf}} \) might be related to leaf shrinkage (Scoffoni et al. 2013), as mentioned above. Consequently, it might cause a reduction in the connections for water flow among cells in the mesophyll. Additionally, leaf shrinkage would reduce evaporative surface within the leaf. The outside-xylem vulnerability could play an important role in driving the initial vulnerability at mild water deficits. Thus, the initial slope of the vulnerability curve, before the bulk of cavitation is expected to occur, has been suggested to be more related to the outside-xylem component, while the behavior of the leaf vulnerability curve at stronger water deficits could be more influenced by the xylem component (Scoffoni et al. 2013). The major influence of the flow path outside the xylem could help to explain small differences observed measuring \( K_{\text{IDRKM}} \) in olive, calculated with either initial flow and initial leaf water potential, or with final flow and final leaf water potential instead (data not shown). It seems that, despite the short period of time that the leaf was connected to the flowmeter, a certain rehydration occurred at the highest water potential values, which is more likely to happen due to a rehydration in the outside part of the xylem than to a refilling process of embolized vessels (Wang et al. 2014; also see Trifilo et al. 2014, Kim et al. 2014).

Role of \( K_{\text{leaf}} \) on the regulation of stomatal conductance
Neither $K_{\text{leaf}}$ or $A_{\text{leaf}}$ unequivocally followed the $g_{\text{smax}}$ trend as $\Psi_{\text{leaf}}$ decreased (Fig. 4). However, the variable most strongly related to $g_{\text{smax}}$ was $K_{\text{leaf}}$, especially in olive. In almond, although this relationship was less strong, it was still highly significant. In both studied species, the decline of $K_{\text{leaf}}$ began immediately with dehydration, whereas that of $g_{\text{smax}}$ began only after a substantial $K_{\text{leaf}}$ loss. This agrees with findings reported for other woody species (Johnson et al. 2009, Pasquet-Kok et al. 2010, Guyot et al. 2012).

However, the reasons for the loss of $K_{\text{leaf}}$ at relatively high values of $\Psi_{\text{leaf}}$ have not been fully elucidated yet. Previous work has suggested that cavitation might be responsible for a major portion of $K_{\text{leaf}}$ decline in response to low leaf water potentials (Sack and Holbrook 2006), although effects in the extra-xylem pathways such as leaf shrinkage (Scoffoni et al. 2013) and aquaporin deactivation (Shatil-Cohen et al. 2011, Pantin et al. 2013) are acquiring larger prominence. Thus, different trajectories of $K_{\text{leaf}}$ decline likely did not implicate cavitation itself as a key signal for stomatal closure (Guyot et al. 2012). A large percentage of $K_{\text{leaf}}$ was lost before stomata started to show an active regulation, ruling out the idea of a protective role of stomata for $K_{\text{leaf}}$ maintenance, as has been suggested for shoots. Thus, our results indicate that stomata would operate at the risk of leaf hydraulic catastrophic failure, with leaves functioning as hydraulic circuit breakers that can be reset overnight, rather than as indicators of their susceptibility to catastrophic hydraulic failure (Bucci et al. 2013). As already reported by different authors (Brodribb and Holbrook 2004, Johnson et al. 2009), our vulnerability curves suggest that $K_{\text{leaf}}$ could be dynamic during the day as $K_{\text{leaf}}$ would vary greatly for the $\Psi_{\text{leaf}}$ range usually observed under field conditions in the study area. These evidences point toward a major contribution of the extra-xylem conductance component of $K_{\text{leaf}}$ (Scoffoni et al. 2012).
The role of ABA in the regulation of $g_{\text{max}}$ did not depict a clear pattern in either almond or olive (Fig. 4d). Although there is a trend for an increasing level of ABA with decreasing $\Psi_{\text{leaf}}$, ABA$_{\text{leaf}}$ was highly variable at low $\Psi_{\text{leaf}}$, suggesting that it might be determined by other variables apart from $\Psi_{\text{leaf}}$. For instance in olive, leaves with $g_{\text{max}}$ as high as 0.23 mol m$^{-2}$ s$^{-1}$ or as low as 0.03 mol m$^{-2}$ s$^{-1}$ presented identical values of ABA of 100 ng g FW$^{-1}$. This does not mean that ABA did not play a role in the response to water stress. Actually, in addition to regulating many processes at the plant and leaf level (Hetherington 2001, Cutler et al. 2010), ABA has been proposed to regulate $K_{\text{leaf}}$ (Pantin et al. 2013). Further studies on the regulation of stomata by hydraulic and non-hydraulic signals are necessary to clarify the actual role of each component in the stomatal control mechanism of the two species considered here.

Conclusions

We found that $K_{\text{leaf}}$ decreased exponentially with $\Psi_{\text{leaf}}$ in both olive and almond. This decrease was steeper for almond than for olive, according to independent leaf functional features, such as lower TLP in olive than in almond. We conclude that neither mechanism analyzed unequivocally drives the $g_s$ response to water stress in these two species. However, $K_{\text{leaf}}$ is the variable most strongly related to the $g_s$ response to water stress, especially in olive, ABA showing no clear effect on $g_s$ regulation. The larger hydraulic capacity of almond at high $\Psi_{\text{leaf}}$ allows $g_s$ to be higher in almond than in olive. This is in agreement with the greater VD values found in almond, which contribute to its higher photosynthetic capacity. We also conclude that, although based on different principles, both EFM and DRKM provide similar $K_{\text{leaf}}$ values. Tree age and the use of terminal parts of shoots instead of leaves do not have any significant effect on measured $K_{\text{leaf}}$ either.
ACKNOWLEDGMENTS

We thank A. Montero for field work assistance, J. Perez-Hormaeche for helping us with the VD images analysis and T. Brodribb and J.M. Torres-Ruiz for discussion. This work was funded by the Spanish Ministry of Science and Innovation (research project AGL2009-11310/AGR). V.H.S. benefited from a Juan de la Cierva research fellowship from Spanish Ministry of Science and Innovation and C.R.D. benefited from a FPDI research fellowship from the Junta de Andalucía.

REFERENCES


FIGURES CAPTIONS

Figure 1. Leaf hydraulic conductance ($K_{leaf}$) obtained with the Dynamic rehydration kinetics method (DRKM) and the Evaporative flux method (EFM) in different olive (a) and almond (b) leaves. Dashed line in represents P50 for each species.
Figure 2. Leaf hydraulic conductance ($K_{\text{leaf}}$) obtained in the same leaves or terminal parts of shoots for olive and almond with the Dynamic rehydration kinetics method (DRKM) and the Evaporative flux method (EFM). Dotted line represents 1:1 line.

Figure 3. Vulnerability curves for leaf hydraulic conductance for olive and almond determined with the Dynamic rehydration kinetics method (DRKM). The fitted curves are exponential and statistically significant ($y = 0.77+2.67*e^{-0.69*x}$, $R^2=0.62$, $P<0.0001$ for olive and $y = 0.75+4.70*e^{-0.74*x}$, $R^2=0.54$, $P<0.0001$ for almond).

Figure 4. Variation of stomatal conductance ($g_{\text{max}}$) (a), leaf hydraulic conductance ($K_{\text{leaf}}$) (b), and leaf ABA ($\text{ABA}_{\text{leaf}}$) (c) with leaf water potential ($\Psi_{\text{leaf}}$) measured when stomatal conductance is considered to be at its maximum (8.30 GMT) for olive and almond. The points represent the average of values for $\Psi_{\text{leaf}}$ intervals of 0.2 MPa and the bars are the standard errors (SE).

Figure 5. Comparison of leaf vulnerability curves for olive (a) and almond (b) where $K_{\text{leaf}}$ was obtained with the Dynamic rehydration kinetics method (DRKM) and $K_{\text{plant}}$ was calculated using Equation 3.