1 **Role of leaf hydraulic conductance in the regulation of stomatal**

2 **conductance in almond and olive in response to water stress**

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4 RUNNING TITLE: LEAF HYDRAULIC FUNCTION IN ALMOND AND OLIVE

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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 (Ψ_{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 Ψ_{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 stess, 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 CO_2 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).

66   Among all the resistances that water has to overcome through the plant, the leaf

67   has been demonstrated to be a major one (Nardini and Salleo 2000, Nardini et al. 2001,

68   Sack et al. 2003, Brodribb and Holbrook 2003) and, thus, it may play an important role

69   in the regulation of stomata (Guyot et al. 2012). Consequently, leaf hydraulic

70   conductance (K_{leaf}) may be a major determinant of plant productivity and growth (Sack

71   and Holbrook 2006). However, the effect of K_{leaf} on stomatal conductance (g_s) is still

72   poorly understood and few studies have addressed the coordination dynamics between

73   both variables (Brodribb and Holbrook 2004, Lo Gullo et al. 2005, Brodribb and Jordan

74   2008, Blackman et al. 2009, Gortan et al. 2009, Johnson et al. 2009). Furthermore, the

75   extent to which K_{leaf} declines with water stress varies from species to species, even

76   within a particular habitat (Sack et al. 2003, Brodribb and Hobrook 2003, Lo Gullo et

77   al. 2005, Johnson et al. 2009, Scoffoni et al. 2012) and the knowledge of its relationship

78   to leaf structure and ecological strategy remains incomplete (Blackman et al. 2010). For

79   these studies, reliable and fast K_{leaf} measurements would be of great help. Although

80   there are new approaches that can provide K_{leaf} measurements within minutes, such as

81   the "dynamic rehydration kinetics method" (DRKM, Blackman and Brodribb 2011), in

82   opposition to more traditional and time-consuming options such as the "evaporative flux

83   method" (EFM), some uncertainties are still unresolved. These include the identification

84   of the flow pathways during leaf rehydration, upon which DRKM measurements are

85   based, and how similar these pathways are to those of transpiration (Flexas et al. 2013).

86   Thus, we conducted a study with the ultimate objective of contributing to the

87   understanding of g_s regulation. We focused on the impact of K_{leaf} on g_s in an evergreen

88   (olive) and a deciduous (almond) Mediterranean species, i.e. two species occurring in

89   the same area but with different ecological strategies. Specifically, our objectives were:

90   (i) to test the impact of DRKM and EFM as well as tree age on the values of K_{leaf}; (ii) to

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91   determine the K_{leaf} response to dehydration in both olive and almond, and potentially

92   related structural and functional leaf traits; and finally (iii) to study the effect of K_{leaf} on

93   g_s in response to water deficit. We further compared the effect of K_{leaf} on g_s with the

94   impact of ABA on g_s. For the first objective, we hypothesized that both methods would

95   produce similar K_{leaf} values, because the flow pathways used in leaf rehydration (basis

96   for DRKM measurements) would be the same as the pathways followed by the water on

97   its way to the leaf evaporation sites (basis for EFM measurements). For the second

98   objective, we hypothesized that we would find differences by comparison between the

99   species in maximum K_{leaf} and leaf traits such as vein density, photosynthetic capacity,

100   and turgor loss point. For the third objective, we expected that a higher K_{leaf} would be

101   associated with a higher g_s, and the reduction in K_{leaf} would predict the reduction in g_s,

102   to a greater extent than ABA level.

103

Materials and methods

105   The olive (*Olea europaea* L., cv Arbequina) and almond (*Prunus dulcis* (Mill.) *D*.A.

106   Webb, cv. Guara) trees used in this study were located in Seville (Spain) (37º 15' N, -5º

107   48' 102 W). The area has a Mediterranean climate with hot and dry weather from May

108   to September, being mild and wet for the rest of the year. Measurements were taken

109   from May to October, both in 2013 and 2014. The sampled plants were regularly

110   irrigated before the beginning of the experiments to replace their water needs.

111

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

113   We aimed to conduct a study comparing K_{leaf} values obtained using different methods,

114   tree age material, and single leaves vs. terminal parts of shoots. The data obtained in

115   these methodological experiments were independent of the data collected in the water

116   deficit experiment. Leaf hydraulic conductance was measured in fully developed,

117   current year and sun-exposed leaves in both species. This was the same for the test

118   comparing plant material, but in this test also the terminal parts of olive shoots were

119   used. Before taking the measurements, we cut branches long enough to avoid any xylem

120   embolism and put them in dark plastic bags with wet paper towel inside to equilibrate

121   for at least 30 min. Leaves and terminal parts of shoots were cut from the branches

122   under purified water. They were then rapidly connected under water to a flowmeter

123   consisting of silicon tubing containing purified and degassed water. The tubing

124   connected the leaf to a pressure transducer (PX26-005GV, Omega Engineering Ltd.,

125   UK), which was, in turn, connected to a Campbell data logger CR1000 (Campbell,

126   Campbell Scientific Ltd., UK) to register and store readings every 1 s to calculate the

127   flow rate through the leaf (mmol s^{-1} m^{-2}). Reference tubing of different resistances was

128   used to minimize measurement errors (Sack et al. 2011, Melcher et al. 2012). Some

129   branches were allowed to dehydrate before measurement for increasing periods to

130   obtain a wide range of leaf water potential values (Ψ_{leaf}).

131

132   <u>Comparison of DRKM and EFM methodologies</u>

133   To assess the differences between the K_{leaf} vulnerability curves potentially produced by

134   the use of the two tested methods, a group of leaves (Table 1) was measured with either

135   DRKM (K_{lDRKM}) or EFM (K_{lEFM}). The examination of leaf hydraulic methods was

136   conducted using different types of plant material. With olive, we used leaves from eight

137   6-year-old potted trees and three 8-year-old trees and with almond, we used leaves from

138   eight 6-year-old potted trees and three 13-year-old trees.

139   For the DRKM measurements (Brodribb and Holbrook 2003, Blackman and Brodribb

140   2011) the leaves were connected to the flowmeter, as described in the former section,

141   until flow rate decayed from its maximum as leaves rehydrated. Initial flow was

142   determined by fitting an exponential curve through the first 20 s of the rehydration flow

143   data and extrapolating back to the initial point of leaf excision, considering the time (s)

144   required to connect the leaf to the flowmeter (Blackman and Brodribb 2011). After

145   connecting the leaves to the flowmeter, they were covered with moist paper and had no

146   exposure to light in order to prevent transpiration. Prior to the flow rate determination,

147   initial leaf water potential (Ψ_0) was measured in the neighboring leaf. Final leaf water

148   potential (Ψ_{DRKMf}) was also measured in the leaf used to determine the flow after being

149   allowed to equilibrate for at least 30 min. A test conducted with rehydrated olive leaves

150   at different leaf water potentials demonstrated that after 30 min the leaf water potential

151   hardly changed.

152   For the EFM measurements (Scoffoni et al. 2012), the leaves were connected to the

153   same flowmeter, but they were allowed to transpire above a large box fan and under a

154   light source (> 1,000 μmol m^2 s^{-1} photosynthetically active radiation) for at least 30 min

155   until the flow was stable (coefficient variation <5% in the last 5 min). In EFM, it is

156   essential that the flow rate reaches a steady state, because the method assumes a stable

157   leaf water potential (Ψ_{EFMf}) after flow rate reaches the steady state (Scoffoni et al.

158   2012). Ψ_0 was measured as in DRKM.

159   In addition, and to compare the effect of both methods on K_{leaf} values and not

160   only on Ψ_{leaf}- K_{leaf} curves, direct comparisons were conducted by determining K_{leaf} in 26

161   leaves of both species using both methods. After connecting the leaves to the flowmeter,

162   transpiration was prevented, as previously described for DRKM, until the leaves were

163   rehydrated. Then, initial flow rate was determined following DRKM. Later, and while

164   the leaves were still connected to the flowmeter, they were allowed to transpire under

165   the same conditions described before for EFM, and then steady-state flow rate and

166   Ψ_{EFMf} were obtained following EFM.

167   Thus, for each method, K_{leaf} was calculated as:

168

169   $$K_{lEFM} = \frac{E}{(-\Psi_{\text{EFMf}} - 0)A_{leaf}} \qquad , \qquad (1)$$

170

171   $$K_{lDRKM} = \frac{-I}{\Psi_{\text{o}} A_{leaf}} \qquad . \qquad (2)$$

172

173   In Eq. 1, K_{lEFM} is leaf hydraulic conductance measured using EFM, E is steady-

174   state transpiration determined with the readings of the flowmeter (mmol s^{-1}), and A_{leaf} is

175   leaf area (m^2). In Eq. 2, K_{lDRKM} is leaf hydraulic conductance measured with DRKM, I

176   is the instantaneous initial maximum flow rate into the leaf (mmol s^{-1}), and Ψ_{o} is the

177   initial leaf water potential (MPa). To correct for changes in K_{leaf} induced by temperature

178   dependence of water viscosity, standardized K_{leaf} values at 25°C were calculated

179   (Scoffoni et al. 2012).

180   To construct the vulnerability curve for each species, K_{leaf} was then related to the

181   lowest Ψ_{leaf}, i.e. Ψ_{o} in DRKM and Ψ_{o} or Ψ_{EFMf} in EFM (Scoffoni et al. 2012). All Ψ_{leaf}

182   measurements were made with a Scholander-type pressure chamber (PMS Instrument

183   Company, Albany, Oregon, USA).

184

185   <u>Tree age test</u>

186   For the tree age test, for olive we compared the K_{leaf} data of eight 6-year-old potted trees

187   with K_{leaf} data of three 8-year-old trees. With almond, we compared K_{leaf} of eight potted

188   6-year-old trees with K_{leaf} of three 13-year-old trees. Only DRKM was used in the K_{leaf}

189   determination.

190

Single leaves—terminal shoots test

An additional experiment was performed, with the olive trees only, to assess the effect

on K_{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_{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.

9

215   <u>Leaf hydraulic vulnerability parameters</u>

216   To compare the two species in their hydraulic parameters, K_{leaf} at full hydration (K_{lmax})

217   was determined for both species using the average K_{leaf} for data above -0.5 MPa

218   (Scoffoni et al. 2012). Ψ_{leaf} values, for which K_{lmax} had declined by 50% and 80% (P50

219   and P80, respectively), were calculated. P50 and P80 were used as indicators of

220   vulnerability of leaf hydraulic conductance to decreasing water potential.

221

222   <u>Pressure-volume curves and turgor loss point</u>

223   We used five leaves of the 13-year-old almond and 8-year-old olive trees to calculate

224   the pressure-volume curves. Leaves were sampled in the morning of October 7 of 2014

225   (a few days after the water deficit experiment ended) and were rehydrated for 24 h, then

226   left to desiccate. Leaf weight and leaf water potentials were measured at least nine times

227   during that period until the leaves reached minimum Ψ_{leaf} values of ca. -5 MPa. The

228   turgor loss point (TLP) was calculated as the intersection point of the two curves

229   represented by the inflection point of the relation 1/ Ψ_{leaf} vs. 1-relative leaf water

230   content.

231

232   <u>Photosynthetic response curves</u>

233   Five A-C_i response curves (the response of net CO_2 assimilation to varying intercellular

234   CO_2 concentration) per species were measured between 09:00 and 13:00 GMT during

235   the experimental period. Measurements were made using a Li-cor LI-6400 portable

236   photosynthesis system (Li-cor, Lincoln, NE, USA) at ambient temperature, saturating

237   PPFD (photoshynthetic photon flux density, 1,600 μmol m^{-2} s^{-1}) and an ambient CO_2

238   concentration (C_a) of between 50 and 1,500 μmol mol^{-1}. After steady-state

239   photosynthesis was achieved, the response of A to varying C_i was measured by lowering

240 C_a stepwise from 390 to 50 µmol mol^{-1}, returning to 390 µmol mol^{-1}, and then

241 increasing C_a stepwise from 390 to 1,500 µmol mol^{-1}. Each A-C_i curve comprised 16

242 measurements, each made after at least 3 min at each C_a. Maximum carboxylation rate

243 (V_{cmax}) was estimated by the curve fitting method proposed by Ethier and Livingston

244 (2004). Rubisco kinetic parameters were taken from the literature (Bernacchi et al.

245 2002).

246

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

248 *conductance*

249 This last study consisted of a drydown experiment using potted plants, which aimed at

250 determining whether the decline in K_{leaf} with increasing dehydration matched the

251 decline in g_s. Three potted olive and almond trees of 8 and 13 years, respectively, were

252 gradually stressed by withholding irrigation for 13 days with olive and 24 days with

253 almond. No plant deaths were reported. Measurements of K_{leaf}, g_s, Ψ_{leaf}, and ABA$_{leaf}$

254 were conducted on the terminal parts of shoots and leaves of the two species, sampled

255 from the outer part of the canopy facing S-E at ca. 1.5 m above ground. Two samples

256 from three trees per species were used at predawn (05:00) and at 08:30 GMT, when g_s is

257 at its maximum (g_{smax}). We obtained the time for g_{smax} through daily g_s measurement

258 cycles in olive (Fernández et al. 1997) and almond (Rodriguez-Dominguez et al.,

259 personal communication). Maximum stomatal conductance was measured in the same

260 leaves throughout the experiment, when possible. K_{leaf} and Ψ_{leaf} were measured using

261 leaves or terminal parts of shoots from the same branches. The measurements were

262 conducted on four clear and sunny days in September 2014 (September 2, 5, 10, and 15

263 for olive and 2, 5, 15, and 26 for almond; technical problems impeded same-day

264 measurements in olive and almond). K_{leaf} was measured using DRKM as described

265   before. The maximum stomatal conductance (g_{smax}, mol m^{-2} s^{-1}) was measured with a

266   Li-cor LI-6400 portable photosynthesis system (Li-cor) with a 2×3 cm standard

267   chamber at ambient light and CO_2 conditions.

268   Leaves collected at the same time intervals as the rest of measurements were

269   used to determine ABA_{leaf} concentration. ABA_{leaf} was measured by the liquid

270   chromatography-electrospray/tandem mass spectrometry method of Gómez-Cadenas et

271   al. (2002). Samples of about 400 mg of frozen leaf tissue, midribs not included, were

272   milled, homogenized and extracted in 5 mL of distilled water using a benchtop

273   homogenizer (Polytron PT 1600 E, Kinematica AG, Switzerland). An aliquot of 50 μL

274   of 2-ppm deuterated abscisic acid (dABA) was previously added as an internal standard.

275   Samples were centrifuged (20,000 rpm, 15 min, 4°C), supernatants were acidified to pH

276   3.0 (150 μL acetic acid 30% v/v), and leaf extracts were twice partitioned with 3 mL of

277   diethyl ether. Organic phases were collected in Erlenmeyer flasks and evaporated using

278   a vacuum pump. Tube walls were washed with 1 mL diethyl ether and desiccated again.

279   Dry residues were re-suspended in 500 μL methanol, completed to a total volume of 1

280   mL with Milli-Q quality (reverse osmosis) water and filtered through a 13 mm diameter

281   polypropylene membrane syringe filter (Ø 0.22 μm, RephiQuik PTFE Non-sterile

282   Syringe Filter, RephiLe Bioscience Ltd., China). A calibration line was also prepared

283   with different ABA concentrations (5, 10, 25, 50, and 100 ppb) and the internal standard

284   dABA. Measurements were conducted using an Agilent 1290 Infinity HPLC system

285   (Agilent Technologies Inc., CA, USA) coupled with an electrospray/tandem mass

286   spectrometer (3200 QTRAP® LC/MS/MS System, AB SCIEX, Framingham, MA,

287   USA) and data were analyzed with mass spectrometry software (Analyst® Software, AB

288   SCIEX). Leaf ABA was normalized by fresh weight (g).

289   The main weather variables in the area were monitored by a Campbell weather

290 station (Campbell Scientific Ltd., Shepshed, UK). The station recorded 30 min average

291 values of air temperature (T_a), air humidity (RH_a), global solar radiation (R_s), and

292 photosynthetically active radiation (PAR), among other variables.

293   Leaf hydraulic conductance measured at 8:30 GMT was compared with K_{plant},

294 calculated according to Ohm's law analogy assuming steady-state conditions:

295

296       $$K_{plant} = \frac{g_{smax}D}{\Psi_{s-}\Psi_{leaf}}$$,   (3)

297

298   where g_{smax} (mol m^{-2} s^{-1}) is the stomatal conductance measured at 08:30 GMT,

299 D_a is the air vapor pressure deficit (mmol mol^{-1}) determined from measurements by the

300 weather station mentioned above, Ψ_s is the soil water potential that is assumed to be

301 similar to Ψ_{leaf} at predawn (Ψ_{pd}, MPa), and Ψ_{leaf} is the leaf water potential measured at

302 08:30 GMT. All leaf water potentials were measured with the Scholander-type pressure

303 chamber already mentioned.

304

305 *Data processing and statistical analysis*

306 The most-used functions in plant hydraulic studies (linear, sigmoidal, logistic, and

307 exponential) were fitted to our dataset of leaf vulnerability curves ($\Psi_{leaf} - K_{leaf}$), using

308 maximum likelihood, as described in Scoffoni et al. (2012). The function with the

309 lowest Akaike information criterion (AIC) and highest R^2 was chosen as the best fit

310 function. Outlier tests were conducted for each 0.5 MPa interval using Dixon's test

311 (Sokal and Rohlf, 1995) for the vulnerability curves.

312   Statistical analyses were used to compare g_{smax}, K_{leaf}, and ABA_{leaf} between

313 species for two Ψ_{leaf} ranges: one going from -1.2 to -2.1 MPa (n = 6 and 9 for olive and

314   almond, respectively) and the other from -2.7 to -3.0 MPa (n = 2 and 3, for olive and

315   almond, respectively). We selected these two groups, considering that there were

316   enough data for the comparison of the two species and avoiding the range of Ψ_{leaf},

317   where g_{smax} of both species changed dramatically (between -2.1 and -2.7 MPa). We were

318   also able to compare K_{leaf} by species for higher values of Ψ_{leaf} (<-1.1 MPa) from the leaf

319   vulnerability curves. The Mann–Whitney U-test was used instead of Student's t-test for

320   the comparisons due to the lack of normality in some cases. Significant differences were

321   reported when variations between the groups were P <0.05. The same test was used to

322   analyze the differences on K_{leaf} by method, tree age, and plant material.

323   Binned values by 0.2 MPa intervals of K_{leaf} and ABA_{leaf} were used to analyze

324   their linear effect on binned values of g_{smax} to make the trends and correlations more

325   robust, given that the variables were not measured in the same leaves. Simple

326   regressions were run to determine whether K_{leaf} and ABA_{leaf} were significantly related to

327   g_s ($\alpha = 0.05$).

328   The points at where the slope of the $\Psi_{leaf} - K_{leaf}$ and $\Psi_{leaf} - g_{smax}$ curve changes,

329   were determined with a piecewise regression using the R package "segmented."

330   R software was used for all the analyses (R version 3.1.1) except for the Mann–

331   Whitney U-test, where SigmaPlot (version 12.0, Systat Software, Inc., San Jose

332   California USA) was used.

333

334   **Results**

335   *Effect of methods and tree age on K_{leaf} values*

336   The $\Psi_{leaf} - K_{leaf}$ relationships determined by the two methods were statistically

337   indistinguishable (Table 1 and Fig. 1) and K_{leaf} obtained in the same leaves with both

338   methods rendered similar values (Fig. 2), although with a slight tendency for EFM to

14

339   return higher values of K_{leaf} as compared to DRKM (slope K_{lEFM} vs. K_{lDRKM} = 1.11). The

340   vulnerability curves obtained using leaves of different ages of tree were also statistically

341   similar and, in the case of olive, data from terminal parts of shoots and single leaf were

342   overlaid (Table 1).

343   Due to the lack of differences by tree age or part of the plant, all of the data

344   obtained with DRKM were pooled together for each species. Both olive and almond

345   best fitting curves were exponential (Fig. 3).

346

347   *Structural and functional leaf traits*

348   The higher K_{lmax} in almond (4.70 mmol m^{-2} s^{-1} MPa^{-1}) compared to olive (3.42 mmol m^{-2}

349   ^{2} s^{-1} MPa^{-1}) related well with the leaf VD of each species: 11.33 ± 0.28 mm mm^{-2} for

350   almond and 6.74 ± 0.19 mm mm^{-2} for olive (Table 2). This higher hydraulic capacity in

351   almond than in olive also correlated with a higher photosynthetic capacity of leaves

352   (185.7 µmol m^{-2} s^{-1} in almond and 128.0 µmol m^{-2} s^{-1} in olive). The slope of the

353   transition between maximum and minimum K_{leaf} values was steeper for almond than for

354   olive (Fig. 3). Thus, P50 and P80 were higher for almond than for olive (Table 2).

355   Accordingly, TLP was also higher for almond than for olive (-2.26 for almond and -3.30

356   MPafor olive), corresponding to a K_{leaf} loss of 65% in almond and 69% in olive (values

357   derived from the vulnerability curves for each species). We also calculated the ratio

358   g_{smax}/K_{leaf} as an index of the degree of the stomata's hydraulic buffering against changes

359   in D_a and drought (Brodribb and Jordan 2008, Scoffoni et al. 2015). We observed that

360   this ratio was lower for olive (88.02 ± 12.10) than for almond (140.37 ± 25.7) and for

361   any Ψ_{leaf}, but differences were not significant in the range of Ψ_{leaf} for the number of

362   replicates allowed to conduct statistical comparisons (-1.2/-2.1 MPa).

363

Effect of leaf hydraulic conductance on stomatal conductance in response to water

365 *stress*

366 During the days of September of 2014 when the irrigation withholding study was

367 conducted, air temperature varied between 16°C and 21°C at dawn to 21°C and 26°C at

368 8:30 GMT, when measurements of g_{smax} were made. D_a was 0.04 ± 0.03 kPa at dawn

369 and increased to 0.81 ± 0.07 kPa at 8:30 GMT.

370   The lack of irrigation reduced Ψ_{pd} (taken as proxy of soil water potential) from -

371 0.47 ± 0.05 MPa to -1.20 ± 0.04 MPa in almond, and from -0.76 ± 0.11 MPa to $-4.12 \pm$

372 0.41 MPa in olive. Minimum values of Ψ_{leaf} at 8:30 GMT (Fig. 4a) were reached at the

373 end of the experiment, being of -3.10 ± 0.02 MPa in almond and -6.14 ± 0.01 MPa in

374 olive. Both g_{smax} and K_{leaf} decreased with lowering Ψ_{leaf}, for both species (Fig. 4a and b).

375 However, K_{leaf} started to decrease at higher Ψ_{leaf} values than g_{smax}. Moreover, the Ψ_{leaf}

376 values where g_{smax} reached its minimum values, -2.68 MPa in olive and -2.14 MPa in

377 almond, represented a K_{leaf} reduction of 65% in olive and 63% in almond. These values

378 were higher but not very different from the TLP values (-3.30 MPa in olive and -2.26

379 MPa in almond). The other variable analyzed, ABA_{leaf} (Fig. 4c), did not follow clearly

380 the g_{smax} trend either. Interestingly, in olive, the relationship of Ψ_{leaf} with ABA_{leaf} was

381 not even statistically significant ($P > 0.05$). The rest of the relationships shown in Fig. 4,

382 between Ψ_{leaf} and g_{smax}, and K_{leaf} and ABA_{leaf}, were statistically significant ($P < 0.05$).

383   We further analyzed the linear relationships of K_{leaf} and ABA_{leaf} with g_{smax} and

384 found that K_{leaf} was the only variable significantly correlated to g_{smax} in olive and

385 almond ($r^2 = 0.79$ and 0.47, respectively). Thus, adding ABA_{leaf} as a predictor did not

386 significantly improve the prediction of g_s from K_{leaf}.

387   The results of comparing g_{smax} by species showed that it was significantly lower

388 in olive than in almond ($P < 0.05$) for the two Ψ_{leaf} ranges analyzed in both species (-

389 1.2/-2.1 MPa and -2.7/-3.0 MPa). This same test showed no differences in K_{leaf} between

390 the species. However, almond K_{leaf} was significantly higher than olive K_{leaf} for the Ψ_{leaf}

391 range between -0.4 and -1.1 MPa, using the leaf vulnerability curves (Fig. 3). On the

392 contrary, ABA_{leaf} was significantly higher for olive than for almond in the -1.2/-2.1

393 MPa Ψ_{leaf} range (Fig. 4d). Unfortunately, there were not enough replicates of ABA_{leaf}

394 for the -2.7/-3.0 MPa range comparison.

395 An independent estimate of K_{plant} was calculated from leaf gas exchange and leaf

396 water potential measurements (Eq. 3). Despite the shortcomings of this estimate

397 (transpiration was not measured in the same leaves used for K_{leaf} measurements and root

398 water potential was assumed to be the same at predawn and midday), our data (Fig. 5)

399 confirm that the curve shape found for K_{leaf} in Fig. 3. K_{leaf} and K_{plant} decreased markedly

400 at high leaf water potentials, the decrease for both K_{leaf} and K_{plant} being steeper in

401 almond than in olive.

402

403 **Discussion**

404 *Measuring the vulnerability of K_{leaf} to dehydration: methodological considerations*

405 Values of K_{leaf} determined by both DRKM and EFM showed good agreement, both in

406 absolute values (Fig. 2b) and vulnerability to dehydration (Table 1, Fig. 1). This was

407 despite methodological limitations (common and specific to each method, Scoffoni et

408 al. 2012) and different measurement principles (Scoffoni et al. 2012, Blackman and

409 Brodribb 2011). To our knowledge, this is the first time that both methods have been

410 tested on the same plant material. We did not find any difference due to the tree age or

411 the sampled plant material (Table 1). In the first case, this was likely because all leaves

412 were of the current year, exposed to sun and all trees had been well-irrigated until the

413 beginning of the water withholding experiment, and, presumably, no cavitation episodes

17

414 occurred (Hacke et al. 2001). Regarding the sampled plant material experiment, the lack

415 of differences between the measurements made in single leaves and terminal parts of

416 shoots with a few leaves could have been due, as suggested before (Blackman and

417 Brodribb 2011, Nardini and Salleo 2000) and tested here, to the major resistance being

418 located in the leaves and, thus these organs and not the shoots, being responsible for the

419 total conductance (Nardini et al. 2003, Sack and Holbrook 2006).

420

421 *K_{leaf} response to water stress and interspecific differences in structural and functional*

422 *leaf traits*

423 The high hydraulic capacity in almond, likely related to its higher VD, agrees with its

424 high growth and water use rates, when soil water conditions are not limiting. Maximum

425 K_{leaf} has been found to be related to maximum stomatal conductance and photosynthesis

426 across different species (Sack et al. 2003, Sack and Holbrook 2006). Species with large

427 photosynthetic capacity must show a high leaf hydraulic capacity to cope with the high

428 g_s values required to avoid diffusional limitations to photosynthesis (Flexas et al. 2004).

429 In agreement with that, our data show that the photosynthetic capacity in almond leaves,

430 estimated as V_{cmax}, was 1.44-fold that of olive (Table 2). These data are in agreement

431 with a potential trade-off between hydraulic safety and efficiency. The steeper slope

432 between K_{leaf} and Ψ_{leaf} in almond at high Ψ_{leaf} values (Fig. 3) shows that almond is more

433 vulnerable to dehydration than olive, as its P50 and P80 values also suggest (Table 2).

434 According to its higher leaf hydraulic vulnerability, TLP in almond was higher

435 than in olive (Table 2). Indeed, deciduous species have been shown to exhibit far more

436 rapid transitions from high to low K_{leaf} values than evergreen ones (Brodribb and

437 Holbrook 2003), as well as higher TLP (Corcuera et al. 2002). Moreover, the lower

438 ratio g_{smax}/K_{leaf} for olive than almond indicates that olive had a higher degree of

439   hydraulic buffering of g_s against declines in Ψ_{leaf} during leaf dehydration. As TLP was

440   measured in leaves following the drought-response experiment, higher TLP values

441   would have been found before due to osmotic adjustment.

442   High cavitation thresholds in leaves have been reported for species belonging to

443   the same genus as almond (*Prunus mahaleb*, -0.75 MPa) and the same family as olive

444   (*Phillyrea angustifolia*, -0.9 MPa) (Kikuta et al. 1997). In general, however, lower P50

445   values and shallower, more linear in shape Ψ_{leaf} declines would have been expected for

446   drought-tolerant species (Scoffoni et al. 2012) such as olive and almond. The P80

447   values of this study (-4.21 MPa in almond and -5.35 MPa in olive) were in the range of

448   previously published P80 values of drought-tolerant species. For example, Scoffoni et

449   al. (2012) reported P80 values for species of dry habitats ranging from -4.12 MPa for

450   *Heteromeles arbutifolia* to -5.25 MPa for *Cercocarpus betuloides*. P80 has been

451   reported to be more useful for comparison of species' vulnerabilities, because P50

452   values often occur in the middle of the steepest decline (Scoffoni et al. 2012). Indeed, as

453   P50 and P80 values are a function of K_{lmax}, these values may be artifactually skewed.

454   Maximum K_{leaf}, contrary to what happens with stems, cannot be measured and it is

455   usually estimated from the highest K_{leaf} values or extrapolating the Ψ_{leaf}- K_{leaf} adjusted

456   curve to $\Psi_{leaf} = 0$ (Scoffoni et al. 2012).

457   Apparently, the loss of hydraulic conductance at high Ψ_{leaf} could be surprising

458   since the functional range of Ψ_{leaf} for both species is usually lower, with minimum Ψ_{leaf}

459   around -3.5 MPa in almond (Egea et al. 2012) and -4.5 MPa in olive (Torres-Ruiz et al.

460   2013). Although steep decreases of K_{leaf} to high Ψ_{leaf} have been often reported

461   (Blackman et al. 2009, Scoffoni et al. 2012), methodological artifacts in Ψ_{leaf}

462   measurement should be considered. When leaves are well-hydrated, Ψ_{leaf} is high and the

463   gradient of water potential during measurements is small. Mistakes made in the correct

464   determination of high Ψ_{leaf} would lead to large errors in the estimation of K_{leaf}.

465   However, absolute errors in the measurement of Ψ_{leaf} at high water potentials, 0.1 MPa

466   for example, cannot explain the high values of K_{leaf} observed and thus, a Ψ_{leaf}

467   measurement artifact can be disregarded. Additional evidence suggesting that our

468   results were not affected by a methodological artifact comes from the comparison of the

469   response of K_{leaf} and K_{plant} to Ψ_{leaf} (Fig. 4), since both curves show a similar shape.

470   The early loss of K_{leaf} might be related to leaf shrinkage (Scoffoni et al. 2013), as

471   mentioned above. Consequently, it might cause a reduction in the connections for water

472   flow among cells in the mesophyll. Additionally, leaf shrinkage would reduce

473   evaporative surface within the leaf. The outside-xylem vulnerability could play an

474   important role in driving the initial vulnerability at mild water deficits. Thus, the initial

475   slope of the vulnerability curve, before the bulk of cavitation is expected to occur, has

476   been suggested to be more related to the outside-xylem component, while the behavior

477   of the leaf vulnerability curve at stronger water deficits could be more influenced by the

478   xylem component (Scoffoni et al. 2013). The major influence of the flow path outside

479   the xylem could help to explain small differences observed measuring K_{lDRKM} in olive,

480   calculated with either initial flow and initial leaf water potential, or with final flow and

481   final leaf water potential instead (data not shown). It seems that, despite the short period

482   of time that the leaf was connected to the flowmeter, a certain rehydration occurred at

483   the highest water potential values, which is more likely to happen due to a rehydration

484   in the outside part of the xylem than to a refilling process of embolized vessels (Wang

485   et al. 2014; also see Trifilo et al. 2014, Kim et al. 2014).

486

487   *Role of K_{leaf} on the regulation of stomatal conductance*

488 Neither K_{leaf} or ABA_{leaf} unequivocally followed the g_{smax} trend as Ψ_{leaf} decreased (Fig.

489 4). However, the variable most strongly related to g_{smax} was K_{leaf}, especially in olive. In

490 almond, although this relationship was less strong, it was still highly significant. In both

491 studied species, the decline of K_{leaf} began immediately with dehydration, whereas that of

492 g_{smax} began only after a substantial K_{leaf} loss. This agrees with findings reported for

493 other woody species (Johnson et al. 2009, Pasquet-Kok et al. 2010, Guyot et al. 2012).

494 However, the reasons for the loss of K_{leaf} at relatively high values of Ψ_{leaf} have not been

495 fully elucidated yet. Previous work has suggested that cavitation might be responsible

496 for a major portion of K_{leaf} decline in response to low leaf water potentials (Sack and

497 Holbrook 2006), although effects in the extra-xylem pathways such as leaf shrinkage

498 (Scoffoni et al. 2013) and aquaporin deactivation (Shatil-Cohen et al. 2011, Pantin et al.

499 2013) are acquiring larger prominence. Thus, different trajectories of K_{leaf} decline likely

500 did not implicate cavitation itself as a key signal for stomatal closure (Guyot et al.

501 2012). A large percentage of K_{leaf} was lost before stomata started to show an active

502 regulation, ruling out the idea of a protective role of stomata for K_{leaf} maintenance, as

503 has been suggested for shoots. Thus, our results indicate that stomata would operate at

504 the risk of leaf hydraulic catastrophic failure, with leaves functioning as hydraulic

505 circuit breakers that can be reset overnight, rather than as indicators of their

506 susceptibility to catastrophic hydraulic failure (Bucci et al. 2013). As already reported

507 by different authors (Brodribb and Holbrook 2004, Johnson et al. 2009), our

508 vulnerability curves suggest that K_{leaf} could be dynamic during the day as K_{leaf} would

509 vary greatly for the Ψ_{leaf} range usually observed under field conditions in the study area.

510 These evidences point toward a major contribution of the extra-xylem conductance

511 component of K_{leaf} (Scoffoni et al. 2012).

512   The role of ABA in the regulation of g_{smax} did not depict a clear pattern in either

513   almond or olive (Fig. 4d). Although there is a trend for an increasing level of ABA with

514   decreasing Ψ_{leaf}, ABA_{leaf} was highly variable at low Ψ_{leaf}, suggesting that it might be

515   determined by other variables apart from Ψ_{leaf}. For instance in olive, leaves with g_{smax} as

516   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

517   of 100 ng g FW^{-1}.This does not mean that ABA did not play a role in the response to

518   water stress. Actually, in addition to regulating many processes at the plant and leaf

519   level (Hetherington 2001, Cutler et al. 2010), ABA has been proposed to regulate K_{leaf}

520   (Pantin et al. 2013). Further studies on the regulation of stomata by hydraulic and non-

521   hydraulic signals are necessary to clarify the actual role of each component in the

522   stomatal control mechanism of the two species considered here.

523

524   **Conclusions**

525   We found that K_{leaf} decreased exponentially with Ψ_{leaf} in both olive and almond. This

526   decrease was steeper for almond than for olive, according to independent leaf functional

527   features, such as lower TLP in olive than in almond. We conclude that neither

528   mechanism analyzed unequivocally drives the g_s response to water stress in these two

529   species. However, K_{leaf} is the variable most strongly related to the g_s response to water

530   stress, especially in olive , ABA showing no clear effect on g_s regulation. The larger

531   hydraulic capacity of almond at high Ψ_{leaf} allows g_s to be higher in almond than in olive.

532   This is in agreement with the greater VD values found in almond, which contribute to

533   its higher photosynthetic capacity. We also conclude that, although based on different

534   principles, both EFM and DRKM provide similar K_{leaf} values. Tree age and the use of

535   terminal parts of shoots instead of leaves do not have any significant effect on measured

536   K_{leaf} either.

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702

703 **FIGURES CAPTIONS**

704 Figure 1. Leaf hydraulic conductance (K_{leaf}) obtained with the Dynamic rehydration

705 kinetics method (DRKM) and the Evaporative flux method (EFM) in different olive (a)

706 and almond (b) leaves. Dashed line in represents P50 for each species.

707

708   Figure 2. Leaf hydraulic conductance (K_{leaf}) obtained in the same leaves or terminal

709   parts of shoots for olive and almond with the Dynamic rehydration kinetics method

710   (DRKM) and the Evaporative flux method (EFM). Dotted line represents 1:1 line.

711

712   Figure 3. Vulnerability curves for leaf hydraulic conductance for olive and almond

713   determined with the Dynamic rehydration kinetics method (DRKM). The fitted curves

714   are exponential and statistically significant ($y = 0.77+2.67*e^{-0.69*x}$ $R^2=0.62$, *P*<0.0001

715   for olive and $y = 0.75+4.70*e^{-0.74*x}$, $R^2=0.54$, *P*<0.0001 for almond).

716

717   Figure 4. Variation of stomatal conductance (g_{smax}) (a), leaf hydraulic conductance

718   (K_{leaf}) (b), and leaf ABA (ABA_{leaf}) (c) with leaf water potential (Ψ_{leaf}) measured when

719   stomatal conductance is considered to be at its maximum (8.30 GMT) for olive and

720   almond. The points represent the average of values for Ψ_{leaf} intervals of 0.2 MPa and the

721   bars are the standard errors (SE).

722   Figure 5. Comparison of leaf vulnerability curves for olive (a) and almond (b) where

723   K_{leaf} was obtained with the Dynamic rehydration kinetics method (DRKM) and K_{plant}

724   was calculated using Equation 3.

725