Research paper

Role of hydraulic and chemical signals in leaves, stems and roots in the stomatal behaviour of olive trees under water stress and recovery conditions

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The control of plant transpiration by stomata under water stress and recovery conditions is of paramount importance for plant performance and survival. Although both chemical and hydraulic signals emitted within a plant are considered to play a major role in controlling stomatal dynamics, they have rarely been assessed together. The aims of this study were to evaluate (i) the dynamics of chemical and hydraulic signals at leaf, stem and root level, and (ii) their effect on the regulation of stomatal conductance (g_s) during water stress and recovery. Measurements of g_s, water potential, abscisic acid (ABA) content and loss of hydraulic functioning at leaf, stem and root level were conducted during a water stress and recovery period imposed on 1-year-old olive plants (*Olea europaea* L.). Results showed a strong hydraulic segmentation in olive plants, with higher hydraulic functioning losses in roots and leaves than in stems. The dynamics of hydraulic conductance of roots and leaves observed as water stress developed could explain both a protection of the hydraulic functionality of larger organs of the plant (i.e., branches, etc.) and a role in the down-regulation of g_s. On the other hand, ABA also increased, showing a similar pattern to g_s dynamics, and thus its effect on g_s in response to water stress cannot be ruled out. However, neither hydraulic nor non-hydraulic factors were able to explain the delay in the full recovery of g_s after soil water availability was restored.

Keywords: ABA, hydraulic conductivity, hydraulic segmentation, leaf hydraulic conductance, stomatal conductance.

Introduction

In vascular plants, only a portion of the water taken up by roots is used for photosynthesis; most water flows back to the environment through stomata due to plant transpiration. Plants control this amount of water lost by regulating their stomatal aperture (i.e., stomatal conductance (g_s)), allowing them to avoid levels of water stress that could affect their growth and survival. The importance and benefits of controlling stomatal behaviour have been previously reported in a large number of studies (Buckley 2005, Galmés et al. 2007, Torres-Ruiz et al. 2013), yet an integrated understanding of stomatal control remains elusive. In this sense, it has been reported how stomata respond to both chemical and hydraulic signals emitted within the plant (Comstock 2002, Tardieu et al. 2010), but their relative importance in the responses of plants to drought and recovery is still poorly understood.

On one hand, the loss of hydraulic functioning by the plant constitutes a signal involved in stomatal regulation. Cavitation, i.e., the change from liquid to water vapour induced by 'air seeding' caused by high tension during water transport (Salleo et al. 2000, Tyree and Zimmermann 2002), is one of the most

common events that generate such hydraulic signals. Cavitation breaks the water columns in xylem conduits and, therefore, reduces plant water transport capacity. Indeed, the risk of conduit collapse due to the negative pressure of xylem sap would also affect the hydraulic efficiency of the plant and, in turn, would also constitute a hydraulic signal (Hacke et al. 2001, Cochard et al. 2004). The vulnerability to cavitation or collapse of the xylem conduits not only varies among and within species in different habitat conditions (Holbrook and Zwieniecki 2005), but it can also change among organs within a plant, in agreement with the theory of hydraulic segmentation (Tyree et al. 1993, Tsuda and Tyree 1997). Therefore, the role of hydraulic signals in stomatal behaviour is expected to vary not only among species, but also among the different plant organs in which they are generated.

On the other hand, the phytohormone abscisic acid (ABA) has been described as the major mediator in the chemical signalling mechanism involved in the stomatal response of plants during drought (Assmann and Shimazaki 1999, Bauer et al. 2013). Although it has been considered traditionally as a root-to-shoot signal transported in the transpiration stream to the leaves (Dodd 2005), the control of g_s in some species is not related to xylem ABA concentrations or to the ability of roots to produce ABA (Holbrook 2002). Indeed, considering the stem-mediated hydraulic redistribution hypothesis, the redistribution of water from the wet to the dry organs would reduce the water available for transpiration and, therefore, the strength of a possible ABA signalling mechanism triggered at root level (Nadezhdina et al. 2009). The response of the stomata to drought conditions has also been related to significant ABA synthesis at leaf and guard cell levels and, therefore, to an increase of foliar ABA (Christmann et al. 2005). Although the importance of ABA in stomatal behaviour is clear, there are few absolute relationships between the level of ABA and g_s (Brodribb and McAdam 2013).

As the interaction between the stomata and the environment varies between species (Tardieu and Simonneau 1998, Pou et al. 2012), the influence of hydraulic and chemical signals on stomatal behaviour may also vary under increasing water stress and water recovery conditions. Thus, leaves and roots could act as safety valves to prevent xylem dysfunction at the stem level under water stress conditions (Bucci et al. 2013). In other cases, high ABA concentrations at leaf level have been reported after a drought episode, being related to gradual embolism repair in rehydrated plants (Lovisolo et al. 2008). Therefore, understanding the dynamics of the hydraulic and chemical signals holds great potential for explaining plant stomatal behaviour and, therefore, the control of transpiration under conditions of low soil water availability.

In this context, the main aim of this study was to evaluate the effect of hydraulic and chemical signals on the regulation of g_s under water stress and recovery conditions in olive plants.

Both hydraulic functioning and ABA concentrations at leaf, stem and root level were evaluated during a gradual imposition of water stress and a subsequent water recovery period in olive, a species well adapted to drought and with a tight control of g_s to avoid critical water potential values (Fernández et al. 1997, Cuevas et al. 2010, Torres-Ruiz et al. 2013). We hypothesized that stomatal behaviour in olive plants would be largely explained by the changes in hydraulic functioning and ABA in distal organs (i.e., leaves and roots) instead of in stems. Results, therefore, would provide valuable information to advance the state-of-the-art in the coordination between ABA and plant hydraulics and stomatal function.

Materials and methods

Plant material and water treatments

Experiments were carried out on 1-year-old 'Arbequina' olive plants (*Olea europaea* L.) grown in a greenhouse located at 'La Hampa' experimental farm (37°17′N, 6°3′W, altitude 30 m), near Seville (SW Spain), in October and November 2012. Plants were grown in 3-l pots filled with a mixture of 75% soil and 25% organic matter and randomly arranged to cover possible environmental heterogeneity. On 9 October 2012, after maintaining all the plants for 3 months under well-watered conditions, they were separated into two groups to apply two different water regimes: Control (C plants) in which plants were daily irrigated to non-limiting soil water conditions; and Stress (S plants) in which plants were under increasing water stress by withholding irrigation. After 35 days of water withholding, a group of S plants which showed values of leaf water potential at predawn (Ψ_{pd}) of approximately −3.5 MPa were irrigated to field capacity conditions (recovery irrigation, R plants) and, then, daily irrigated as C plants up to the end of the study (i.e., for 6 days). All measurements described below were conducted every 7–10 days during the water stress period in two to four C plants and in four to six S plants. During the water recovery phase, measurements were taken at 18 h and 6 days after applying the recovery irrigation (RI) in five to seven R plants and one to two C plants, assuming that the C plants would show similar behaviour during the whole experiment.

Microclimate, plant water status and stomatal conductance

In the greenhouse, air temperature (T^a), relative humidity of air and vapour pressure deficit were determined at ~1.5 m height among the plants (HMP45C, Vaisala, Inc., Helsinki, Finland). The sensor was connected to a CR10X datalogger (Campbell Scientific, Logan, UT, USA) which stored data every 15 min. Both the Ψ_{pd} and the leaf water potential (Ψ_l) at ~10.30–11.30 GMT were determined in two fully expanded leaves per plant with a Scholander-type pressure chamber (Soilmoisture Equipment Corp., Santa Barbara, CA, USA), following recommendations by

Turner (1988) and Koide et al. (1989). The g_s was determined simultaneously with the Ψ_l measurements in two leaves per plant with a portable gas analyser Li-6400 (Li-Cor, Lincoln, NE, USA) using a 2×3-cm standard chamber under ambient light and CO_2 conditions.

Leaf, stem and root hydraulic conductance

The dynamics of the hydraulic functioning on leaves, stems and roots were evaluated in those plants used for water status and gas exchange measurements. Thus, the leaf hydraulic conductance (k_l) was determined using the dynamic rehydration kinetics method (Brodribb and Holbrook 2003, Blackman and Brodribb 2011). We used the terminal parts of stems with 6–10 leaves due to the small size of olive leaves. Previous studies (Sack and Holbrook 2006, Blackman and Brodribb 2011) have demonstrated that stems and single leaves have similar k_l values (expressed per leaf area). The hydraulic resistance in the stem is assumed to be negligible as long as the stems are short and contain water-filled open vessels (Blackman and Brodribb 2011), which was the case in our study. Thus, the stems were cut under water and rapidly connected to a handmade flow meter to determine the variation in the uptake of water. The flow meter consisted of a pressure transducer (Omega Engineering Ltd, Manchester, UK) connected to a Campbell datalogger CR1000 (Campbell Scientific Ltd, Shepshed, UK). The readings were stored every 1 s. 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 as described in Blackman and Brodribb (2011). The kinetics of the Ψ_l relaxation were determined by measuring the Ψ_l in two adjacent leaves before and after the rehydration. The k_l was then calculated as

$$k_l = \frac{-l}{\Psi_0 \times A} \qquad (1)$$

where Ψ_0 is Ψ_l before rehydration (MPa), l is the instantaneous initial maximum flow rate into the leaf (mmol s^{-1}) and A is the leaf area of the stem (m^2). The total leaf area of each sample was determined using a Li-Cor 3000-A area meter (equipped with a LI-3050C Transparent Belt Conveyor; Li-Cor).

The percentage loss of conductivity of the stem (PLC$_{stem}$) was determined in two current-year stems per plant. Stems were cut under water, wrapped in plastic bags for 1.5 h with wet paper towel inside to allow equilibrium between Ψ_l and xylem water potential (Ψ_x), and transported to the laboratory. Stem Ψ_x was determined in two leaves per stem and a 3-cm-long sample was then excised under water from each stem, debarked and plugged to a XYL'EM apparatus (Bronkhorst, Montigny-les-Cormeilles, France) for the hydraulic measurements. To account for possible passive uptake of water by the sample and increase the accuracy of the measurements, the hydraulic conductance (k) was determined by perfusing a filtered (0.22 µm) and degassed 20 mM KCl solution at pressure gradients of 2.5 and 3.5 kPa. Previous tests in olive have shown that these water heads are below the threshold for refilling xylem vessels opened at both ends during measurement (data not shown). The k value was then determined by measuring linear flow by the pressure gradient relationship at the two pressure gradients used and deriving it directly from the slope as described by Torres-Ruiz et al. (2012). The sample maximum k (k_m) was then calculated similarly to k but after perfusing the samples at 0.15 MPa for 20 min to remove the embolism. PLC$_{stem}$ was then determined as

$$\text{PLC}_{stem} = 100(1 - k/k_m). \qquad (2)$$

After collecting the stems, the entire plants were also bagged with wet paper towel inside to prevent transpiration upon determination of the PLC of roots (PLC$_{root}$) the day after. For PLC$_{root}$, the k value of the root system was determined by the vacuum chamber method (Kolb et al. 1996). Briefly, the entire root system of each plant was cut under water and inserted into a vacuum canister with its proximal end protruding. The root system was connected by plastic tubing to a beaker of degassed solution (20 mM KCl) on a digital balance (Mettler Toledo, Greifensee, Switzerland). Pressure in the vacuum canister was reduced to -20 kPa and flow was computed by monitoring the weight of the beaker at 5-s intervals with a computer. The canister was then allowed to reach atmospheric pressure to compute possible flows with no pressure gradients (F_0) (Torres-Ruiz et al. 2012). The flows at -20 kPa and at atmospheric pressure were determined for a second time to test whether F_0 had shifted during the measurements. When F_0 deviation was $>10\%$, measurements were repeated. The k value was determined from the slope of the flow vs pressure relationship of the four data points. Samples were then vacuum infiltrated for 1 h to remove the embolism and k_m was determined as k for calculating PLC$_{root}$ according to Eq. (1).

The PLC and Ψ_x values determined on stems and roots were plotted to determine the vulnerability curves for both plant levels by fitting a Weibull function (Neufeld et al. 1992) with an additional independent factor to consider the levels of embolism measured at $\Psi_x = 0$ (Torres-Ruiz et al. 2013):

$$\text{PLC} = (100 - y_0) - (100 - y_0)e^{-(x/b)^c} + y_0 \qquad (3)$$

with x being the Ψ_x, b the Ψ_x for a PLC of 63%, c a dimensionless parameter controlling the shape of the curve and y_0 the PLC at Ψ_x of 0 MPa. For roots, Ψ_x was assumed to be similar to Ψ_{pd} since plants were under non-transpiring conditions (Choné 2001).

Abscisic acid determination

Leaf ABA was determined in leaves collected in the early morning and frozen in liquid nitrogen. Leaf samples were stored frozen at −80 °C until ABA measurement using the liquid chromatography–electrospray/tandem mass spectrometry method of Gómez-Cadenas et al. (1996). Samples of ~400 mg of frozen leaf tissue without midribs were milled with liquid nitrogen and homogenized and extracted in 5 ml of distilled water. An aliquot of 50 μl of 2 μg ml^{-1} deuterated abscisic acid (dABA) was added as an internal standard. Samples were centrifuged (31,000 g; 15 min; 4 °C), supernatants were acidified to pH 3.0 (150 μl acetic acid 30% (v/v)) and leaf extracts were two-times partitioned with 3 ml of diethyl ether. Organic phases were collected and evaporated using a vacuum pump. Tube walls were washed with 1 ml of 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 water (reverse osmosis) and filtered through a polypropylene membrane syringe filter (∅ 0.2 μm, VWR® International, Philadelphia, PA, USA). Analyses were conducted using an Agilent 1290 Infinity HPLC system (Agilent Technologies, Inc., Santa Clara, CA, USA) coupled with an electrospray/tandem mass spectrometer (3200 QTRAP® LC/MS/MS System, AB SCIEX, Framingham, MA, USA) and data were processed with mass spectrometry software (Analyst® Software, AB SCIEX). Leaf ABA was normalized by fresh weight.

Stem and root xylem sap for ABA determination was extracted using a Scholander-type pressure bomb applying a maximum overpressure of 0.2 MPa until 10–15 μl of sap were collected in 0.5-ml vials. The sap samples were then frozen in liquid nitrogen and stored in a −80 °C freezer until ABA determination following the method of Gómez-Cadenas et al. (1996). Two microlitres of 2 μg ml^{-1} dABA were added as an internal standard to an aliquot of 30 μl obtained from each sap sample and centrifuged (15,000 g; 15 min; 4 °C). Twenty microlitres of the supernatants were used for the ABA determination by liquid chromatography–tandem mass spectrometry (UHPLC179 MS-MS, Agilent 1290 Infinity, Waldbronn, Germany) and data were analysed with mass spectrometry software (Analyst® Software, AB SCIEX).

Statistics

Linear models using restricted maximum likelihood (R package 'nlme'; Pinheiro et al. 2011) were used to assess the overall effect of the different water treatments applied (i.e., Control, Stress and Recovery) on Ψ, g_s, PLC (root and stem), k_l and ABA at leaf, stem and root level. Multiple comparisons were conducted when an overall effect was detected with the R package 'multcomp' (Hothorn et al. 2008). The average of each plant was used when more than one measurement per plant was taken. Differences were considered significant when $\alpha = 0.05$. Dixon's test was used to determine outliers (Sokal and Rohlf

1995). The analyses were conducted with the R packages mentioned and Sigmaplot (SPSS, Inc., Chicago, USA).

Results

The greenhouse mean daily temperature was ~22 °C for most of the study period and vapour pressure deficit was never >1.40 kPa. The lack of irrigation reduced Ψ_{pd} in S plants from −0.43 MPa measured on Day 0 of the experiment to values closer to −4.00 MPa at the end of the water stress period on Days 25 and 35 (Figure 1). In contrast, C plants showed a nearly constant Ψ_{pd} of −0.57 ± 0.03 MPa for the entire experimental period. Ψ_{pd} values of S plants were significantly lower than those of C plants from the sixth day after water withholding and until the application of irrigation recovery. Ψ_{pd} recovered quickly in R plants after 18 h of irrigation recovery, showing similar Ψ_{pd} to the C plants (Figure 1). A similar trend was observed for Ψ_l (data not shown).

As a consequence of the water deficit imposition, a marked g_s reduction was observed in S plants with Ψ_l lower than −1.5 MPa (Figure 2). After 18 h of initializing the recovery irrigation, R plants still showed lower g_s values than C plants for the whole experimental period. However, a marked increase in g_s was observed 6 days after the RI (Figure 2), resulting in g_s values significantly higher than those after 18 h of the RI (Table 1).

Regarding plant hydraulic functionality, different patterns were observed at the leaf, stem and root level (Figure 3). Whereas marked hydraulic losses in roots and leaves were observed in S plants at Ψ_x and Ψ_l lower than −1.5 MPa,
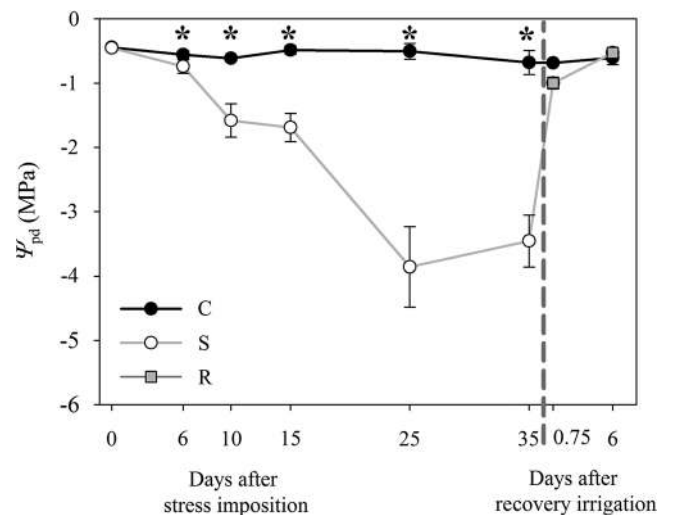

Figure 1. Time courses of leaf water potential measured at predawn (Ψ_{pd}) along the experimental period in Control (C), Stress (S) and Recovery (R) plants. Data points are the average of three to five values; vertical bars represent ±SE. Asterisks indicate a statistically significant difference ($P < 0.05$). The dashed line indicates the beginning of the recovery irrigation. The x-axis represents the days after the beginning of the water stress imposition and recovery irrigation.

respectively, PLC_{stem} started to increase slightly only when Ψ_x reached lower values than approximately −1.5 MPa. This agrees with the calculated air entry pressure (P_e) of the stems, which was about −1.3 MPa (Figure 3). Abscisic acid also showed different trends at the root, leaf and stem level (Figure 4). Sap ABA increased progressively at both the root and stem level as Ψ_{pd} decreased, although this increase was steeper in stems than in roots (Figure 4a and b). In contrast, leaf ABA showed a marked increase up to Ψ_{pd} values of about −1.0 MPa, and from −1.5 MPa onwards it remained nearly constant (Figure 4c).

Both k_l and PLC_{root} showed a strong decline with g_s at Ψ_{pd} values lower than −1 MPa during the progression of water stress (Figure 5a and c), suggesting some coordination among them. Leaf ABA depicted a relationship with g_s, decreasing exponentially as water stress progressed (Figure 5b). During the water recovery phase, R plants showed similar or even lower levels of ABA at the root, stem and leaf levels than C plants 18 h after applying the RI (Table 1). A similar recovery time was observed in k_l. Although mean k_l in R plants 18 h after applying the RI
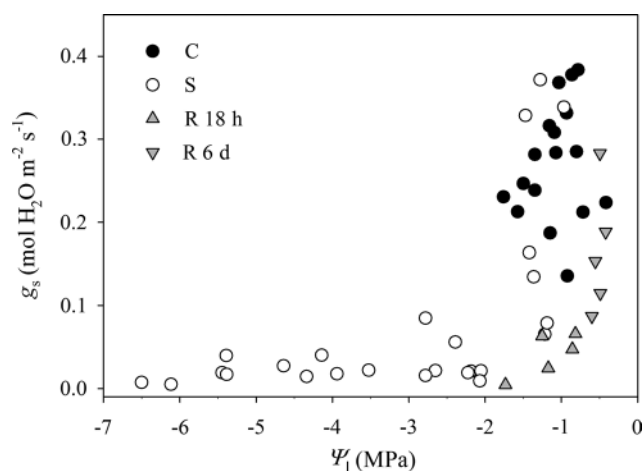
was lower than mean k_l in C plants (probably due to a single k_l value, Figure 3), the difference was not significant.

Discussion

Water stress phase

The steep exponential decay of g_s with soil water availability (Figure 5) or Ψ_l (Figure 2) indicates strong stomatal regulation above a threshold of water potential. The trend of the results suggests that the plant tuned a wide range of values of g_s before achieving complete stomatal closure in order to maintain Ψ_l above −1.5 MPa. Such a threshold agrees with previous studies in olive trees which reported a tight control of stomatal behaviour to maintain Ψ_l around −1.4 and −1.6 MPa and avoid critical Ψ_l values for this species (Sofo et al. 2008, Torres-Ruiz et al. 2013). This value coincides with the P_e value at the stem level, which indicates the threshold xylem pressure at which PLC_{stem} begins to increase rapidly (Meinzer et al. 2009). Hence, the stomatal closure observed in this study would allow the plant to maintain Ψ_l and, therefore, Ψ_x around the P_e value to preserve stem hydraulic functioning. A similar P_e has been previously reported for mature olive trees (Torres-Ruiz et al. 2013), suggesting that this value is characteristic of this species and not easily modified by different growing conditions or plant age and size. When the water stress becomes more severe, beyond the value of P_e, the plant overcame its capacity for regulating g_s, and Ψ_l dropped to more negative values. Despite the low Ψ_l reached in this study, the high resistance to cavitation of olive stems preserved their functionality for most of the water potentials observed (Figure 3b). Therefore, no significant changes in PLC_{stem} were recorded in the range of water potential above −1.5 MPa where the regulation of g_s occurred (Figure 2). This suggests that the loss of hydraulic conductivity in the stem is not the signal triggering stomatal closure. In contrast, the progress of k_l, leaf ABA and PLC_{root} matched the pattern observed for g_s as a function of Ψ_{pd} (Figure 5). The different dynamics observed in the loss of hydraulic functioning between leaves, stems and roots indicate

Figure 2. Relationships between leaf water potential (Ψ_l) and stomatal conductance (g_s) of Control (C), Stress (S) and Recovery plants 18 h (R 18 h) and 6 days (R 6 d) after the recovery irrigation.

Table 1. Mean ± SE values of the main physiological variables measured in R plants during the water recovery phase 18 h and 6 days after applying the RI, and in C plants during the recovery period (for ABA, C plants of the rest of the experimental period were also used due to the lack of samples). Different letters and the asterisks indicate a statistically significant difference ($P < 0.05$); ns, no significant difference. FW, fresh weight.

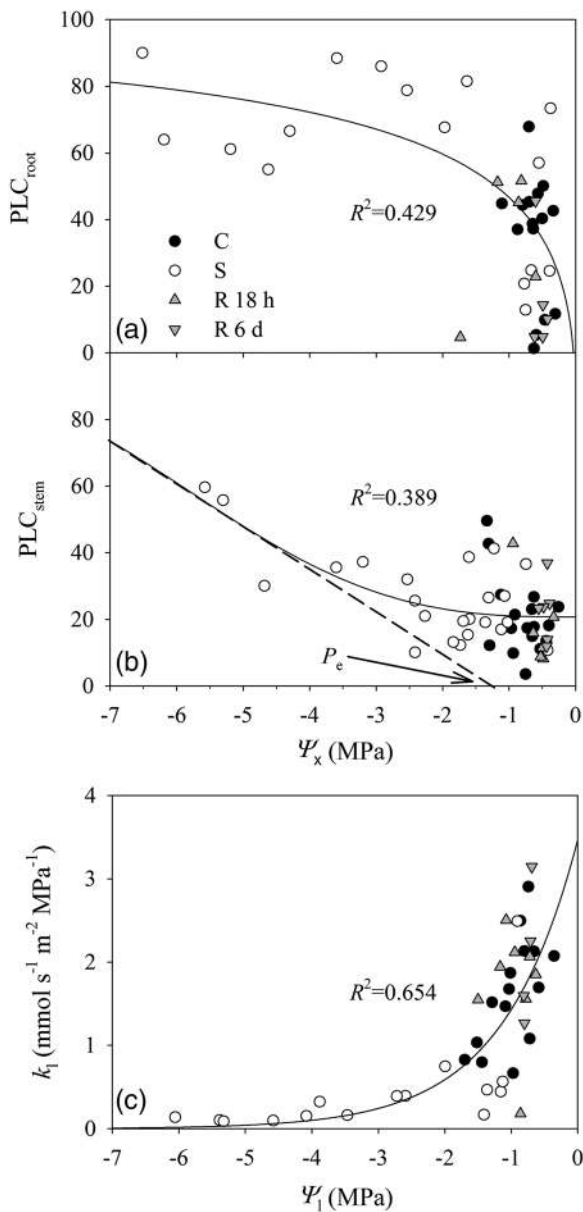| Variables	R plants		C plants	
	Time after RI			
	18 h	6 days		
g_s (mol H_2O m^{-2} s^{-1})	0.043 ± 0.007a	0.162 ± 0.016b	0.171 ± 0.044b	*
k_l (mmol s^{-1} m^{-2} MPa^{-1})	1.72 ± 0.25	2.06 ± 0.41	2.69 ± 0.20	ns
PLC_{stem}	18.0 ± 5.3	22.5 ± 3.6	14.27 ± 3.1	ns
PLC_{root}	35.1 ± 9.2	16.0 ± 7.6	34.9 ± 4.9	ns
ABA_{root} (ng ml^{-1})	6.7 ± 0.9a	8.3 ± 0.7a	16.9 ± 4.6b	*
ABA_{stem} (ng ml^{-1})	20.7 ± 5.8	11.7 ± 0.7	19.0 ± 6.6	ns
ABA_{leaf} (ng gFW^{-1})	80.1 ± 32.3a	32.2 ± 1.8b	46.2 ± 5.7a	*

Figure 3. Relationships between xylem water potential (Ψ_x) and percentage loss of conductivity at root (PLC_{root}) (a) and stem (PLC_{stem}) (b) level, and between leaf water potential (Ψ_l) and leaf hydraulic conductance (k_l) of Control (C), Stress (S) and Recovery plants 18 h (R 18 h) and 6 days (R 6 d) after the recovery irrigation. The solid lines represent the Weibull equation (Eq. 3) (a and b) and the exponential curve (c) fitted to the C and S data for each plant level. The dashed line represents the tangent through the midpoint of the fitted curve for the stems and its x-intercept represents the air entry pressure (P_e) following Meinzer et al. (2009).

an important hydraulic segmentation in olive trees which allows them to confine the hydraulic dysfunction to the distal organs of the plant (fine roots and leaves) in favour of the larger ones (stems and trunk) (Tsuda and Tyree 1997, Vilagrosa et al. 2012). This is consistent with other findings which have also reported that the stem is more resistant to hydraulic dysfunction than other plant organs in several species (e.g., Choat et al. 2005,
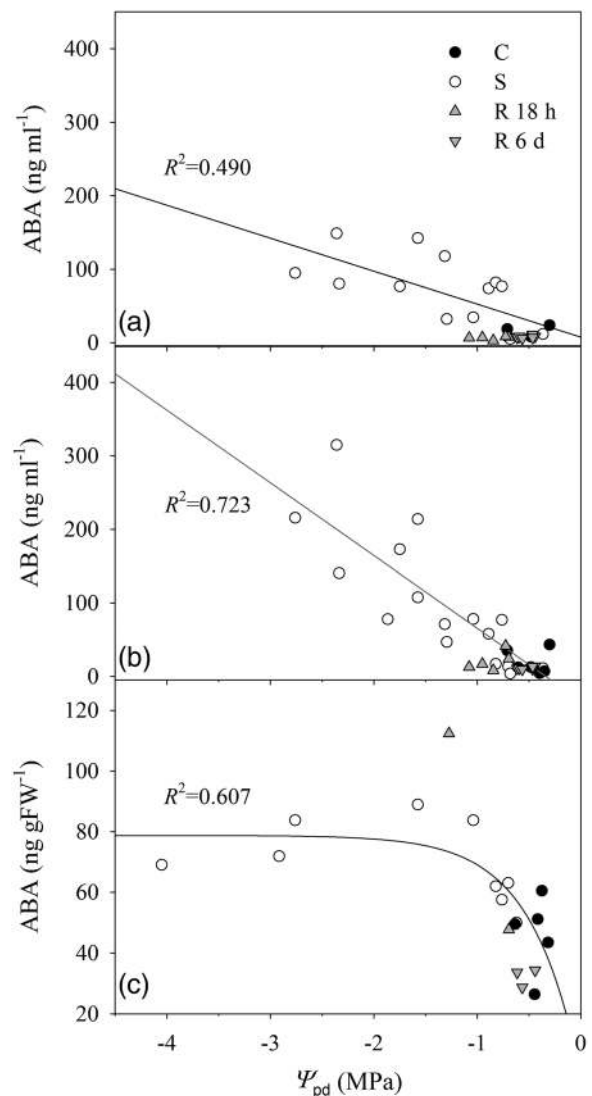

Figure 4. Relationships between predawn water potential (Ψ_{pd}) and ABA content at root (a), stem (b) and leaf level (c) of Control (C), Stress (S) and Recovery plants 18 h (R 18 h) and 6 days (R 6 d) after the recovery irrigation. The solid lines represent the linear regression (a and b) and the exponential curve (c) fitted to the C and S data for each plant level.

Hao et al. 2008, Johnson et al. 2011, Bucci et al. 2013). As has been noted by Bucci et al. (2013), whereas leaves would act as 'safety valves' to protect the integrity of the upstream hydraulic pathway, roots would decouple parts of the plant from drier soil layers. The dynamic seasonal and diurnal evolution of k_l (Brodribb and Holbrook 2004, Zufferey et al. 2011, Scoffoni et al. 2012) makes this variable appropriate for a fine modulation of the response of stomatal conductance to changes in the atmospheric demand. Indeed, our results show how the high reductions in g_s agree with the high losses of hydraulic conductance in leaves and roots. Interestingly, a wide range of k_l values were reached by the olive species for Ψ_l higher than −1.5 MPa, which indicates its capacity to regulate stomatal behaviour and, therefore, to control transpiration. Similar curve shapes and k_l
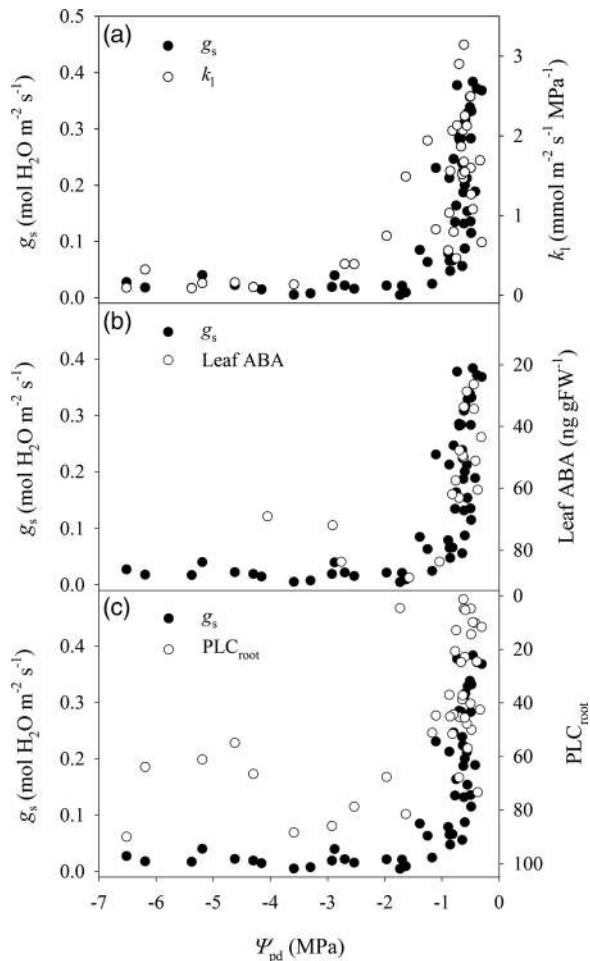
Figure 5. Evolution of maximum stomatal conductance (g_s) and leaf hydraulic conductance (k_l) with predawn water potential (Ψ_{pd}) (a), g_s and leaf ABA with Ψ_{pd} (b) and g_s and percentage loss of conductivity at root (PLC$_{root}$) level with Ψ_{pd} (c), considering the data of the whole study period (C, S and R plants).

values have been previously reported for other species (Domec et al. 2009, Scoffoni et al. 2012). However, considering that the occurrence of daily cycles of embolism and refilling is actually under debate (Delzon and Cochard 2014, Wang et al. 2014), it is important to highlight that the exponential decay on k_l and root k observed at Ψ values near zero might be linked not only to cavitation events, but also to a possible reduction in the permeability of extra-xylary tissue (Sack and Holbrook 2006, Pou et al. 2013) or to the xylem implosion. Interestingly, Scoffoni et al. (2014) have recently reported that the extra-xylary pathway may be more vulnerable than the xylem pathway, delaying, therefore, the onset of xylem embolism or collapse. Thus, more experiments are required to elucidate the processes leading to such exponential decays observed at relatively high Ψ_l. In any case, the reduction in k_l observed at Ψ_l below −1.5 MPa would constitute an important mechanism for olive to reduce its water losses by transpiration and it would be, therefore, closely related to its resistance to drought.

As discussed by Buckley (2005), the homoeostatic control of Ψ_l, widely known as isohydric behaviour, can be explained by a simple feedback regulation of stomatal conductance in response to Ψ_l and a reduction in the hydraulics which would act as an amplifier of the signal. This would be the case in our study due to the important decreases in the hydraulic functioning observed at leaf and root level as the plant approaches the target fixed by P_e. Furthermore, the coordination between roots and leaves in response to water stress is noteworthy. If both ends of the hydraulic pathway have a key role in the maintenance and regulation of the hydraulic system in plants, it is desirable that the progress of hydraulic loss follows similar patterns. As a consequence, co-limitation is achieved, avoiding disequilibrium between the hydraulic supply of water in roots and leaves. This coordination of the hydraulic functionality of roots and leaves has been observed in grapevines as well (Lovisolo et al. 2008), and not only during the progress of water stress, but also during the restoration of functionality after re-watering. This functional coordination of the hydraulic components of these two distal parts of the plant deserves further attention in the future.

This coordination of roots and leaves was not found with the ABA results. Neither stem ABA nor root ABA showed significant relationships with stomatal behaviour (Figure 4). Although both hydraulic and non-hydraulic signals (leaf ABA) would be able to partially describe the regulation of stomatal conductance as suggested in Figure 5, the hydraulic limitation imposed by the distal organs of the plants (i.e., roots and leaves) would be sufficient to explain the regulation of g_s under water stress progression in our study. This agrees with previous experiments of response to water stress and recovery that have recognized hydraulic signalling as the main effector of the observed response of stomata (Brodribb and Cochard 2009, Resco et al. 2009). However, the effect of leaf ABA on stomatal behaviour should not be dismissed and relationships between g_s and ABA, like those found in Figure 5, have been used to claim the central role played by ABA in the stomatal control of transpiration (Tardieu and Simonneau 1998). Recently, Pantin et al. (2013) have suggested the dual role played by ABA exerting an independent effect in both k_l and g_s. One is a direct biochemical action on guard cells and the other an indirect hydraulic action through a decrease in leaf water permeability triggered within vascular tissues. This dual effect of ABA deserves further research in the future, since it appears to be a critical point to unravel the regulation of transpiration by stomata as well as the dichotomy between hydraulic and non-hydraulic signalling. The discussion on which signal is more important in the regulation of stomatal conductance has drawn much attention in plant ecophysiology (Hetherington 2001, Schachtman and Goodger 2008, Brodribb 2009, Brodribb and McAdam 2011), but it is beyond the scope of this work.

Water recovery phase

Re-watered plants (i.e., R plants) recovered their water status only 18 h after irrigation, showing Ψ_l and Ψ_{pd} similar to C plants. This agrees with previous studies on olive which reported a good recovering capacity from water stress for this species (Moriana et al. 2007, Torres-Ruiz et al. 2013). However, g_s was still lower than the control 18 h after irrigation ($P = 0.002$), and it reached values of C plants 6 days later ($P = 0.823$), meaning that the g_s recovery happened some time after 18 h and before 6 days after the irrigation recovery. As previously reported for other species, there was a limitation for g_s due to a factor different from the plant water status per se (Lovisolo et al. 2008, Resco et al. 2009). Lovisolo et al. (2008) found in grapevines that during the first stages of recovery after a water stress period, the delay in the recovery of stomatal conductance was related to the presence of ABA in leaves. These authors proposed that the slower elimination in the plant of the non-hydraulic stress-derived ABA signal could facilitate the recovery of leaf gas exchange by promoting gradual embolism repair in different organs of the plants. However, this does not seem to be the case in our study. A quick reduction of ABA was found in all plant organs studied, reaching similar or even lower values than the C plants (Figure 4). Although our results are not robust enough to draw a clear conclusion (Table 1), they are in accordance with the results found by Pou et al. (2008) in grapevine who reported that although during a water stress imposition period there was a close relationship of stomatal conductance with ABA concentration, after re-watering the correlation did not hold. In their case, k_l was used to explain the recovery of g_s after water application, and therefore the delay in the recovery of g_s was explained by a hydraulic factor. Once again, this was not our case either since k_l recovered to control values just 18 h after re-watering (Figure 3). Scoffoni et al. (2012) found that the success in the recovery of k_l after water stress imposition was species dependent, and dependent on the degree of stress suffered by the plants. In any case, the quick recovery of k_l seems to have a prime role in the dynamics of whole-plant hydraulic recovery and tolerance to dynamic water regimes.

The regulation and response to water stress and the recovery of g_s, k_l and plant water relations are known to be strongly mediated by aquaporins (Kaldenhoff et al. 2008), which at the same time are regulated by ABA. A key crossroad of both hydraulic and chemical signalling might be located in the bundle sheath cells. Shatil-Cohen et al. (2011) have suggested that these cells act as valves during drought by converting ABA signals from the vein xylem into decreases in k_l by deactivating aquaporins. After a water stress imposition and recovery study, Pou et al. (2013) concluded that this regulation is complex and involves the participation of different families of aquaporins, like PIPs and TIPs, with different responses to stress. The roles of aquaporins extend beyond just facilitating water flow across cellular membranes and, in relation to water stress and coordination with g_s, they may be involved in either osmoregulation or CO_2 transport (Pou et al. 2013, Perez-Martin et al. 2014). Therefore, a possible explanation for the delay in the recovery of g_s in our study might have involved restoration of the activity of certain aquaporins not affecting k_l directly but, for instance, the balance of osmolytes in the cells. Another possibility is that a metabolic limitation occurred. Recently, Kelly et al. (2012) reported that high levels of hexokinase expression reduced the stomatal conductance and transpiration of *Arabidopsis* plants. Under conditions of water stress, the increase of ABA in guard cells induces the expression of hexokinases which accelerates the stomatal closure. As the activity of hexokinases is involved in sugar sensing and stimulation of the balance of osmolytes, restoration of this balance after the recovery of water status might explain the low stomatal conductance at the initial stage of the recovery process. Further studies are needed to evaluate the presence of hexokinases in leaves after re-watering and the balance of sugars between guard cells and apoplast and mesophyll.

Conclusions

The reduction in g_s observed in 'Arbequina' olive trees during the imposed water stress was more related to the loss of hydraulic functioning at the most distal organs of the plant (i.e., roots and leaves) than to the increase of ABA levels at leaf, stem and root level. However, the g_s restrictions observed in re-watered plants, even after recovering their water status, were not related to a reduced hydraulic functioning or to the presence of chemical signals produced during the water stress phase. Results showed a marked hydraulic segmentation in olive plants which allows us to confine the hydraulic dysfunction at leaf and root level in favour of larger organs of the plant (i.e., stems and trunk).

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Conflict of interest

None declared.

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