Physiological and Genetic Response of Olive Leaves to Water Stress and Recovery: Implications of Mesophyll Conductance and Genetic Expression of Aquaporins and Carbonic Anhydrase

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Keywords: *Olea europaea*, drought, re-irrigation, *Oe*PIP1.1, *Oe*PIP2.1, stomatal conductance, water relations

Abstract

Drought is considered to be the main environmental factor limiting photosynthesis (A_N) and, consequently, plant growth and yield worldwide. During photosynthesis, the pathway of CO_2 from the atmosphere to the site of carboxylation in the chloroplast stroma has two main components: stomatal (g_s) and mesophyll (g_m) conductances. Both are finite and dynamic, responding to many abiotic factors, therefore reducing CO_2 concentration. However, little is known about g_m regulation in the short term, where a possible role of aquaporins (AQP) and carbonic anhydrase (CA) has been proposed. Five-year-old olive trees growing in 50 L pots were used to evaluate the acclimation and recovery of A_N to drought and subsequent re-watering. Control trees were well-irrigated, while in stressed trees irrigation was withheld for 13 days and then resumed. We made a simultaneous analysis of the genetic expression of two AQP, *Oe*PIP1.1 and *Oe*PIP2.1, and of CA, on the one hand, and leaf water status, leaf gas exchange and shoot hydraulic conductivity on the other. This is the first time that genetic expression in olive is related to main physiological variables. Two days after withholding irrigation (a.w.i.), the g_s and g_m values in Stress tress were lower than in Control trees. This limited photosynthesis. Leaf water status decreased from day 4 a.w.i. Midday leaf water potential dropped from -1.2 on the day before withholding irrigation to -6.0 MPa on day 9 a.w.i. CA expression decreased during drought and there was a peak on *Oe*PIP1.1 expression on day 4 a.w.i. Leaf water status recovered in ca. 36 h after resuming irrigation. Both g_m and A_N did not fully recover until 46 days after rewatering. Stomatal conductance, however, did not recover in that period, probably because of an irreversible loss of shoot hydraulic conductivity. Both *Oe*PIP1.1 and *Oe*PIP2.1 peaked 36 h after rewatering. We found significant correlations between g_m and both *Oe*PIP2.1 and CA expression.

INTRODUCTION

Water stress may have a great effect on photosynthesis, causing serious limitations on plant growth and yield worldwide, especially in semi-arid areas like those in the Mediterranean basin (Lawlor, 1995) where *Olea europaea* grows.

Photosynthesis requires the diffusion of CO_2 from the atmosphere to the site of carboxylation in the chloroplast stroma. In this path, CO_2 has to cross the stomata pore (stomatal conductance, g_s) and the air spaces and membranes of mesophyll cells (mesophyll conductance, g_m). Although an infinite and constant g_m is assumed in most studies, it is now known that g_m is finite and sufficiently low to cause a lower CO_2 concentration in the chloroplast compared to that in the substomatal cavity. Consequently,

g_m may limit photosynthesis (Flexas et al., 2008). Moreover, g_m is not constant, but responds rapid and reversibly to changing ambient conditions like those causing drought (Flexas et al., 2002; Perez-Martin et al., 2009).

On the basis of a temperature response coefficient (Q_{10}) of approximately 2.2 for g_m in tobacco leaves, Bernacchi et al. (2002) claimed that an enzymatic or protein-facilitated diffusion of CO_2 controls g_m. The most likely candidates have been proposed to be carbonic anhydrase (CA) and aquaporins (AQPs). Thus, some AQPs have been related to CO_2 transport in the leaf mesophyll during photosynthesis (Galmés et al., 2007). Within the multiple indirect evidence of the AQP involvement in g_m regulation are the in vivo variation of g_m caused by different expression levels of *Nt*AQP1 in transgenic tobacco plants or the increased CO_2 permeability in oocytes membranes expressing *Nt*AQP1 (Flexas et al., 2006). However, the potential relationship between the role of AQPs in the regulation of g_m and the regulation expression of their genes remains unclear. It has been suggested that CA activity is closely associated with g_m in C_3 plants (Flexas et al., 2008). Some authors have proposed that CA-mediated CO_2 diffusion may be more important when g_m is low because of structural properties of the leaves. This applies to woody species, where cell wall conductance is much lower than chloroplast conductance (Gillon and Yakir, 2000). Recent evidence suggests that AQPs could be involved in the regulation of photosynthesis by affecting CO_2 transport from the atmosphere to the chloroplasts, both indirectly through the regulation of water balance and stomata control, and directly through the regulation of mesophyll conductance to CO_2 (Kaldenhoff et al., 2008). However, most of the studies showing AQP expression under water stress lack a simultaneous analysis of the most common physiological responses to water shortage, including plant water status, A_N, g_s and hydraulic conductivity (Galmés et al., 2007).

Our hypotheses are that g_m has a major role in photosynthesis limitations during acclimation to water stress and subsequent recovery, and that the variations in g_m can be related to the genetic expression of AQP or CA. The aims of this work were 1) to characterize, for the first time in olive, the physiological and genetic response to both soil water deficit and subsequent recovery of irrigation, and 2) to derive a relationship between protein expression and g_m.

MATERIALS AND METHODS

The experiment was made in the summer of 2009. We used 5-year-old olive trees (*O. europaea* L. 'Manzanilla') growing in 50 L pots near Seville, southwest Spain (37° 17'N, 6°3'W, 30 m). Two different water treatments were established, with three olive trees per treatment: 1) Control, in which the trees were kept well-watered, and 2) Stress, in which irrigation was withheld for 13 days, followed by a 6 weeks period of daily irrigation. Before imposing the treatments all plants were well-watered. Measurements of g_s, A_N, and chlorophyll fluorescence were made at midday (12:00 GMT) with an open gas exchange system Li-6400 (Li-Cor Inc., Lincoln, NE, USA), with saturating light conditions in the chamber. From these measurements we estimated g_m, using the "variable *J* method" of Harley et al. (1992). For these measurements, two fully developed, sun-exposed leaves per each tree and treatment (n=6) were sampled. Leaf water potential was also measured at midday (Ψ_{mid}) in the same number and type of leaves, with a pressure chamber (PMS Instrument Company, Albany, Oregon, USA). Additional sets of leaves (same characteristics) were sampled for determining relative water content (RWC).

Leaves in which gas exchange measurements were made, were harvested to analyse genetic expression of AQPs *Oe*PIP1.1 and *Oe*PIP2.1, and of CA. The leaves were frozen in liquid N and welted, for RNA extraction with the RNeasy Plant Mini Kit (Qiagen, Venlo, The Netherlands). The extracted RNA was treated with the RNase-Free DNase Set (Qiagen) to avoid genomic DNA contamination during RNA purification. RNA purity and concentration were determined by measuring the absorbance at 260, 280 and 320 nm with a spectrophotometer (Lambda 6 UV-VIS; Perkin-Elmer, Bucks, UK). From 1 μg of the extracted RNA, cDNA was synthesized with the QuantiTect Reverse Transcription Kit (Qiagen). The used specific primers were designed by Applied

Biosystems (Applied Biosystems, Foster City, CA) and were all characterized by FAM reporter. Finally, Real-time PCR (Applied Biosystems 7300 Real-Time PCR System) was performed on the samples and relative gene expression was determined using the standard curve procedure.

The percentage loss of hydraulic conductivity (PLC) was determined in ca. 2 cm long segments taken from the base of 8 current-year shoots per treatment, both at 11 and 46 days after rewatering. Cautions were taken during sampling, following Ennajeh et al. (2008). Hydraulic conductance measurements were made with a xylem embolism meter (XYL'EM, Xylem Embolism Meter, Bronkhorst, Montigny les Cormeilles, France), after Sperry and Tyree (1988).

Volumetric soil water contents (θ_v) were measured with a TDR system (FOM, Institute of Agrophysics, Lublin, Poland), consisting of two 0.15 m long TDR probes per pot, at 0.05 and 0.20 m depths. From the θ_v values we calculated the relative extractable water of the soil (REW), defined by Granier as $REW = (R - R_{min})/(R_{max} - R_{min})$, being R (mm) the actual soil water content, R_{min} (mm) the minimum soil water content measured during the experiments, and R_{max} (mm) the soil water content at field capacity.

Differences between treatments (Student's t-test, $P \leq 0.05$) were analysed with Statistica software package for Windows v. 6.0. ANOVA analyses were performed to evaluate differences between dates (Tukey test, $P \leq 0.05$). Pearson coefficients were calculated to assess correlation between different variables.

RESULTS AND DISCUSSION

In Control trees, REW values showed non-limiting soil water conditions from soon after the beginning of the experiment. In Stress plants, REW decreased rapidly and markedly after withholding irrigation. Soon after resuming irrigation, REW values in Stress plants became close to initial values (Fig. 1a).

The first variables to respond to suspended were g_s and g_m. On day 2 after withholding irrigation (a.w.i.), both variables showed values significantly lower in the Stress plants than in the Control plants (Fig. 2a and c). As a consequence, lower values of A_N were also measured in the Stress plants (Fig. 2b). A fall in g_s and g_m under drought conditions was also described in olive by Diaz-Espejo et al. (2007). As stress developed, mesophyll conductance limitations of photosynthesis became bigger than stomatal limitations (data not shown). Leaf water status measurements showed increasing water stress from day 4 a.w.i. Thus, midday leaf water potential (Ψ_{mid}) dropped from -1.2 MPa on the day before withholding irrigation to -6.0 MPa on day 9 a.w.i. (Fig. 1b). Both Pre-dawn leaf water potential (Ψ_{pd}) and RWC followed a similar pattern (data not shown).

The *Oe*PIP1.1 expression in Stress plants peaked on day 4 a.w.i., and returned to initial values on day 11 a.w.i. (Fig. 2d). Galmés et al. (2007) interpreted the upregulation in some AQPs as a mechanism to promote water movement inside leaves via simplasto by increasing membrane permeability to water when this is less available for the plant. There was no effect of withholding irrigation in *Oe*PIP2.1 expression (Fig. 2e). Secchi et al. (2007a) observed a down-regulation of this protein in olive during drought, and the opposite after resuming irrigation. CA expression decreased gradually along the period without irrigation (Fig. 2f). Similar results were reported by Jones (1973).

After resuming irrigation, leaf water status recovered before gas exchange. Thus, Ψ_{mid} (Fig. 1b), Ψ_{pd} and RWC (data not shown) recovered completely in ca. 36 h. Stomatal conductance, however, did not fully recover in the 46-day experimental period after resuming irrigation (Fig. 2a), probably because of an irreversible loss of shoot hydraulic conductivity. No significant differences were found between the PLC values determined on days 11 and 46 after resuming irrigation (data not shown). The average PLC value for these two days was 32.6%. An excess in ABA production during the stress period might also account for this limited recovery of g_s (Davies et al., 2002). Nevertheless, since the ABA content was not measured, the validity of this hypothesis remains to be ascertained. Neither g_m nor A_N recovered fully until 46 days after resuming irrigation (Fig. 2b and c). Figure 2a and c suggest a faster recovery in g_m than in g_s. This was supported by lower

values for mesophyll conductance limitations of photosynthesis than for stomatal conductance limitations as re-irrigation takes place (data not shown). It was found that g_m and g_s were positively correlated along the experiment (data not shown).

After resuming irrigation, similar patterns were found for *Oe*PIP1.1 and *Oe*PIP2.1 expression. These expressions peaked ca. 36 h after re-watering and returned to initial values afterwards (Fig. 2d and e). This up-regulation could explain the rapid recovery of leaf water status (Fig. 1b). In this regard, there is evidence that AQP function could alter plant water balance, either directly by changing membrane permeability for water, or indirectly by facilitating the transport of gaseous substances like CO_2, thereby affecting stomata opening (Uehlein et al., 2003). CA expression showed a trend to increase after resuming irrigation (Fig. 2f).

The relationship between expression patterns of AQPs and physiological responses to water stress is a complex issue because the expression of different AQP genes may be stimulated, decreased, or unchanged under abiotic stress (Galmes et al., 2007). Probably, this differential regulation allows plants to respond to environmental changes while maintaining their water status (Secchi et al., 2007b). Significant positive correlations were found between g_m and expression of *Oe*PIP2.1 (r^2=0.34; P<0.03) and CA (R^2=0.77; P<0.01). These correlations, however, are not conclusive arguments for a direct protein role in g_m regulation, because possible post-transcriptional modifications of these proteins that could influence protein activity have not been taken into account.

CONCLUSIONS

Leaf gas exchange variables (g_s, g_m and A_N) responded earlier than leaf water status variables to withholding irrigation. Once irrigation was resumed, leaf water status variables were the first to recover.

Mesophyll conductance had a main role in photosynthesis limitations during the period of withholding irrigation. This was supported by two findings: 1) mesophyll conductance limitations of photosynthesis become bigger than stomatal conductance limitations as stress developed, and 2) mesophyll conductance limitations were bigger than stomatal limitations during the first hours after resuming irrigation.

Despite the fact that expression patterns are variable and complex, significant correlations between g_m and expression of *Oe*PIP2.1 and CA were found. Activity level and post-transcriptional modifications of the proteins are needed to obtain sound arguments for assuming a role of these proteins in g_m regulation.

Stomatal conductance did not recover even after 46 days of re-irrigation, probably due to a loss of shoot hydraulic conductivity. However, the recovery of g_m to initial values was enough to restore A_N.

ACKNOWLEDGEMENTS

This work was partly supported by grants for projects AGL2005-00220/AGR and AGL-2009-11310.

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Figures

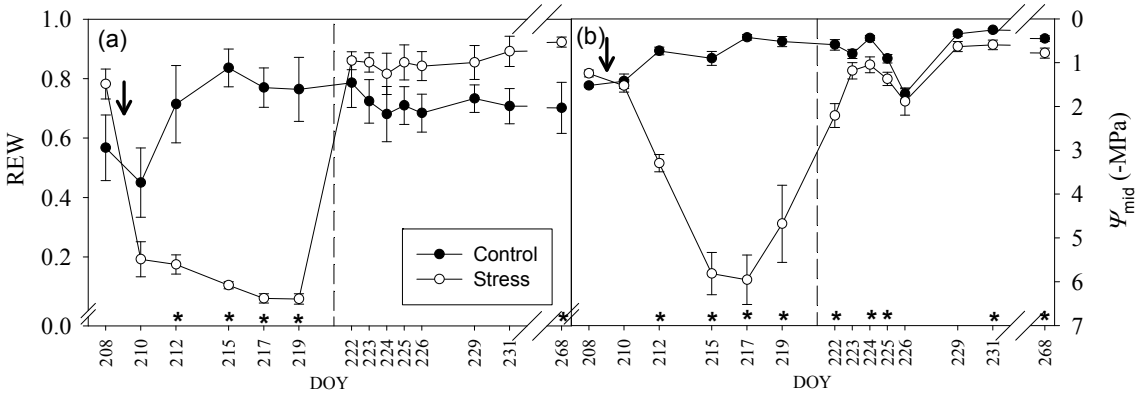

Fig. 1. Time courses, for each treatment, of (a) the relative extractable water (REW) and (b) the midday leaf water potential (Ψ_{mid}). Arrows and dashed lines indicate the day of withholding and resuming irrigation, respectively. Asterisks mean significant differences between treatments (*t*-test, $P \leq 0.05$). See text for details on the measurements.

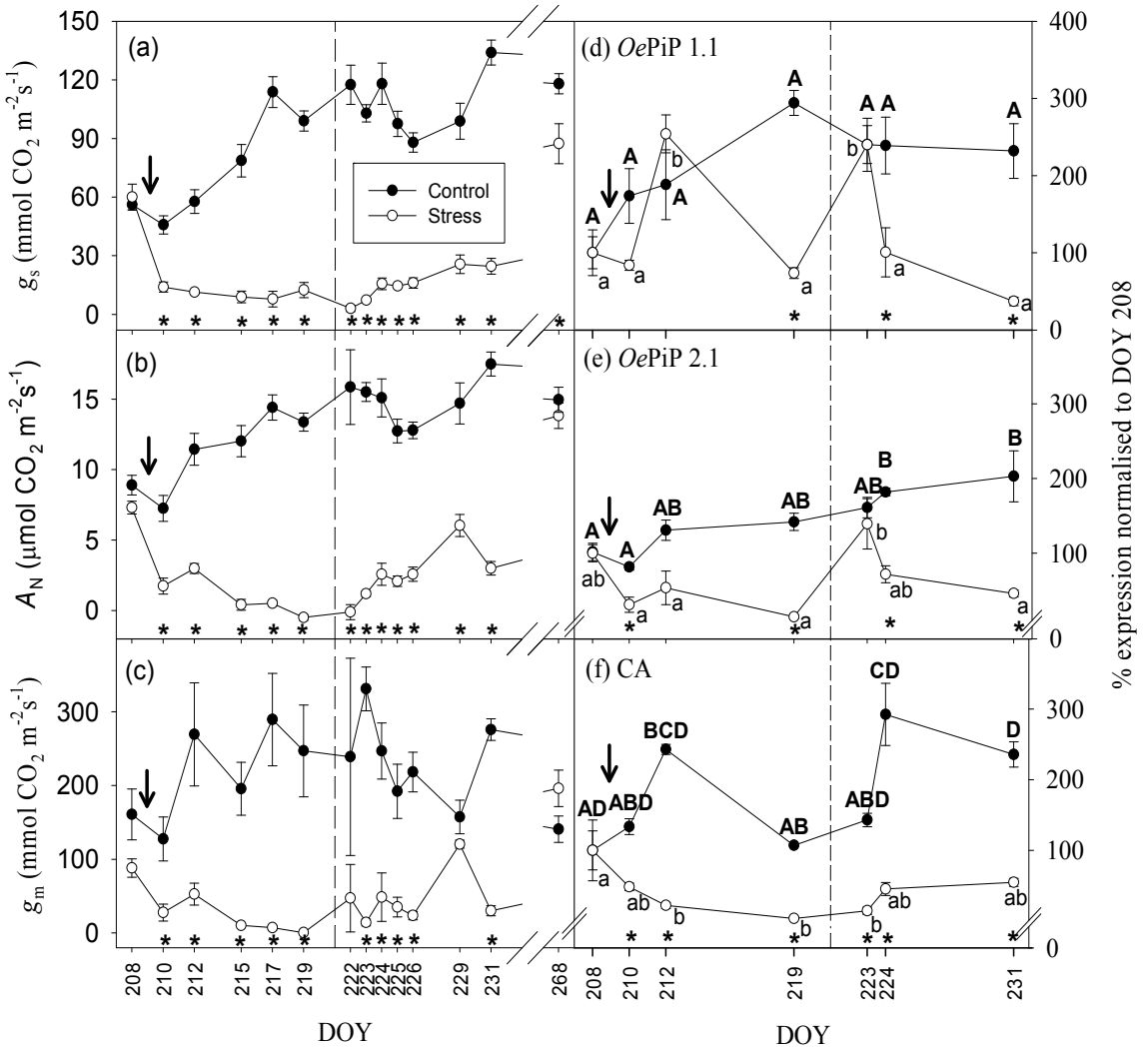

Fig. 2. Time courses of (a) stomatal conductance, g_s, (b) net assimilation rate, A_N and (c) mesophyll conductance, g_m, all referred to CO_2. Also shown are the time courses of the expression of (d) *Oe*PIP1.1, (e) *Oe*PIP2.1 and (f) CA. Values of genetic expression represent the percentage of mRNA transcripts referred to that in the first day of measurement. Data are means ± SE of 4-6 replicates for a-c and 3 replicates for d-f. Different letters mean differences between dates within each treatment following ANOVA (Tukey, $P \leq 0.05$). Capital letters for Control plants and lower letters for Stress plants. Arrows, dashed lines and asterisks like in Figure 1.