Adjustments of water use efficiency by stomatal regulation during drought and recovery in the drought-adapted Vitis hybrid Richter-110 (V. berlandieri × V. rupestris)

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The hybrid Richter-110 (Vitis berlandieri × Vitis rupestris) (R-110) has the reputation of being a genotype strongly adapted to drought. A study was performed with plants of R-110 subjected to water withholding followed by re-watering. The goal was to analyze how stomatal conductance (gs) is regulated with respect to different physiological variables under water stress and recovery, as well as how water stress affects adjustments of water use efficiency (WUE) at the leaf level. Water stress induced a substantial stomatal closure and an increase in WUE, which persisted many days after re-watering. The gs during water stress was mainly related to the content of ABA in the xylem and partly related to plant hydraulic conductivity but not to leaf water potential. By contrast, low gs during re-watering did not correlate with ABA contents and was only related to a sustained decreased hydraulic conductivity. In addition to a complex physiological regulation of stomatal closure, gs and rate of transpiration (E) were strongly affected by leaf-to-air vapor pressure deficit (VPD) in a way dependent of the treatment. Interestingly, E increased with increasing VPD in control plants, but decreased with increasing VPD in severely stressed plants. All together, the fine stomatal regulation in R-110 resulted in very high WUE at the leaf level. This genotype is revealed to be very interesting for further studies on the physiological mechanisms leading to regulation of stomatal responsiveness and WUE in response to drought.

Abbreviations – AN/E, instantaneous leaf WUE; AN/gs, intrinsic leaf WUE; AWA, amount of water available in the substrate; s, bulk modulus of elasticity; δ¹³C, carbon isotope ratio; DOY, day of year; E, rate of transpiration; ET₀, potential evapotranspiration; gs, stomatal conductance; Khplant, whole plant hydraulic conductivity; Ψ, leaf water potential; ΨMD, midday leaf water potential; ΨPO, predawn leaf water potential; PV, pressure-volume; R-110, Richter-110 (hybrid of Vitis berlandieri × Vitis rupestris); VPD, vapor pressure deficit; Ψtp, water potential at turgor loss point; WUE, water use efficiency; RWC, relative water content.
Introduction

Water stress is the most limiting factor for agriculture worldwide (Boyer 1982). Global agriculture accounts for 70% of the amount of water used by humans, but this amount is expected to increase in the near future because of increasing human population and reductions of availability because of global climate change (Bacon 2004). Therefore, to secure a sustainable and efficient use of water, more information on methods and practices to improve plant water use efficiency (WUE) are needed.

Besides agronomic approaches (Gregory 2004) to enhance crop WUE, this can also be improved at the physiological level (Boyer 1996, Parry et al. 2005, Tambussi et al. 2007, Tuberosa et al. 2007). There is evidence for variation in WUE among species, cultivars and populations, as observed in wheat (Farquhar and Richards 1984), sugar beet (Rytter 2005) and grapevines (Bota et al. 2001). Differences in WUE have a genetic basis and often correlate with DNA restriction fragment length polymorphisms or quantitative trait loci (Handley et al. 1994, Martin et al. 1989, Masle et al. 2005, Saranga et al. 2004). Therefore, breeding for high WUE has become a main objective for many crops (Araus et al. 2002).

Despite its obvious interest, the physiological basis for the regulation of WUE is not fully understood because WUE depends on complex arrangements and interactions of physiological mechanisms such as stomatal behavior, photosynthetic type, photosynthetic capacity and leaf and plant anatomy (Parry et al. 2005, Tambussi et al. 2007). Nevertheless, there is general agreement on the important role of stomatal regulation in modulating leaf level WUE (Bacon 2004, Parry et al. 2005), and stomatal closure, in particular, is responsible for the well-known increased leaf and plant level WUE when plants are subjected to moderate water stress (Bota et al. 2001, Chaves et al. 2004, Flexas et al. 2004).

The regulation of stomatal closure under water stress is a complex feature, involving chemical signals, of which ABA is considered the most important (Christmann et al. 2005, Wilkinson 2004), hydraulic signals and cavitation of xylem vessels (Brodribb and Holbrook 2003, Christmann et al. 2007, Cochard et al. 2002) and even electrical signals (Grams et al. 2007). Studies combining several of these signals are scarce (Lovisolo et al. 2002). Therefore, a better understanding of drought-induced regulation of stomatal closure and leaf and plant level WUE is needed. It may be particularly interesting to analyze such responses in species adapted to water stress conditions, such as those found in Mediterranean regions, where plant growth and survival are threatened and, therefore, plants may have developed different strategies to respond to drought, including morphological, physiological and phenological adaptations. Among well-adapted crops, grapevine (Vitis vinifera L.) is especially interesting because the vines perform most of their phenological cycle during summer (Medrano et al. 2003). Grapevine is a traditionally non-irrigated crop that occupies an extensive agricultural area in semi-arid regions, although recently, irrigation has been introduced to increase crop yield. The hybrid rootstock of Vitis Richter-110 (Vitis berlandieri × Vitis rupestris) (R-110) is well adapted to water stress conditions (Galet 1988). Growing as a complete plant, R-110 shows a completely isohydric behavior under several water stress intensities, i.e. it is able to maintain homeostasis in its leaf water relations regardless of decreased soil water availability (Galmés et al. 2007). Therefore, this genotype presents interesting characteristics to study the complex relationships between responses to water stress and recovery with respect to WUE, as it has recently been shown to be an interesting material to evaluate aquaporin expression during drought and re-watering (Galmés et al. 2007).

The aims of the present work were to analyze how stomatal conductance ($g_s$) in drought-adapted R-110 variety is regulated in relation to different physiological variables under water stress and recovery, and how this affects leaf level WUE. Our hypothesis were (1) that stomatal regulation during water stress is a complex and multilevel phenomenon, hence not correlated with a single signaling factor [ABA, leaf water potential ($\Psi$) or hydraulic conductivity], (2) that stomatal regulation may not be necessarily driven by the same factors (or their combination) during water stress and recovery and (3) that, overall, fine stomatal regulation in drought-adapted R-110 would lead to improved WUE under water stress.

Materials and methods

Plant material and water stress treatments

Plants of R-110 were subjected to water withholding followed by re-watering. Plant height was about 1.5 m at the onset of the experiments, with a basal stem diameter of 2–3 cm and a total leaf area of 1.3 ± 0.3 m$^2$. The experiment was performed from June to August 2005 at the Universitat de les Illes Balears (Mallorca, Spain). Sixty plants were grown outdoors in 30 l pots filled with a mixture of soil and organic substrate. One-year-old plants were irrigated daily from April to mid-July, supplemented weekly with 50% Hoagland’s solution (Hoagland and Arnon 1950). On July 28, day of year (DOY 209), 20 plants were kept as controls, while irrigation was stopped for the remaining 40 plants. Two
levels of water stress were established, defined by the leaf maximum daily gₜ as suggested by Flexas et al. (2002): moderate water stress (gₜ about 0.15 mol H₂O m⁻² s⁻¹) and severe water stress (gₜ about 0.05 mol H₂O m⁻² s⁻¹). These values of gₜ corresponded to approximately 55 and 20% that of control plants for moderate and severe water stress, respectively. After the first level of gₜ was achieved (DOY 213), 20 plants were maintained at similar gₜ of approximately 0.15 mol H₂O m⁻² s⁻¹ for a week by daily replacing the amount of water consumed, as determined by weighting of pots every evening. No water was added to the remaining 20 plants until gₜ was approximately 0.05 mol H₂O m⁻² s⁻¹ (DOY 217) and sustained for a week at that level of stress as previously described. After 1 week at the established soil water deficit, all plants were irrigated to field capacity.

Gas exchange measurements were taken daily, while the rest of physiological measurements were performed only on five specific sampling days per each treatment (Fig. 1): the day the desired gₜ was first achieved (day 0), 7 days after sustaining the plants at constant soil moisture, just before re-watering (day 7) and then 1, 3 and 7 days after re-watering, i.e. days 8, 10 and 14, respectively.

Gas exchange measurements

Gas exchange measurements were performed on 10–12 leaves from different plants per treatment, between 12:00 and 13:00 h using an open gas exchange system (Li-6400; Li-Cor, Inc., Lincoln, NE). All measurements were performed at 1500 μmol m⁻² s⁻¹ to ensure light saturation with a CO₂ concentration in the cuvette of 400 μmol CO₂ mol⁻¹ air. Temperature and vapor pressure deficit (VPD) were not controlled.

Leaf water status

Predawn water potential (Ψₚₚ) and midday leaf water potential (Ψₚₕₚ) were determined with a Scholander pressure chamber (Soilmoisture Equipment Corp., Santa Barbara, CA). Five replicates per treatment were obtained from five different plants.

Leaf relative water content (RWC) at predawn Ψₚₚ and midday was determined as follows: RWC (%) = [(fresh weight – dry weight)/(turgid weight – dry weight)] × 100 (Slavik 1974, Turner 1981). Turgid weight was determined by placing samples in distilled water and maintaining them at 8°C in darkness until they reached a constant weight (Slavik 1974, Turner 1981), typically after 12 h. Dry weight was obtained after placing the samples in an oven at 60°C for 48 h. Five replicates per treatment and sampling day were obtained from different individuals.

On DOY 218 and 224, mid-canopy and sun-exposed mature leaves were randomly selected in the morning from each of the plots. On day 218, both moderately and severely stressed plants were still under water stress, while on day 224 severely stressed plants were on their seventh day of water stress and moderately stressed plants were on their fourth day of recovery. On each of these days, four leaves per cultivar were collected to develop pressure–volume (PV) curves (Alsina et al. 2007, Tyree and Richter 1981). Leaves were excised, immediately sealed in plastic bags containing water and transported to the laboratory. Petioles were recut under water and set in water-filled beakers. After this, the beaker was enclosed in a plastic bag to rehydrate the leaf for 24 h at 8°C in the dark. Four water-saturated leaves were measured for each treatment. Each leaf was weighed and allowed to dehydrate by transpiration at a constant temperature for a period during which they were repeatedly placed in the pressure chamber to determine Ψ. Data for initial saturated weight, intermediate fresh weight corresponding to values for Ψ and final dry weight were used to calculate the RWC. The RWC and the corresponding Ψ were plotted as a ‘Type II’ (Ψ⁻¹ × RWC) transformation (Tyree and Richter 1981, 1982). Osmotic potential at full turgor, osmotic potential at turgor loss point, water potential at turgor loss point (Ψₚₕₚ) and leaf bulk modulus of elasticity (c) were obtained from the PV curves (Turner 1988).

Hydraulic conductivity

Whole plant hydraulic conductivity (Kₚₚ) was calculated considering Kₚₚ from the Ohm’s law analogy for the soil–plant–atmosphere continuum (Lovisolo et al. 2002):
\[ E = K_{\text{hplant}} \times (\Psi_{\text{soil}} - \Psi_{\text{leaf}}), \]

where \( E \), \( \Psi_{\text{leaf}} \) and \( \Psi_{\text{soil}} \) are transpiration rate, leaf water potential and soil water potential, respectively. \( \Psi_{\text{PP}} \) was taken as a proxy for \( \Psi_{\text{soil}} \) and \( \Psi_{\text{MD}} \) was taken as \( \Psi_{\text{leaf}} \).

**Concentration of ABA in the xylem sap**

The concentration of ABA in the xylem sap was determined at midday in five plants per treatment and sampling day. Fully expanded and sun-exposed young leaves were excised, and xylem exudates were collected by pressure application with a leaf pressure chamber (Soilmoisture Equipment Corp.). After discarding the first droplet, sap was collected and immediately submerged in liquid nitrogen and kept at −80°C. ABA concentrations [ABA] in the xylem sap were measured with the PhytoDeket ABA enzyme immunoassay test kit (Agdia Inc., Elkhart, IN) following manufacturer's instructions.

**Carbon isotope composition in leaf dry matter**

Some of the most recently developed and initially marked leaves from each treatment were sampled for carbon isotope analysis after keeping the plants at the desired stress level for 7 days, just before re-watering. Samples were dried for 48 h at 70°C, ground into powder and subsampled for C-isotope ratio analysis. Samples were combusted in an elemental analyzer (Thermo, Bremen, Germany); CO₂ was separated by chromatography and directly injected into a continuous flow isotope ratio mass spectrometer (Thermo Finnigan Delta Plus, Bremen, Germany). Peach leaf standards (NIST 1547) were run for every six samples. The calculation of carbon isotope ratio (δ¹³C) was \( \delta^{13}\text{C}_{\text{sample}} (\%) = (R_{\text{sample}}/R_{\text{standard}} - 1) \times 1000 \) (Farquhar and Richards 1984), where \( R_{\text{sample}}/R_{\text{standard}} \) were referred to a Pee De Belemnite (PDB) standard.

**Amount of water available in the substrate and whole plant water use biomass**

The amount of water available in the substrate (AWA) was calculated as (pot weight − minimum pot weight/maximum pot weight − minimum pot weight) × 100. Minimum pot weight was considered at the wilting point. For its measurement, two plants per treatment were not irrigated until a constant weight value was achieved. Maximum pot weight was considered as the pot weight at field capacity.

Plant water use was determined by quantifying pot weight losses on a daily basis. All plants were weighted every evening, before and after irrigation. Their weight difference was considered as the total daily water use. A superficial layer of perlite was added in each pot to avoid evaporation.

Whole plant biomass was determined at the end of the experiment. At this time, eight plants per treatment were harvested, and for each one of them, leaf, stems, and main and fine roots were separated and dried in an oven at 60°C to obtain dry weight. The sum of all fractions was the total plant dry weight at the end of the experiment. Because plant dry weight was not determined at the beginning of the experiment (i.e. before applying the treatments), we cannot give an estimate of plant production during the experiment. Initially, plants were selected to have a similar weight and size. Therefore, differences in dry weight at the end of the experiment are because of the effects of treatments on plant production during the experiment. Hence, while we cannot provide absolute estimates of whole plant WUE, estimations of the relative change in whole plant WUE caused by the different water stress treatments with respect to controls can be attained.

Relative whole plant WUE was defined as the treatment-induced relative maintenance of whole plant dry weight with respect to the relative maintenance of water losses, defining the ‘relative maintenance’ as the value of treated plants divided by that of control plants:

\[ \text{Relative WUE} = (\text{dry weight treatment/dry weight control})/(\text{water losses treatment/water losses control}). \]

**Statistical analysis**

Standard errors were calculated directly from crude data or, in the case of \( K_{h_{\text{plant}}} \), from the standard errors of means of transpiration and water potential, according to standard methods (Taylor 1982). Regression coefficients and correlations were calculated with the 8.0 SIGMAPLOT software package (SPSS, Chicago, IL). Differences between means were assessed by Duncan test \((P < 0.05)\), performed with the spss 14.0 software package (SPSS).

**Results**

**Experimental conditions and plant water status**

Climate conditions during the experiment (July to August 2005) were those typical for Mediterranean regions, with mean daytime temperatures above 25°C (Fig. 2A), night temperatures above 10°C (not shown) and daily irradiance frequently reaching 24.5 MJ m⁻² day⁻¹ (Fig. 2B). Peak photosynthetic photon flux density (PPFD) at midday was usually 1500–1700 μmol m⁻² s⁻¹ (not shown). VPDs were moderate, because of the proximity of the sea, with most days ranging between 1.5 and 2.5 kPa (Fig. 2C).
Potential evapotranspiration (ET0) showed average values of 5 mm day\(^{-1}\), with some variability among days (Fig. 2D). There were only three cloudy days as reflected by the lower radiation, temperature and ET0 (Fig. 2). A small amount of rain on August 4 (not shown) did not affect substrate water content because all plants were preventively placed inside a greenhouse during that day. Because transpiration and photosynthesis were measured under Li-Cor chamber conditions, the relationships between the chamber and free-air VPD at the measuring time were analyzed, and a good agreement was observed between them (data not shown). However, a certain heating in the sun-exposed leaf chamber led to slightly higher VPD than in free air. These differences were higher for stressed leaves (not shown). The modification of leaf temperature by the leaf chamber may have also contributed to these slight differences that were, however, of minor importance.

Treatments produced clear differences in AWA (Fig. 3A), which were kept stable along the 8 days of deficit irrigation. After re-watering, AWA of stressed pots did not fully recover, possibly because of substrate compression during water stress reducing their water storage capacity. Compression was visually apparent at the end of the experiment, particularly in the severely stressed treatment. By contrast, \(\Psi_{PD}\) of moderately and severely stressed plants was not significantly different compared with control plants, although severely stressed plants showed lower values on two of the days (DOY 217 and 232), notably after re-watering (Fig. 3B). Despite non-significant differences in \(\Psi\), \(g_s\) strongly responded to water withholding, and the desired levels of moderate and severe stress were reached 4 and 8 days after stopping irrigation, respectively (Fig. 3C). Differences between treatments were almost constant during water stress; thus, re-watering was applied on days 220 and 224 for moderately and severely stressed plants, respectively. Recovery of \(g_s\) after re-watering was slower than expected. Initially, \(g_s\) was lower in control plants for up to the first 6 days in severely stressed plants and for up to 10 days in moderately stressed plants. It was not until day 236 (16 days after re-watering) that \(g_s\) fully recovered (Fig. 3C). Reduced \(g_s\) resulted in an increased intrinsic leaf WUE (\(A_{WUE}/g_s\)) even after re-watering (Fig. 3D). \(\Psi_{MD}\) remained within a narrow range regardless of treatments (Fig. 3E), but \(K_h^{\text{plant}}\) was substantially reduced by water stress, and part of the reduction persisted upon re-watering (Fig. 3F).
No differences were observed between treatments in PV curves when they were performed (data not shown). Among treatments and days, \( \Psi_{\text{tlp}} \) averaged \(-1.98 \pm 0.13\) MPa, \( \pi_{100} \) averaged \(-1.52 \pm 0.05\) MPa and \( \pi \) averaged \(9.76 \pm 0.70\) MPa. The fact that \( \Psi_{\text{tlp}} \) was approximately \(-2\) MPa, while \( \Psi_{\text{MD}} \) never dropped below \(-1.5\) MPa means that the \( \Psi_{\text{tlp}} \) was never reached during the experiment regardless of water stress and is consistent with the fact that no midday depression of \( g_s \) was observed between 11:00 and 14:00 h (local time) in any of the treatments (data not shown).

**Dependency of \( g_s \) on physiological variables**

Although \( \Psi_{\text{MD}} \) remained largely unchanged during the experiment regardless of the treatment (Fig. 3E), the lowest values were attained at intermediate \( g_s \) values in both control and moderately stressed plants, but not in severely stressed plants. Therefore, no general correlation was found between \( \Psi_{\text{MD}} \) and \( g_s \) (Fig. 4A, B). Hence, while in control plants the relationship between both parameters appears positive, under water stress the reverse is true. Contrarily, a highly significant correlation was found between \( g_s \) and xylem [ABA] during the periods of water withholding and AWA maintenance (Fig. 4C) despite a large variability in xylem [ABA] within treatments. However, no significant correlation \((P > 0.05)\) was observed between \( g_s \) and [ABA] during recovery (Fig. 4D). The correlation between \( g_s \) and \( K_{\text{hplant}} \) estimated from the Ohm’s law analogy for the soil–plant–atmosphere continuum was less strong than that with [ABA] during water stress (Fig. 4E), but the only significant one during recovery (Fig. 4F).

**Variations of \( g_s \) and WUE**

As expected, a curvilinear relationship was observed between net photosynthesis and \( g_s \) when combining all data from this experiment (data not shown). Consequently, \( A_{\text{net}}/g_s \) increased from 60 to 150 \( \mu \)mol CO\(_2\) mol\(^{-1}\) H\(_2\)O when \( g_s \) decreased as water stress intensified (Fig. 3D). Leaf level instantaneous WUE \( (A_{\text{net}}/E) \) also increased with increasing water stress (data not shown).

Throughout the experiment, large changes in VPD were recorded, with daily average values ranging from 0.2 to 2.5 kPa, and most values around 1.8 kPa (Fig. 2C). stomatal conductance clearly responded not only to induced changes in AWA but also in response to VPD (Fig. 5A, B). In control plants and during water stress, \( g_s \) tended to decrease with increasing VPD (Fig. 5A). Interestingly, the slopes of \( g_s \) vs VPD were similar regardless of the treatment. During recovery, only plants that had been submitted to severe water stress kept a strong response of \( g_s \) to VPD (Fig. 5B). Consequently, rate of transpiration \( (E) \) showed very different patterns of response to VPD among treatments and during re-watering. During water stress, this relationship ranged from the expected positive response in control plants, through no response in moderately stressed plants, to a negative response in severely stressed plants (Fig. 5C). Upon re-watering, moderately stressed plants displayed a positive relationship between \( E \) and VPD, while no clear relationship was observed in previously severely stressed plants (Fig. 5D).
Leaf level AN/E not only increased with increasing water stress but also became more independent of VPD (Fig. 5E). Hence, while in control plants AN/E clearly decreased as VPD increased, this dependency was less clear in water-stressed plants, where the slope was lower (Fig. 5E). The negative relationship was restored upon re-watering (Fig. 5F), although AN/E remained higher in previously stressed than in control plants. Integrating AN/E through the entire experimental period revealed significant increases of 25 and 40% in moderately and severely stressed plants, respectively (Table 1). Differences in WUE between treatments were also reflected in $\delta^{13}C$ values in the dry matter of leaves, mostly expanded during the treatment (Table 1). $\delta^{13}C$ was significantly lower ($P < 0.05$) in control plants ($-26.9\%_o$) than in stressed plants with the highest values ($-24.7\%_o$) for severely stressed plants and intermediate values for moderately stressed plants ($-25.1\%_o$). Contrarily, the treatments seem not to affect whole plant relative WUE during the study period (Table 1).

**Discussion**

Stomatal closure is among the first responses of leaves to water stress. In grapevines, a good relationship between $g_s$ and $\Psi$ and/or leaf RWC has been observed in some genotypes (Liu et al. 1978, Rodrigues et al. 1993) but not in others (Flexas et al. 2002, Schultz 2003). R-110 shows an almost isohydric behavior, strongly reducing $g_s$ in response to water stress without a significant change in leaf $\Psi$ (Fig. 3). When AWA was reduced by 70%, $g_s$ was almost halved, without changing $\Psi_{PD}$ or $\Psi_{MD}$, and when soil water content was reduced by approximately 80%, $g_s$ decreased below 0.05 mol H$_2$O m$^{-2}$ s$^{-1}$ with only a slight decrease in $\Psi_{PD}$ but not in $\Psi_{MD}$ (Fig. 3). Because leaf $\Psi_{PD}$ is expected to be in equilibrium with substrate $\Psi$, this may be indicative that substrate surface layers dehydrate rapidly, while deep layers do it slowly (Henson 1982, Medrano et al. 2002). Because the volume that can be occupied by roots is restricted in pots, a significant portion of roots is present at the very superficial substrate layers. Although the plant has still enough water extractable so as to keep $\Psi_{PD}$ unchanged, these superficial roots sense water stress and provoke stomatal closure. Henson (1982) showed in rice that fast drying rates in pot experiments resulted in a higher [ABA] and lower $g_s$ at any given $\Psi$. Similarly, Medrano et al. (2002) showed in grapevines that $g_s$ at any given $\Psi_{PD}$ was lower in potted than in field-grown plants. This effect is somewhat similar to that occurring in partial root-drying experiments (Stoll et al. 2000), except that the spatial heterogeneity in substrate desiccation occurs vertically and not laterally.

The degree of stomatal closure was compared with changes in leaf water potential ($\Psi_{MD}$), xylem [ABA] and hydraulic conductivity ($K_{Hplant}$). The dependency of $g_s$ on any of these parameters was not general (Fig. 4), possibly indicating a multiscale regulation of $g_s$. First, $g_s$ did not correlate with $\Psi_{MD}$ because the latter was kept within a narrow range throughout the entire experiment. Contrarily, a highly significant inverse relationship was found between $g_s$ and [ABA] for data corresponding to irrigated and water-stressed plants, as already shown in grapevines (Liu et al. 1978, Lovisolo et al. 2002, Stoll et al. 2000) and other species (Liu et al. 2005). However, this correlation disappeared during re-watering, when ABA recovered to control values, while $g_s$ remained somewhat low. In agreement with evidences that xylem embolism triggers stomatal closure (Salleo et al. 2000), a significant correlation was found between $g_s$ and $K_{Hplant}$ under water.

**Fig. 5.** The relationship between VPD and (A and B) $g_s$, (C and D) transpiration (E) and (E and F) AN/E. Data for plants during water stress (A, C and E) and recovery (B, D and F) are plotted separately. For control plants, all values are plotted in the four panels. Data are average ± se of 10–12 replicates. Black circles represent control plants, gray squares represent moderately stressed plants and white triangles represent severely stressed plants.
stress. During recovery, the correlation between gs and Khplant was stronger and highly significant. Therefore, stomatal regulation during water stress in R-110 is complex, differing during water stress and recovery. Regulation of gs during water stress involves both ABA and, secondarily, hydraulic signaling. Conversely, during recovery, only hydraulic signaling persists. Additional regulatory mechanisms cannot be discarded, such as other chemical messengers like cytokinins or xylem sap pH (Wilkinson 2004), electrical signals (Grams et al. 2007) or to the formation of stomatal occlusions of unknown nature that persist longtime after re-watering (Gallé and Feller 2007).

Besides the complex physiological regulation of stomatal closure, the important daily variation in VPD clearly affected gs in a range of variation determined by the treatment (Fig. 5). In other drought-adapted species, such as olive (Moriana et al. 2002) or Aleppo pine (Maseyk et al. 2008), water stress results in a reduced slope of the gs–VPD relationship. Contrarily, in R-110, the relationships between gs and VPD for the three treatments presented similar slopes, which allowed a greater adjustment of gs and E to substrate water availability and atmospheric demand. For instance, in control plants, gs decreased by about 15% per kPa of increased VPD, similar to olive and pines, which decreased gs by 12 and 18% per kPa, respectively, at a similar range of VPDs (Maseyk et al. 2008, Moriana et al. 2002). However, in moderately stressed olive and pines, the gs reduction was only 8–10% per kPa, while in severely stressed plants it was almost 0% (Maseyk et al. 2008, Moriana et al. 2002). By contrast, in R-110, gs decreased by 31 and 41% per kPa in moderately and severely stressed plants, respectively. Therefore, in R-110, stomatal responsiveness to VPD was more sensitive as water stress intensified. This led to a progressive change in the response of leaf transpiration to VPD, from increased E at high VPD in control plants, through almost no response to VPD in moderately stressed plants and to slightly decreased E at high VPD in severely stressed plants. A negative relationship between E and VPD may reflect cavitation-induced changes in the slope but not the tendency (i.e. direction) in the whole E–VPD relationship (Buckley 2005), but in R-110, it seems that the whole tendency is modified because the stomatal response to VPD compensates the expected increase in water loss, limiting or even reducing water loss by evapotranspiration. Although the mechanism remains unknown, this response is clearly beneficial for water saving in semiarid conditions, and reflects the reputed high degree of drought adaptation of R-110.

Irrespective of the physiological mechanisms involved, both tight stomatal regulation in response to water stress and VPD and sustained stomatal closure after re-watering are beneficial for leaf level WUE. In stressed plants, Aν/gs rapidly increased and was kept above control values during the entire experiment, even approximately 2 weeks after re-watering. The values obtained (up to 150–200 μmol mol⁻¹) are among the highest found for any species. In drought-adapted crops, such as olives or grapevines, maximum reported values of Aν/gs are 120–140 μmol mol⁻¹ (Bota et al. 2001, Moriana et al. 2002). Similar maximum values, 110–150 μmol mol⁻¹, are found among native Mediterranean species (Faria et al. 1998, Flexas et al. 2004, Maseyk et al. 2008). Only in the xeromorphic Olea europaea var. sylvestris values higher than those reported here (up to 215 μmol mol⁻¹) have been described (Faria et al. 1998). In addition to presenting very high Aν/gs, R-110 seems capable of maintaining high Aν/E at elevated VPD (Fig. 5). Consistent with the optimization theory (Cowan 1977), Aν/E is expected to decline with increasing VPD that has been experimentally confirmed in numerous species, irrespective of water stress (Farquhar et al. 1980, Maseyk et al. 2008, Moriana et al. 2002). However, when subjected to water stress, R-110 displayed a much lower dependency of Aν/E on VPD, keeping Aν/E within a narrower range, and always higher than in irrigated plants, regardless of VPD. Although the underlying mechanisms are unknown, it is clearly advantageous for water use in drought-prone areas and deserves more attention in the near future.

High instantaneous Aν/gs does not necessarily result in longer term whole plant high WUE. In the present study, direct evidence of long-term adjustments of leaf level WUE is provided by the significant variations of δ¹³C on

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**Table 1. WUE of control, moderately stressed and severely stressed plants.** Aν/E values were averaged from mid-morning values for the entire experimental period and expressed in g m⁻². Data are average ± s.e of 10–12 replicates. δ¹³C values are from leaf dry matter of leaves expanded during the experimental period. Water expenses are shown as averaged daily for the approximated entire plant growth period (May 1, 2005 to August 31, 2005). Whole plant relative WUE was calculated as described in Materials and methods. All data are average ± s.e of six replicates. Different letters denote significant differences within each treatment at P < 0.05 by Duncan’s analysis. Values without letters denote no statistical differences within treatments at P < 0.05 by Duncan’s analysis.

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<tbody>
<tr>
<td>Aν/E (g l⁻¹)</td>
<td>3.2a ± 0.1</td>
<td>3.97b ± 0.1</td>
<td>4.56c ± 0.2</td>
</tr>
<tr>
<td>Leaf δ¹³C (‰)</td>
<td>−26.9a ± 0.1</td>
<td>−25.1b ± 0.2</td>
<td>−24.7c ± 0.1</td>
</tr>
<tr>
<td>Total biomass (g)</td>
<td>316 ± 23</td>
<td>275 ± 32</td>
<td>253 ± 29</td>
</tr>
<tr>
<td>Average daily water expense (l)</td>
<td>2.26a ± 0.04</td>
<td>2.16b ± 0.04</td>
<td>2.00c ± 0.05</td>
</tr>
<tr>
<td>Relative whole plant WUE</td>
<td>1</td>
<td>0.91</td>
<td>1.04</td>
</tr>
</tbody>
</table>

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the dry matter of leaves expanded during the experimental period (Condon et al. 2004, Farquhar and Richards 1984). Plant dry weight showed a declining, although non-significant tendency from control to severely stressed plants of approximately 20% (Table 1). Similarly, and because water was restricted only during 10–12 days over a total growing period of 4–5 months, the expected increases in dry mass and the water use integrated during the growth cycle was only slightly decreased compared with control plants. Consequently, the large increases in leaf level WUE were not reflected in significant increases in the whole plant WUE. However, in many studies conducted over longer time periods, a drought-induced decrease in $^{813}$C of about 2% as found here, reflected increases in whole plant WUE of 20–30%, depending on the species and conditions (Boyer 1996, Condon et al. 2004, Ryutter 2005). Therefore, it may be expected that significant increases in whole plant WUE would be attained for longer water stress periods.

In summary, we have found that R-110 adjusts stomatal closure very tightly in response to water stress (AWA and VPD), while maintaining almost constant leaf water relations. This results in very high level WUE after water stress and high VPD, which is kept high even many days after alleviating water stress. Stomatal regulation in this species is a complex and multilevel phenomenon, not correlated unequivocally with any single signaling factor (ABA, Ψ or hydraulic conductivity). Moreover, the mechanisms leading to stomatal regulation seem to differ during water stress and recovery. This remarkable stomatal regulation makes R-110 a good model plant adapted to drought and a potential target to characterize molecular mechanisms for tight stomatal adjustments and improved WUE, devoted to future genetic improvements of WUE in crops from semiarid regions.

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References


