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Grapevine physiological responses during water stress and re-watering: implications for Water-Use-Efficiency

PhD Thesis

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CERTIFIQUEM :

Que el present treball titulat "*Grapevine physiological responses during water stress and re-watering : Implications for Water-Use-Efficiency* ", presentat per Alícia Pou Mir per optar al TÍTOL univesitari oficial de DOCTOR per la Universitat de les Illes Balears dins del programa de doctorat en Biologia de les Plantes en Condicions Mediterrànies, s'ha realitzat sota la nostra direcció al Departament de Biologia de la Facultat de Ciències de la Universitat de las Illes Balears.

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Palma de Mallorca, 29 d'Abril del 2011

Director	Director	Autora
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SYMBOLS AND ABREVIATIONS LIST

Symbol	Meaning
Δe	isotopic enrichment at the site of evaporation
$\Delta F/F_m'$	operating quantum efficiency of PSII photochemistry
Δ_L	isotopic enrichment of mean lamina leaf water enrichement
Δ_{Lnss}	non-steady-state isotopic enrichment of mean lamina mesophyll water
Δ_{Lss}	steady-state isotopic enrichment of mean lamina mesophyll water
Δ_v	isotopic enrichment of atmospheric water vapour
$\wp$	Péclet number
ABA	abscisic acid
A_c	photosynthesis limited by carboxylation
A_N	net CO_2 assimilation rate
A_q	photosynthesis limited by RuBP regeneration
AQP(s)	aquaporin(s)
Asc	ascorbate
AWA	amount of water available
B_L	biochemical limitations to photosynthesis
C	molar concentration of water
C_a	atmospheric CO_2 concentration
C_c	chloroplastic CO_2 concentration
cDNA	complementary deoxyribonucleic acid
Chl	chlorophyll
C_i	sub-stomatal CO_2 concentration
Ci*	apparent CO_2 photocompensation point
CTAB	cetyl trimethyl ammonium bromide
D	tracer-diffusivity
DEPC	diethyl pyrocarbonate
DHAsc	dehydroascorbate
DNA	deoxyribonucleic acid
DTT	dithiothreitol
E	leaf transpiration rate
e_a/e_i	ratio of ambient to intercellular vapour pressure
ETR (J_{flu})	electron transport rate
F_{leaf}	flow rate through the leaf
F_m	maximum fluorescence in the dark-adapted state
F_m'	maximum fluorescence in the light-adapted state
F_o	basal fluorescence of the dark adapted leaf
F_s	steady-state fluorescence emission
F_v/F_m	maximum quantum efficiency of PSII photochemistry
g_c	cuticular conductance
g_m	mesophyll conductance
g_s	stomatal conductance
g_t	total conductance for water vapour of stomata and boundary layer
HCFM	hydraulic conductance flow meter

HPTS	trisodium 3-hydroxy-5,8,10- pyrenetrisulphonate
HPTS-acetate	8-acetoxypyrene-1,3,6-trisulfonic acid, trisodium salt
J_{max}	electron transport driving regeneration of RuBP
K_c	Michaelis constant for the carboxylase activity of Rubisco
Kh	hydraulic conductance
Kh_{lamina}	lamina hydraulic conductance
Kh_{leaf}	leaf hydraulic conductance
$Kh_{leaf\text{-}max}$	maximum leaf hydraulic conductance
Kh_{plant}	whole-plant hydraulic conductance
K_o	Michaelis constant for the oxygenation activity of Rubisco
Ks-l	leaf specific hydraulic conductance from soil-to-leaf
L	scaled effective pathlength
LA	leaf area
LAI	leaf area index
LAVPD	leaf-to-air vapour pressure deficit
L_{ss}	steady-state scaled effective pathlength
MC_L	mesophyll limitations to photosynthesis
NPQ	non-photochemical quenching
NS_L	non-stomatal limitations to photosynthesis
P	pressure
PAR	Photosynthetic active radiation
PCR	polymerase chain reaction
PEG	polyethylene glycol
PGA	phosphoglycerate
PPFD	photosynthetic photon flux density
P_r	photorespiration rate
PRD	partial rootzone drying
PSI	photosystem I
PSII	photosystem II
P-V	pressure-volume
PVP	polyvinylpyrrolidone
R-110	Richter 110
rbcL	Rubisco large subunits encoding genes
rbcS	Rubisco small subunits encoding genes
R_d	rate of mitochondrial respiration in the light
RDI	regulated deficit irrigation
RGR	relative growth rate
R_{leaf}	leaf hydraulic resistance
R_n	rate of mitochondrial respiration in the dark
RNA	ribonucleic acid
$R_{outside\ ylem}$	extra-vascular hydraulic resistance of the leaf
R_{plant}	plant hydraulic resistance
RT	reverse transcription reaction
RT-PCR	real time-polymerase chain reaction
Rubisco	ribulose-1,5-biphosphate carboxylase/oxygenase
RuBP	ribulose-1,5-biphosphate
RWC	relative water content
R_{xylem}	xylem hydraulic resistance of the leaf
S_L	stomatal limitations to photosynthesis
T	Temperature
V_c	carboxylation activity of Rubisco

$V_{c,max}$	maximum rates for the carboxylation activity of Rubisco
VPD	vapour pressure deficit
w_i	mole fraction of water vapour in the leaf intercellular air spaces
WUE	water use efficiency
WUE_i (A_N/g_s)	intrinsic water use efficiency
WUE_{inst} (A_N/E)	instantaneous water use efficiency
WUEp	water use efficiency at the plant level
α	leaf absorbance
β	fraction of absorbed light that reaches photosystem II
Γ*	CO_2 compensation point in the absence of mitochondrial respiration
δ13C	Carbon 13 isotope discrimination
ε	leaf bulk modulus of elasticity
ε^+	equilibrium fraction between liquid water and vapour
ε_k	kinetic fraction from the leaf to the atmosphere
Π	osmotic potential
Ψ	water potential
Ψ_{leaf}	leaf water potential
Ψ_{MD}	midday leaf water potential
Ψ_{PD}	Predawn leaf water potential
Ψ_{soil}	soil water potential
Ψ_{stem}	stem water potential
Ψ_{tlp}	water potential at turgor loss point
$\Psi_{\pi 0}$	osmotic potencial at zero turgor
$\Psi_{\pi 100}$	osmotic potencial at full turgor
Φ_{CO2}	apparent quantum efficiency of CO_2 fixation

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Chapter 1

INTRODUCTION

1.1. THE IMPORTANCE OF WATER STRESS IN PLANTS

Water is the main resource for plant growth and ecosystem primary production around the world, thus soil water availability results as the main determinant of the plant physiology. In semi-arid areas, or when soil water is limited, vegetation type and distribution relate directly to the amount of water that plants can extract from the soil. In that sense, many studies have focused their attention on the importance of drought as one of the most important constrains limiting growth in plants and crops and ecosystem productivity around the world (Passioura, 1996; Aussenac, 2000).

Water is also a key element for many plant physiological processes and, hence, for plant distribution and survival. However, survival and distribution of plants strongly depend on their ability to adjust to environmental variations; therefore, different genotypes may rely to different extents on avoidance and tolerance strategies to cope with different degrees of acclimation to drought stress. Indeed, plants may be limited to moist sites by such diverse physiological characteristics as extreme water stress sensitivity of stomata, poor stomatal control of water loss, low photosynthetic water use efficiency, or poor recovery after drought, or contrarily might compete successfully on drier sites showing high photosynthetic water use efficiencies at high levels of water stress and/or delayed stomatal opening following re-watering, so there are large variations among species in photosynthetic response to water stress (Sullivan *et al.*, 1974), thus affecting the distribution of the species (Wuenscher and Kozlowski, 1971; Chaves *et al.*, 2007).

1.1.1. The special interest of Mediterranean climate areas

Climate characteristics (i.e. water availability) of a particular area influence the type of crop varieties grown in a region and the type of agricultural practices that will be used. Moreover, the presence of adequate sun, heat and water are vital for healthy growth and development of crops during the growing season.

Mediterranean climate is the typical climate of most of the territories in the Mediterranean Basin, which represents about 60% of the world's total Mediterranean areas. It's characterized by a hot, dry summer and a cool wet period in winter, and

can be considered as the transition zone between dry tropical and temperate climates as described by Aschmann in 1973. As a result, this climate areas receive almost all of their yearly rainfall out of the summer season, and may go anywhere from 4 to 6 months during summer without having any significant precipitation. Thus, in these areas, progressive soil water deficits and high leaf-to-air vapour pressure gradients, together with high irradiance and temperatures, exert large constraints on crop yield and quality. Moreover, in addition to water limiting conditions during the dry season, plant growth is also constrained by cold temperatures during winter, since the growth of many Mediterranean species is limited when daily mean temperatures are below 10ºC (Rambal, 2001), a common event in the Mediterranean Basin. This climatic trade-off within the Mediterranean region greatly affects its plant species distribution.

In the Balearic Islands, were most of this Thesis research has been performed, summer drought is the most important constraint for plant performance, although cold winter stress can affect plant performance in the mountain areas of Mallorca (Flexas *et al.*, 2003; Gulías, 2004). Therefore, in the present Thesis drought stress will be considered as the most determinant factor to determine both short term and adaptive plant physiological responses.

Nowadays, it is known that drought may affect whole countries over several years and may result in serious social, economical and environmental costs. In the Mediterranean-climatic regions of the world, water is the major limitation for plant productivity. This situation is compounded by the predicted change in climate with increased temperatures and decreased precipitation as a result of global warming (Mannion, 1995; Houghton, 2001). Furthermore, drought events are expected to strengthen in terms of intensity, frequency and geographic expanse as a consequence of global climate change (IPCC 2007). This issue will become even more important because world water supply is limiting, while demand for food and water for irrigation will raise along with the human population (Somerville and Briscoe, 2001). There is thus an increasing need to anticipate the consequences of drought on crop plants, with the objective to design more efficient and water-saving cropping systems. Therefore, studies on plants including identification and selection of physiological traits that increase plant water use efficiency (WUE) and drought tolerance under

water-limited conditions are important to better understand plant physiological characters and to take physiological water saving measures.

1.2. PLANT RESPONSES TO WATER STRESS

Properly, drought refers to a water deficit in the soil that is sustained in time (days-months). By contrast, water stress occurs in plant tissues as a consequence of any water deficit. This can occur very frequently even in well-irrigated plants in a short term (minutes-hours), whenever the evaporative demand is higher than the xylem capacity for refilling leaves. For instance, the incidence of dry wind can cause a temporary water deficit in the leaf. Therefore, plant responses to water scarcity are complex and encompass a myriad of changes in physiological processes, including stress sensing and signaling, changes in growth and biomass allocation patterns, water status homeostasis, decreased stomatal conductance and CO_2 assimilation, osmoregulation, and detoxification processes (Passioura, 1996; Chaves *et al.*, 2003).

Classically, plant resistance to drought has been divided into escaping, avoidance and tolerance strategies (Levitt, 1980; Turner, 1986). Escape strategies rely on successful reproduction before the onset of severe stress, so they exhibit a high degree of developmental plasticity, being able to complete their life cycle before physiological water deficits occur. Plants can also endure drought conditions by avoiding tissue dehydration, while maintaining tissue water potential as high as possible, or by tolerating low tissue water potential. Dehydration avoidance is associated with a variety of adaptive traits, which involve minimizing water loss and maximizing water uptake (Chaves *et al.*, 2003). Minimizing water loss is strongly related to tight stomatal regulation, and on the other hand, maximized water uptake is regulated by adjusting the allocation pattern, namely increasing investment in the roots (Jackson *et al.*, 2000). Finally, drought tolerance is determined by a number of mechanisms that increase the tolerance of plant tissues to dehydration (Kramer, 1988). Dehydration tolerant species function under low plant water potentials to facilitate water uptake from drying soils by maintaining a soil-to-leaf water potential gradient, which also facilitates a rapid recovery after drought (Tschaplinski *et al.*,

1998). Both high osmotic potential and low elasticity help in rapid decreases of water potential given a change in water content (Abrams, 1990).

Regarding leaf-level responses, stomatal closure, together with leaf growth inhibition, are among the earliest responses to drought, protecting plants from extensive water losses, which may result in cell dehydration, runaway xylem cavitation and death. Cell expansion has been shown to be the most sensitive process to water stress in many crops (Boyer, 1970; Hsiao, 1973; Bradford and Hsiao, 1982). The implication of this sensitivity is that, during crop development, leaf area may be reduced with consequent reduction in light interception, and thus in the whole 'source size' of assimilates and limitation of the rate of transpiration (Lu and Neumann, 1998). This leaf area reduction may be quite strong even at mild water stress, and with no effect at all on stomatal closure. Though, when water stress is severe enough to induce stomatal closure, the source intensity for assimilates (the photosynthetic rate) would also be reduced, and consequently the resulting biomass too.

More than 90% of plant dry matter, and almost any process involved in crop growth and productivity, depend on assimilates derived from photosynthesis. While total leaf area represents the 'source-size' for assimilates, the leaf photosynthetic rate represents the 'source-intensity' for assimilates. The effect of water stress, then, may lead to stomatal and/or non-stomatal limitations to photosynthesis (Farquhar and Sharkey, 1982; Jones, 1985; Grassi and Magnani, 2005; Galmés *et al.*, 2007a).

Stomatal aperture are the major pathway for the movement of CO_2 from the atmosphere into the photosynthetic mesophyll leaves, and appears to be controlled by complex mechanisms which operate to maintain a variable balance between allowing CO_2 uptake to proceed, while restricting the loss of water vapour, and preventing leaf desiccation (Schulze and Hall, 1982). Further evidences indicated that stomatal closure is likely to be mediated by chemical signals traveling from the dehydrating roots to shoots. Thus, abscisic acid (ABA) was identified a chemical signal involved in the regulation of stomatal closure (Schulze 1986; Davies and Zhang, 1991). Hence, stomatal traits are of key interest in the study of drought-adaptation to water stress conditions and drought recovery. However, although restricted CO_2 diffusion across leaves is likely to be the most usual cause for decreased photosynthesis rates under

water stress, metabolic impairment may also occur, particularly under severe water stress (Flexas and Medrano, 2002; Lawlor and Cornic, 2002; Flexas *et al.*, 2006a). Furthermore, photosynthetic capacity also depends on the efficiency of the process of CO_2 fixation into organic compounds, which is related to the affinity of Rubisco for CO_2 with respect to O_2, i.e. the Rubisco specificity factor (Roy and Andrews, 2000; Lawlor, 2001). In that sense, numerous studies have reported a myriad of changes in physiological processes by water stress (Fig. 1.1).

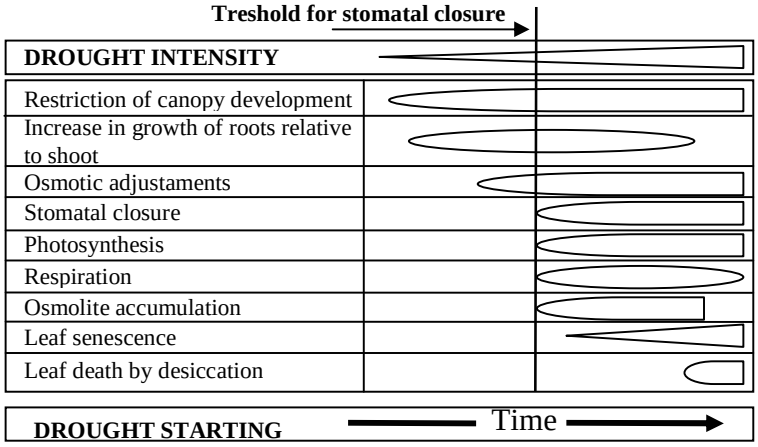

Figure 1.1. General time course of gross and adaptive changes in crop plants in response to the gradual development of water stress in the field. The width of a band represents the relative magnitude of the response. The shape of a band reflects the variation of responses with increasing stress intensity and duration. The starting position of a band on the time scale indicates the water stress threshold for eliciting the response. Redrawn from Bradford and Hsiao (1982) and modified by Steduto (1997).

1.2.1. Carbon and water flow balance: water use efficiency

Water use efficiency (WUE) provides the simplest mean of assessing whether yield is limited by water supply or other factors and is considered an important component of adaptation to water deficit conditions (Edhaie and Waines, 1993).

WUE depends on complex interactions between environmental factors and physiological mechanisms such as stomatal behavior, photosynthetic capacity, and leaf and plant anatomy (for a review see Bacon, 2004). Changes in plant water use efficiency (WUE) related to water availability present two interacting components: a plastic response, evident when individuals of the same genotype are compared (e.g. wet versus dry years) (Lambers *et al.*, 1998), and an interspecific response, evident

when different species living in habitats with different water availability are compared.

Regardless of its nomenclature, the concept of WUE always reflects a balance between gains (moles of carbon, crop yield) and costs (moles of water, volume of water used). This balance can be measured at different levels from instantaneous fluxes in the leaf (photosynthesis *vs* transpiration rates) to values concerning whole plant or crop, although in a wider context this concept is also applied to whole agricultural systems. WUE values can therefore be analyzed at different space and time scales and all of them are significant for the evaluation and optimization of water use.

Water use efficiency at the plant level (WUEp) is a key parameter for research as it allows deepening in the understanding of the physiological and ecophysiological mechanisms. Accurate measurements of WUEp need a simplified system where water loss by soil water evaporation, percolation or runoff is minimized. For this reason, leaf-level measurements (often determined from single leaf gas-exchange measurements) have commonly been used as proxy for WUEp. At the leaf level, the instantaneous net CO_2 assimilation (A_N), transpiration (E) rates, as well as the determination of stomatal conductance (g_s) permits determining the ratio (A_N/E) defined as "instantaneous water-use-efficiency (WUE_{inst})", and the ratio A_N/g_s as the "intrinsic water-use-efficiency (WUE_i)" (Fischer and Turner, 1978).

WUE_i largely excludes the effects of changing evaporative demand on water flux out of the leaf (Bierhuizen and Slatyer, 1965) and it has been predominantly used in studies of water stress effects on grapevines (Düring, 1987; Schultz, 1996; Flexas *et al.*, 1998; Escalona *et al.*, 1999; Bota *et al.*, 2001; Gaudillère *et al.*, 2002; Souza *et al.*, 2005; Chaves *et al.*, 2004, 2007; Pou *et al.*, 2008; Zsófi *et al.*, 2009). Furthermore, differences in WUE_i between genotypes have been reported to have a genetic basis in grapevines (Düring and Scienza 1980; Eibach and Alleweldt, 1984; Chaves *et al.*, 1987; Bota *et al.*, 2001; Gibberd *et al.*, 2001; Schultz, 2003; Flexas *et al.*, 2004; Soar *et al.*, 2006; Satisha *et al.*, 2006; Flexas *et al.*, 2008). In general, an increase in WUE_i under drought or deficit irrigation strategies (mild to moderate water deficits) has been observed in these studies. WUE usually decreases as water

availability increases because stomatal conductance (g_s) increases linearly with water availability, and so does E (under a constant water vapour gradient between leaf and atmosphere). In contrast, A_N follows a saturation curve as g_s increases, because photosynthesis becomes limited by factors other than water (e.g. temperature or nutrients) (Toft *et al.*, 1989). However, this model does not take into account that water supply could trigger a pulse of N availability and a consequent increase in enzymatic activity. Such a reduction of carboxylation resistance could overshadow the reduction of stomatal resistance and increase rather than decrease WUE as a response to higher water availability. WUE_{inst} is less frequently used than WUE_i is (Farquhar *et al.*, 1989), the former includes losses of carbon because of respiration at night or from non-photosynthetic organs such as roots, and losses of water at night because of incomplete stomatal closure or high cuticular conductance.

1.3. GRAPEVINE CROP: A CLASSICALLY ADAPTED MEDITERRANEAN CROP

1.3.1. Water stress as the main determinant of fruit production and quality

Grapevines are one of the oldest cultivated plants that, along with the process of making wine, have resulted in a rich geographical and cultural history of development (Johnson, 1985; Penning-Roswell, 1989; Unwin, 1991). Present viticultural regions are located in six out of seven continents, between latitudes 4° and 51° in the Northern Hemisphere (NH) and between 6° and 45° in the Southern Hemisphere (SH) across a large diversity of climates (oceanic, warm oceanic, transition temperate, continental, cold continental, Mediterranean, subtropical, attenuated tropical, arid and hyper arid climates) (Peguy, 1970, Tonietto and Carbonneau, 2004) (Fig. 1.2). Thus, nowadays, grapevine is grown widely around the world, with its production making the top agriculture lists in many countries (Bisson *et al.*, 2002). According to FAOSTAT time-series and cross-sectional data (http://faostat.fao.org, accessed September 2009) there were 66.271.676 tones of grapes produced on 7.501.872 Ha in 2007. Within all the countries, Spain accounts for the greatest amount of grape-producing areas (1.100.000 Ha) with a prolific economy (1.000-1.500 M€), representing more than 10% of its total agricultural

production, a socioeconomic profit (with about 400.000 wine producers) and an environmental-landscape profit.

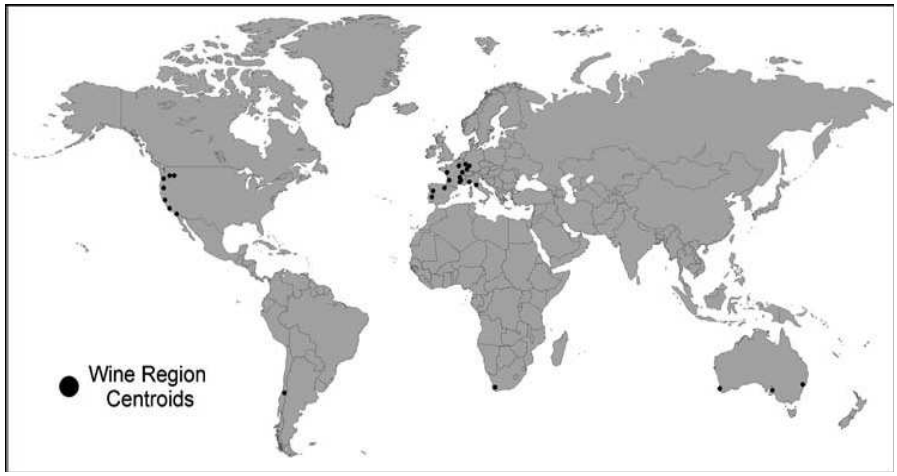

Figure 1.2. Wine region centroids used to extract the appropriate grid cells for both the 0.5◦ × 0.5◦ 1950–1999 observed climatology data and the 2.5◦ ×3.75◦ 1950–2049 HadCM3 climate model data. From Jones *et al.* (2005).

Accordingly, the range and magnitude of environmental factors (e.g., solar radiation, heat accumulation, temperature extremes, precipitation, wind, and extreme weather events such as hail), differ considerably from region to region and so do the principal environmental constraints for grape production and wine quality. However, within the existing production areas, water shortage is probably the most dominant environmental constraint (Williams and Matthews, 1990), and even in moderate temperate climates, grapevines often face some degree of drought stress during their growing season (Morlat *et al.*, 1992; Van Leeuwen and Seguin, 1994; Gaudillère *et al.*, 2002; Gruber and Schultz, 2009). Moreover, the combined effect of drought, high air temperature and high evaporative demand during summer in these semi-arid areas is known to limit grapevine yield and berry and wine quality (Escalona *et al.*, 1999; Chaves *et al.*, 2007; Costa *et al.*, 2007) and may promote dramatic reductions in plant carbon assimilation due to severe decline in photosynthesis under supra-optimal leaf temperatures combined with water deficits, as well as to a partial loss of canopy leaf area (Flexas *et al.*, 1998, 2002; Maroco *et al.*, 2002; Chaves *et al.*, 2003, 2007; Souza *et al.*, 2003; Santos *et al.*, 2007). Consequently and also because in the last decades grape cultivars native from cool climates have been progressively introduced in drier

and warmer areas, irrigation is frequently required and it is becoming an important issue for viticulture, since grapevine production and quality is fully dependent on water availability (Santesteban and Royo, 2006).

Grapevines are able to survive over a range of soil moisture conditions, due to the large and deep root system and physiological drought avoidance mechanisms, such as an efficient stomatal control of transpiration and of xylem embolism (Lovisolo *et al.*, 2002), and/or the ability to adjust osmotically (Rodrigues *et al.*, 1993; Patakas and Noitsakis, 1999), their growth and yield is determined by their total water use (McCarthy *et al.*, 2001). Thus, although **t**he relationship between grapevine water use and grape quality for wine elaboration is not totally established. The most generalized tendency shows that generous watering leads to reduced quality through decreases in colour and sugar content and imbalanced acidity (Matthews *et al.*, 1990; Medrano *et al.*, 2003; Keller *et al.*, 2008; Romero *et al.*, 2010).

1.3.2. Irrigation as a generalized tool to control grapevine WUE and grape quality

Even traditionally, grapevine was a rainfed crop for most of the typical viticulture areas. Nowadays, there is an increasing management control of grapevine crop which includes irrigation as a common practice for modern viticulture for most of semi-arid cropping areas, thus irrigation is important from an agricultural as well as an environmental point of view (Medrano *et al.*, 2010). Moreover, according to climate change predictions (Schultz, 2000; Jones, 2010), increasing temperatures and shifts in precipitation will result in greater drought severity and frequency (Cook *et al.*, 2004; IPCC, 2007; Seager *et al.*, 2007). Therefore, water scarcity could lead to more frequent use of irrigation for an affordable crop productivity (Chaves *et al.*, 2007) and to important changes in the optimum areas for different grape varieties (Schultz, 2000). There are both economic and environmental reasons for the improvement of irrigation efficiency, including, the sustainable use of water resources, prevention of rising water tables and salinity, reducing irrigation costs and sustainable grape production. Consequently, a more accurate use of available water,

i.e. improvement of water use efficiency (WUE), is necessary for a more environmentally sustainable viticulture.

The best way to increase WUE in the vineyard is the precise control of irrigation. Monitoring grapevine water stress is crucial for the ongoing management of vineyard irrigation (Dry and Loveys, 1998), and an increased knowledge of the physiological mechanisms that influence shoot growth and transpiration rates in plants has allowed the development of novel irrigation techniques such as regulated deficit irrigation (RDI) and partial rootzone drying (PRD) to control vine vigor. Under RDI plant water status is maintained within predefined limits of deficit (with respect to maximum water potential) during certain phases of the seasonal development, normally when fruit growth is least sensitive to water reductions (Kang and Zhang, 2004). The rationale underlying this practice is that optimization of fruits numbers, size and quality will be achieved by keeping grapevine vigour in balance with potential production. In PRD, irrigation is scheduled such that the root system is partially dried, stimulating the release of a chemical signal (ABA) the plant roots that ultimately results in a decrease in shoot growth and an increase in water use efficiency (Dry and Loveys, 1998; Santos *et al.*, 2003; Costa *et al.*, 2007).

RDI has been widely studied and, to present, is the most direct technique to improve WUE in the vineyard (Sadras, 2009; Romero *et al.*, 2010), although there are contrasting results in the literature. Thus, several studies in grapevine reported no significant differences between PRD and RDI (Bravdo *et al.*, 2004; Gu *et al.*, 2004; Baeza and Lissarrague, 2005). These apparent contradictions may be related to differences in the intensity of the chemical signaling under PRD irrigation. Moreover, others studies criticized the narrow range of values in which water status should be maintained to avoid undesirable losses of water and grape quality together with undesirable yield losses under variable, and often unpredictable, climate conditions (Schultz, 2003; Medrano *et al.*, 2003; Chaves *et al.*, 2007; Romero *et al.*, 2010).

The impacts of water shortage on crop yield and quality are numerous and can be studied at different spatial scales, ranging from canopy to molecular processes. Approaches at finer scales are expected to improve the understanding of the processes recorded at larger scales. Therefore, studying water stress responses of grapevine is

not only necessary in order to match irrigation to the particular necessities of different soil and climate regions, but to gain new knowledge on general grapevine responses. Understanding the physiological mechanisms and the genetic background underlying the interactions between plants and the environment, and to pay attention to research fields will be pivotal for the development of sustainable viticulture under changing conditions.

1.3.3. Genotype dependent responses to water deficit in grapevine

Genotype-related differences in water stress response are widely reported (Masle *et al.*, 2005, Nilson and Assmann, 2007; Lake and Woodward, 2008), and may arise from constitutive peculiarities of a determined variety as well as from differences in the plant's capacity to osmoregulate, to regulate plant hydraulics, or the fine control of water and carbon flow in the plant and the leaf. Photosynthesis, stomatal conductance and WUE were shown to vary with grapevine variety (Chaves *et al.*, 1987; Schultz, 1996, 2003; Bota *et al.*, 2001; Soar *et al.*, 2006; Palliotti *et al.*, 2009). In grapevine, yet variation in photosynthetic efficiency seems to be small (Bota *et al.*, 2001), suggesting that genotypic variation in WUE is largely linked to diversity in stomatal conductance, under both well-watered and water deficit conditions (Escalona *et al.*, 1999; Gaudillère *et al.*, 2002; Chaves and Oliveira, 2004).

In respect to the control of water flow, grapevines have been classified as an isohydric species based on their ability to maintain leaf water potential (Ψ_{leaf}) at a maximum through stomatal control of transpiration (Düring, 1987; Winkel and Rambal, 1993). However, a large diversity in drought tolerance is observed in different *Vitis vinifera* L. (Smart and Coombe, 1983) by their varied ability to maintain Ψ_{leaf} under conditions of water stress and by their differences in leaf morphology and anatomy (Ben Salem-Fnayou *et al.*, 2005; Gómez del Campo *et al.*, 2003), leaf lipid composition, shoot conductivity and vulnerability to cavitation (Schultz, 2003), ability for osmotic adjustment (Düring, 1984) and sensitivity of photosynthesis, transpiration and water-use efficiency to increasing water deficit (Bota *et al.*, 2001; Gomez-Del-Campo *et al.*, 2005; Medrano *et al.*, 2003; Schultz, 2003). This suggests that a classification of isohydric and anisohydric may be

appropriate within the grapevine cultivars (Chaves *et al.*, 1987; Winkel and Rambal, 1993; Schultz, 1996). Schultz (2003) compared the hydraulic architecture of two different grapevine cultivars, Grenache and Syrah. Grenache demonstrated near-isohydric behavior and Syrah anisohydric behavior when subjected to water stress. Soar *et al.* (2006) further investigated the stomatal response of these two varieties by exposing them to varying vapour pressure deficit (VPD). The same isohydric/anisohydric behaviour was apparent in response to high VPD, as seen for soil moisture deficit. It was further suggested that ABA physiology may be a key process in determining this stomatal response thus, the capacity for ABA biosynthesis and ABA-mediated stomatal closure depends on the cultivar, being higher in isohydric cultivars like Grenache and lower in anysohydric cultivars like Shiraz (Soar *et al.*, 2006). Stomatal closure is thought to control drought induced cavitation in grapevine (Lovisolo and Schubert, 1998).

Differences between grapevine varieties in the response to drought and/or VPD may be mediated by aquaporins (AQP) (Sade *et al.*, 2009; Vandeleur *et al.*, 2009). A recent study exploring the nature of the isohydric and anisohydric response pattern of different grapevine cultivars (thus, differing in their water use strategies) suggested that physiological and anatomical differences in the roots played a major role in water transport and that differences in root hydraulic conductance could be related to the differential expression of the two most highly expressed plasma membrane intrinsic protein (PIP) AQP (VvPIP1;1 and VvPIP2;2) (Vandeleur *et al.*, 2009).

Differences among grapevine cultivars in water use efficiency (WUE) have been reported, based on instantaneous gas-exchange data (Bota *et al.*, 2001; Schultz, 2003; Gómez-del-Campo *et al.*, 2003; Soar *et al.*, 2006), isotopic composition ($^{13}C/^{12}C$) of leaf and/or fruit dry matter (Gibberd *et al.*, 2001; Gaudillère *et al.*, 2002) or biomass accumulation per unit of water used (Gibberd *et al.*, 2001). Substantial evidence for genetic variability of WUE was also shown in grapevine rootstocks (Satisha *et al.*, 2006). Thus, to achieve a better understanding at the physiological level of such variation in relation to WUE, the aim is to research key, poorly characterized aspects of WUE including transpiration control (leaf hydraulic

conductivity) and CO_2 assimilation (stomatal and mesophyll conductance) and to deepen in the less known grapevine responses to water stress such as recovery after re-watering.

1.4. THE REGULATION OF WATER FLOW UNDER WATER STRESS AND DURING RE-WATERING

1.4.1. Water transport and hydraulic conductivity: from roots to leaves

Water moves through the plant via the water conducting xylem vessels forming a continuous system from the roots to the cell walls of the leaf mesophyll (known as the soil-plant-air-continuum). Thus, the xylem vessels and xylem parenchyma cells have evolved an intricate structure – function relationship to satisfy the demands of water flow within higher plants; by controlling and facilitating the movement of water and ions (loading) and out (unloading) of the xylem vessels. The ascent of water flow in plants can be explained by the Cohesion-Tension theory. This theory is usually ascribed to Dixon (1914) but the idea of the xylem being under negative pressure was first proposed by Bohm (1893). The ascent of sap in higher plants is driven by transpiration (*E*) in the leaves, providing a pressure gradient, typically around -1 to -2 MPa, for water to flow, although pressure can fall as low as -10 MPa (Tyree and Sperry, 1989). Thus, *E* can be explicitly described via the steady-state formulation of the soil–plant–atmosphere hydraulic continuum (modified from Whitehead and Jarvis, 1981; Whitehead, 1998):

$$E = K_l (\Psi_{soil} - \Psi_{leaf} - hp_w g)$$

where K_l is the leaf-specific hydraulic conductance of the soil-plant continuum, Ψ_{soil} and Ψ_{leaf} are the soil and leaf water potentials, respectively, and $hp_w g$ is the gravitational pull on a water column of height *h* and density *pw*. The tension difference across the plant ($\Psi_{soil} - \Psi_{leaf}$) increases in proportion to *E* as long as K_l remains constant, for example when no cavitation occurs. The water conducting vessels are maintained under constant negative pressure such that the water column remains in a metastable state. According to the Cohesion-Tension theory, this is deemed possible by the physical properties of water and the hydraulic architecture of plants (Tyree, 1997; Tomos and Leigh, 1999; Steudle, 2001). However, *E* has an

upper limit (E_{crit}) because increasing tension causes decreased K_l as a result of air entry through pit pores into conduits, thereby initiating cavitation (nucleation of vaporization) and producing an embolized, or air-filled conduit.

The validity of the Cohesion-Tension theory has been questioned resulting in a lively ongoing debate (Wei *et al.*, 1999; Zimmermann *et al.*, 2000).

1.4.2. Leaf hydraulic conductance and vulnerability

Leaf hydraulic conductance (Kh_{leaf}) is a measure of how efficiently water is transported through the leaf, determined as the ratio of water flow rate (F_{leaf}) through the leaf (through the petiole and veins, and across the living tissues in the leaf to the sites where water evaporates into the airspaces) to the driving force for flow, the water potential difference across the leaf ($\Delta\Psi_{\text{leaf}}$). Kh_{leaf} is typically normalized by leaf area (i.e., $F_{leaf}/\Delta\Psi_{leaf}$ is further divided by lamina area; units of mmol water m^{-2} s^{-1} MPa^{-1}). Kh_{leaf} is the more commonly used metric. However, because resistances are additive in series, the hydraulic resistance of the leaf (R_{leaf}) is used in discussion of the leaf as a component of whole-plant resistance, or when partitioning the resistances within the leaf.

Maximum leaf hydraulic conductance ($Kh_{max\text{-}leaf}$) across a range of species and life forms were reviewed in Sack and Holbrook (2006). Measurements of leaf hydraulic conductance for hydrated leaves ($Kh_{max\text{-}leaf}$), made with several methods (Sack *et al.*, 2005), indicate a dramatic variability across the 107 species so far examined (Sack and Holbrook, 2006). Thus, $Kh_{max\text{-}leaf}$ ranges 65-fold from the lowest value (for the fern *Adiantum lunulatum*; 0.76 $mmolm^{-2}$ s^{-1} MPa^{-1}) to the highest (for the tropical tree *Macaranga triloba*; 49 mmol m^{-2} s^{-1} MPa^{-1}). Regarding grapevine (*Vitis vinifera L.*), classified as one of the temperate woody angiosperms (Fig. 1.3), its $Kh_{max\text{-}leaf}$ values are usually high (around 3 to 10 mmol m^{-2} s^{-1}), due to the presence of large xylem vessels (Scholander *et al.*, 1995; Essau, 1965). However those values undergo large fluctuations directly related to different cultivars and environmental conditions (Schultz, 2003; Sack *et al.*, 2003). Interspecific variation in $Kh_{max\text{-}leaf}$ reflects differences in the anatomy of the petiole and venation, as well as pathways beyond the xylem through living tissues to sites of evaporation.

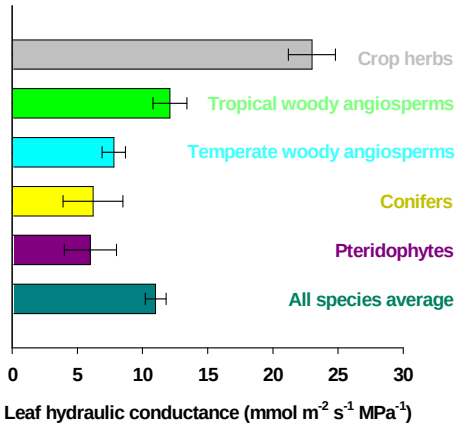

Figure 1.3. Leaf hydraulic conductance averaged for contrasting life forms. Error bars = 1 SE. From Sack and Holbrook (2006).

Kh_{leaf} is also highly dynamic over a range of time scales (from minutes to months), showing circadian and developmental trajectories, and responding rapidly, often reversibly, to changes in temperature, irradiance, and water supply. It has been determined that Kh_{leaf} generally declines with increasing water stress (Nardini *et al.*, 2001; Lo Gullo *et al.*, 2003), but that the extent of the decline and the water potentials corresponding to the decline, vary from species to species, even within a particular habitat (Salleo *et al.*, 2001, Brodribb and Holbrook, 2003a; Hao *et al.*, 2008). An important factor contributing to the decline of Kh_{leaf} at low Ψ_{leaf} is xylem cavitation (Kikuta *et al.*, 1997, Nardini *et al.*, 2003; Zwieniecki *et al.*, 2004; Johnson *et al.*, 2009). Understanding of the mechanisms responsible for the desiccation-induced decline in Kh_{leaf} is still far from complete, and complicated by the interactions of light, temperature and water status with Kh_{leaf}. Voicu *et al.* (2008), Scoffoni *et al.* (2008) and Sellin *et al.* (2008) have all found increases in Kh_{leaf} with increasing light, and Sack *et al.* (2004) observed increases in Kh_{leaf} that were greater than would be expected only due to the reduced viscosity of water with increasing temperature. Even less well understood is the phenomenon of Kh_{leaf} recovery while still under negative pressures (Clearwater and Goldstein, 2005; Nardini *et al.*, 2008). Recent research has suggested that these changes may be partially due to variation at the molecular scale, including aquaporin gene expression and protein conformation (Nardini *et al.*, 2005; Cochard *et al.*, 2007; Kaldenhoff *et al.*, 2008), that result in

reductions in leaf hydraulic conductance (Kh_{leaf}), thus affecting the efficiency of water flow through the leaves.

Adequate Kh_{leaf} is critical for preventing transpiration-induced desiccation and subsequent stomatal closure that would restrict carbon gain. Leaves would close their stomata at water potential thresholds at which Kh_{leaf} begins to decline sharply, thus preventing substantial losses of Kh_{leaf}. From different published studies, two different strategies regarding daily maintenance of Kh_{leaf} were considered: (1) substantial loss and subsequent recovery or (2) a more conservative strategy of loss avoidance. Thus, evidence of embolism reversal (refilling) under tension is reported in leaves (Canny, 2001; Lo Gullo *et al.*, 2003), as well as in roots (McCully, Huang and Ling, 1998), stems and petioles (Zwieniecki and Holbrook, 1998; Bucci *et al.*, 2003; Hacke and Sperry, 2003). These reports sustain the possibility that embolism repair is a way for some plants to be able to operate close to the hydraulic limit of their water-conducting system without risking the compounding effects of frequent xylem cavitation. Recently, the vulnerability of leaf venation has been investigated, and the results of these studies suggest that leaf xylem operates very close to its cavitation threshold (Nardini and Salleo, 2000; Brodribb and Holbrook, 2003; Lo Gullo *et al.*, 2003). Although several studies have observed diurnal cycles of cavitation and recovery in the petiole (Zwieniecki *et al.*, 2000; Bucci *et al.*, 2003), only a few studies have focused on diurnal patterns of leaf hydraulics in the field. Brodribb and Holbrook (2004a) found that in *Simarouba glauca*, Kh_{leaf} decreased by about half by midday (under conditions of moderate evaporative stress) compared to predawn values and recovered completely by the end of the day.

In general rapid recovery of whole leaf hydraulic conductance (Kh_{leaf}) after drought stress has been reported by several authors (Linton and Nobel, 2001; Lo Gullo *et al.*, 2003; Milburn, 1973; Trifiló *et al.*, 2003). Such short-term increases in Kh_{leaf} on re-watering, result from a diversity of mechanisms, such as elastically recover xylem geometry (Brodribb and Holbrook, 2005; Cochard *et al.*, 2004), increasing root pressure (Stiller *et al.*, 2003) and an active mechanism involving ion pumping or transient pressures associated with increasing starch degradation (Bucci *et al.*, 2003; Zwieniecki *et al.*, 2000; Trifiló *et al.*, 2003).

1.4.2.1. Relation to Whole-plant hydraulic conductance

Leaf hydraulic resistance (R_{leaf}) can substantially constrain water transport being a major bottleneck in the whole plant water transport pathway (Sack *et al.*, 1993) and may thus be linked to the enormous variation in leaf structure and function among different plant species.

Studies of plant hydraulic architecture have traditionally focused on characterizing the inherent hydraulic properties of various segments of the root-to-leaf pathway and on the mapping of hydraulic constrictions within the plant (Zimmermann 1978; Tyree and Ewers 1991). Such studies showed that leaf lamina represents one of the least conductive regions of the plant, contributing between 30 and 80% to the whole plant hydraulic resistance (Salleo, Nardini and Lo Gullo, 1997; Nardini and Salleo, 2000; Nardini, 2001; Sack *et al.*, 2003) over a distance of rarely more than 1% of the total hydraulic path length. Therefore, it is important to know how this resistance is allocated and how it varies under different conditions. Additionally, R_{leaf} changes with temperature, water supply, irradiance, and leaf age, as previously seen and can thus increase as a proportion of R_{plant} to become the dominant factor in defining whole plant water transport capacity.

Consequently, it has been described that R_{leaf} may constrain whole-plant hydraulic resistance (R_{plant}) in a higher proportion during peak transpiration. In a given microclimate and soil water supply, R_{plant} determines the leaf water potential at a given transpiration rate (Cowan, 1972; Tyree and Zimmermann, 2002). Thus, R_{plant} constrains maximum stomatal conductance before leaf desiccation, and correlates negatively with maximum stomatal conductance across species (e.g., Nardini and Salleo, 2000).

1.4.2.2. Xylem embolism as impairment of the hydraulic conductance

An increase in the tension of xylem vessels, usually as a result of stress, can cause sudden break of the water column or cavitate. Under high levels of tension, vacuum is formed resulting in air being drawn into the vessels and the formation of embolism. In that case, vessels can no longer be used for water transport, reducing the overall hydraulic conductivity of the stem. Different plant species have a varying vulnerability to cavitation depending on both xylem anatomy and hydraulic properties (Tyree *et al.*, 1994; McElrone *et al.*, 2004) with the objective to protect the stem from extreme xylem tensions during severe drought (Sperry *et al.*, 2006).

Embolism of xylem vessels as a result of water stress has been extensively studied in grapevines (Schultz and Matthews, 1988; Salleo and Lo Gullo, 1989). There have been numerous mechanisms proposed to describe how water stress causes embolism in plants. Zimmermann's (1983) "air seeding" hypothesis has gained most of the experimental support (Cochard *et al.*, 1992; Jarbeau *et al.*, 1995; Pockman *et al.*, 1995). The mechanism of air-seeding relies on the structural properties of the xylem vessels and the associated pit membrane. Air seeding will occur when the tension in the water column (as a result of water stress) increases to a level that forces air through the pits into the water filled vessels, thus resulting in cavitation of the water column.

Water stress in grapevines results in a decrease in stem water potential (Ψ_{stem}). Moderately water stressed vines (Ψ_{leaf} -0.6MPa) showed a reduction in vessel size but had no detectable embolisms. Under more severe water stress (Ψ_{leaf} -0.8MPa), hydraulic conductivity was further reduced due to embolism formation in the xylem vessels (Lovisolo and Schubert, 1998). This observation was similar to that previously reported by Schultz and Matthews (1988).

In order to maintain their hydraulic capacity, plants must have a mechanism to repair or replace embolised vessels. Numerous studies have shown that xylem is able to recover from embolism although the mechanism for recovery is still under debate (Salleo *et al.*, 1996; Canny, 1997; McCully *et al.*, 1998; Zwieniecki and Holbrook, 1998; McCully, 1999; Tyree *et al.*, 1999; Zwieniecki and Holbrook, 2000; Clearwater and Goldstein, 2005). Grapevines have developed different repair mechanisms to

reintegrate vessel functionality including positive root pressure or vine actively transpiration (Holbrook *et al.*, 2001), and in other cases involving active and energy-consuming processes in shoot conductive tissues (Salleo *et al.*, 2004)

Although grapevine petioles and roots are more vulnerable to embolism than shoots under water stress (Lovisolo *et al.*, 2008a), either root or shoot and petioles recover ~35-40% of hydraulic conductivity within 24 h after re-watering, suggesting that a common and coordinated mechanism of recovery among all plant organs must occur. Furthermore, some experiments point to the presence of an active mechanism involving the contribution of living cells in repair of embolism in grapevine (Lovisolo and Schubert, 2006), and also possibly involving the contribution of some aquaporins (Galmés *et al.*, 2007b; Lovisolo *et al.*, 2008b). Presently, all known aquaporins in grapevines are much more abundant in roots than in any other tissue, although they are also present in shoots and leaves.

1.4.2.3. Pathways of water movements in leaves: movements throughout leaf xylem and outside the xylem

The diversity of leaf venation and mesophyll structure might be associated with leaf hydraulic resistance (R_{leaf}). Water enters the leaf through the petiole and flows through several orders of leaf veins (i.e. highly conductive medium), across the bundle sheath, and into and/or around the mesophyll cells (i.e. highly resistive medium), before it escapes the leaf via stomata. In recent studies, R_{leaf} was partitioned into hydraulic resistances of the xylem, including petiole and major and minor veins; and the pathways across the bundle sheath and across mesophyll cells to the airspaces (e.g., Cochard *et al.*, 2004, Gascó *et al.*, 2004, Sack *et al.*, 2004, 2005). Similar approximations were studied when determining Kh_{lamina} by the vascular and extra-vascular pathways of transpired water (Yang and Tyree, 1994; Nardini *et al.*, 2001; Sack *et al.*, 2002).

The hydraulic conductance of this low-conductivity mesophyll pathway should be inversely proportional to its path length, which is likely to strongly influence the overall hydraulic conductance of the leaf given the fact that the hydraulic conductivity of living cells in plants is very low relative to xylem cells

(Boyer, 1985; Passioura, 1988; Frensch and Steudle, 1989) leading to a high proportion of extra-vascular resistance in the leaf (Cochard *et al.*, 2004; Nardini and Salleo, 2005b; Sack *et al.*, 2005). Indeed, the potential role of leaf characters such as vein density (Sack and Frole, 2006) and mesophyll thickness (Aasamaa *et al.*, 2001) as determinants of the hydraulic efficiency of the leaf have recently been recognized (Brodribb *et al.*, 2007).

The extra-vascular hydraulic resistance ($R_{outside\ xylem}$) of a leaf has been estimated to build up 50-90% of the whole leaf resistance (Tyree and Cheung, 1977; Tyree, Nardini and Salleo, 2001; Trifilò *et al.*, 2003; Cochard, *et al.* 2004). Although there is no general consensus on this partitioning of *R* in a leaf and data from the literature are sometimes contradictory (Zwieniecki *et al.*, 2002; Cochard *et al.*, 2004; Sack, Streeter and Holbrook, 2004), the hypothesis that changes in leaf hydraulic conductance may be the result of active processes occurring in the mesophyll living cells and including new expression or activation of aquaporins that can greatly enhance the water permeability of cell membranes is gaining force (Eckert *et al.*, 1999; Otto and Kaldenhoff, 2000; Morillon and Chrispeels, 2001).

Nowadays the current consensus is that the hydraulic resistance of the leaf xylem is of the same order of magnitude that in the extra-xylem pathways (Gascó *et al.*, 2004; Nardini *et al.*, 2005ab; Sack *et al.*, 2004, 2005), and that species vary in their partitioning. Among tropical trees, the percentage of R_{leaf} in the xylem differed significantly between species (ranging from 26% to 89%); i.e. species that establish in high-light environments had on average 70% of R_{leaf} in the xylem, whereas those from low irradiance had about 50% (Sack *et al.*, 2005).

How R_{leaf} is partitioned between the xylem and across the extra-xylem pathways will strongly influence the responsiveness of R_{leaf} to changing conditions. If leaf xylem resistance (R_{xylem}) is a major component of R_{leaf}, then the enormous variation in venation architecture across species could reflect strong differences in R_{leaf}, although this would be very unlikely if R_{xylem} were negligible relative to the $R_{outside\ xylem}$; in that case, even large relative differences in R_{xylem} among species would have little impact on overall R_{leaf}. Additionally, the larger R_{xylem} is, relative to $R_{outside\ xylem}$, the greater the effect of changes in R_{xylem} due to environment on R_{leaf} (e.g.,

drought-induced cavitation) as it has been described by Meinzer (2002). Thus, the implications of this finding are that differences in venation architecture reflect strong differences in R_{leaf}, and also that R_{leaf} will be sensitive to changes in the conductance of both xylem and extra-xylem pathways.

Indeed, the ratio of R_{xylem} to $R_{outside\ xylem}$ is dynamic. For example, R_{xylem} will increase when vein xylem embolizes during drought, whereas $R_{outside\ xylem}$ changes according to an endogenous circadian rhythm, and also increases under low irradiance (Nardini *et al.*, 2005; Sack *et al.*, 2002, Tyree *et al.*, 2005); both resistances increase at lower temperatures, with $R_{outside\ xylem}$ increasing more strongly (Matzner and Comstock, 2001; Sack *et al.*, 2004). Each of these leaf compartments has its own hydraulic properties, the former mainly depending on the geometry of the xylem conduits (Canny, 1990; Cochard *et al.*, 2004) and water permeability of the pits (Sperry *et al.*, 2005), whereas the latter are closely dependent on water permeability of cell membranes and, ultimately, on cell metabolism (Morillon and Chrispeels, 2001).

So, the overall leaf hydraulic conductance comprises both axial water transport along xylem vessels and transcellular transport in vascular bundles and the mesophyll.

Water movement through leaf xylem: petiole and venation

Leaf vein systems are enormously variable in many aspects: vein arrangement and density, the number, size, and geometry of the vascular bundles in the veins; and of the xylem conduits within the bundles (Roth-Nebelsick *et al.*, 2001). These structural characteristics play a critical role on how water is distributed across the leaf.

As described by Sack *et al.* (2003a), primary, secondary, and tertiary veins protrude as ridges visible on the abaxial face of the lamina. Primary veins are connected directly to the petiole. Thus, depending on the species, there is only one primary vein, usually referred to as the midrib, or more. Secondary veins branch from the primary one(s), and tertiary veins are smaller in diameter, branching from and sometimes linking the primary and secondary veins. Quaternary veins (henceforth

‘‘minor veins’’) are fully embedded in the mesophyll and consist of several diameter classes, all smaller than the tertiary veins. Fine veins form a continuous mesh with the major vein system, dividing the lamina into regions referred to as areoles.

Higher minor vein density in general corresponds to higher supply capacity (or $Kh_{max\text{-}leaf}$), not by increasing the conductance through the vein xylem system per se (Cochard *et al.*, 2004), but primarily by increasing the surface area for exchange of xylem water with surrounding mesophyll, and secondarily by reducing the distance through which water travels outside the xylem (Roth-Nebelsick *et al.*, 2001; Sack and Frole, 2006). By contrast, the arrangement and density of major veins is not related to $Kh_{max\text{-}leaf}$ (Sack and Frole, 2006). However, major vein arrangement plays an essential role in distributing water equitably across the lamina (Roth-Nebelsick *et al.*, 2001; Zwieniecki *et al.*, 2002), and redundancy of major veins could buffer the impacts of damage and/or cavitation.

Thus, the bulk of transpired water will be drawn out of minor veins, resulting in major and minor veins acting approximately in series (Sack *et al.*, 2003; Yang *et al.*, 1994), so leaf photosynthetic performance was hypothesized to become involved based upon the well supported observation that maximum net assimilation rate is coupled to the capacity of the leaf vascular system to supply water to photosynthesizing mesophyll cells (Brodribb *et al.*, 2005; Sack and Holbrook, 2006). Cavitation within the leaf vasculature appears to be extensive and may play a role in patterns of stomatal closure within the leaf (Salleo *et al.*, 2001, 2000) as does the degree hydraulic contact between leaf aeroles (Mott and Buckley, 1998).

Water movement outside the xylem: bundle sheath and mesophyll

Water movement pathways outside the xylem, though short in distance, are complex and potentially vary strongly across species that diverge in leaf mesophyll anatomy, and generally have relatively low hydraulic conductivities on water length and area basis as mentioned above. However, there is little consensus on the flow path in the leaf mesophyll. At issue is to what extent it is apoplastic *vs*. symplastic; whether the site of evaporation is near the substomatal chamber or deeper within the

leaf and the extent of hydraulic contact between different leaf tissues, in particular epidermis *vs.* mesophyll.

It is known that once water leaves the xylem, it enters the bundle sheath. Bundle sheath extensions (BSE) consist of parenchyma or schlerenchyma cells of the vascular bundle sheath surrounding minor veins, which extend to the epidermis (Wylie, 1952; McClendon, 1992; Kenzo *et al.*, 2007). A number of early studies concluded that water exits the xylem through cell walls, moving around the bundle sheath protoplasts, as membranes were thought to present too much resistance to occur in transpirational pathways (e.g., Boyer, 1974, 1977). However, the presence of aquaporins and the high surface area for water transport across the membranes of bundle sheath cells means that intercellular water movement is, in fact, plausible (Kaldenhoff and Eckert, 1999; Martre *et al.*, 2002; Sack *et al.*, 2004; Schaffner, 1998). Indeed, in some species, the presence of lignified or suberized cell walls, which constitute apoplastic barriers, forces water to cross cell membranes (i.e. entering bundle sheath cells), as do the Casparian strips in the root endodermis (Lersten, 1997; Fricke, 2002; Hachez *et al.*, 2008). Leaves with bundle sheath cells may show stronger Kh_{leaf} light response, as these cells guide light into interior vascular and mesophyll tissues (Karabourniotis *et al.*, 2000; Nikolopoulos *et al.*, 2002; Scoffoni *et al.*, 2008).

In addition to radial water flux, which is controlled by evapotranspiration, water movement across leaf cell membranes is also important for a) water homeostasis, b) increasing cell volume, c) maintaining turgor during expansion, d) regulating the opening and closure of stomata, and e) controlling leaf movement.

Beyond bundle sheath there is the mesophyll. A large portion of the mesophyll volume is airspace, with limited cell-to-cell contact, but spongy mesophyll cells are in contact to a far greater degree than palisade cells, and thus would seem better suited to conduct water (Wylie, 1943, 1946). The epidermis has substantial cell-to-cell contact, and water could move directly there from the minor veins, in species with bundle sheath extensions. In those species, the epidermis can remain hydrated despite having little vertical contact with the photosynthetic mesophyll (LaRue 1931; Warrit *et al.*, 1980). In species lacking bundle sheath extensions, water

must move across the mesophyll. As in the composite transport model in the root, water can take different paths on its way through the leaf, moving through the apoplast (in the cell walls) or from cell to cell (Steudle, 1994; Steudle and Peterson, 1998). The latter route is composed of symplastic (through the plasmodesmata) and the transcellular (across cell membranes) paths. The contribution of each pathway to the overall leaf hydraulic conductance (Kh_{leaf}) is unclear, and probably differs among species and conditions.

Due to its lower resistance to water flux, the apoplastic path is believed to be the main route during transpiration (Sack and Holbrook, 2006). The first experimental studies on sunflower suggested that apoplastic movement dominates during transpiration, and that water crosses mesophyll membranes only during rehydration or growth (Boyer, 1977; Weatherley, 1963). However, in support for a symplastically path, evidence for a role of aquaporins in leaf water transport is emerging. This was first suggested by strong expression of aquaporins in bundle sheath cells (Frangne *et al.*, 2001) or other cell types showing high water permeability (Hachez *et al.*, 2008). In addition, the general aquaporin blocker, mercury, was able to inhibit Kh_{leaf} in sunflower (*Helianthus anuus*) and in six temperate deciduous trees (Aasamaa and Sober, 2005; Nardini *et al.*, 2005). Furthermore, cell pressure probe has indicated significant symplastic water transport among mesophyll cells in succulent Kalanchoë leaves (Murphy and Smith 1998), and more recently, pressure probe measurements in midrib parenchyma cells of corn leaves revealed that the effects of light (in addition to turgor) on leaf water transport were mediated in part through changes in cell hydraulic conductivity (Kim and Steudle, 2007). The hypothesis that rapid changes in Kh_{leaf} involve aquaporin regulation was further substantiated in a study in walnut trees (Cochard *et al.*, 2007). The authors analyzed the abundance of two major PIP2 aquaporin transcripts during a transition from dark to high light and found a very good kinetic correlation between the increase in Kh_{leaf} and the increase in PIP2 aquaporin expression.

1.4.2.4. Coordination with gas exchange

The efficiency of water transport within leaves, or leaf hydraulic conductance (Kh_{leaf}), has been demonstrated to vary between species (Tyree *et al.*, 1999; Brodribb *et al.*, 2005), ecological niches (Nardini and Salleo 2005; Sack, Tyree and Holbrook 2005), seasons (Salleo *et al.*, 2002; Brodribb and Holbrook, 2003b) and in response to environmental factors such as drought (Nardini *et al.*, 2001, Lo Gullo *et al.*, 2003), light (Tsuda and Tyree, 2000; Sack *et al.*, 2002, 2003b), mechanical damage (Hüve *et al.*, 2002; Nardini and Salleo, 2003; Sack *et al.*, 2003a) and senescence (Salleo *et al.*, 2002; Brodribb and Holbrook, 2003) as previously described. Among these factors of variation, Kh_{leaf} probably correlates best with stomatal conductance (Brodribb and Holbrook, 2004a; Brodribb *et al.*, 2005; Nardini, Salleo and Andri, 2005). Considering that these parameters represent conductances of water in the liquid and the gas phase which move in a serial pathway through the leaf, this correlation, as well as similar relationships between stem hydraulic conductance and g_s, indicate that water potential gradients in non-stressed plants are relatively conservative (Nardini and Salleo, 2000; Meinzer, 2002). That is, during their evolution plants increased the conductance of the vascular system in order to accommodate increased transpirational demand rather than operating at increased water potential gradients.

Nowadays, there is an increasing interest in leaf hydraulics, which is partly due to the fact that if leaves represent a large fraction of total plant hydraulic resistance (as reported above), foliage would be the site of important constrains to leaf gas-exchange rates (Nardini and Salleo, 2003; Trifilò *et al.*, 2003; Brodribb and Holbrook, 2003), and ultimately for plant productivity. The gas exchange behaviour of foliage must also be related to the hydraulic characteristics of the leaf xylem due to the series-connection of xylem and stomata along the water path through the plant. Following the cohesion-tension theory, leaves draw water from the soil by a water potential gradient generated by evaporation at the leaf. The difference between soil and leaf water potential is therefore determined by the rate of water loss at the leaf, and the resistance to water flow imposed by the xylem vessels and non-xylem pathways radial to the vasculature.

In numerous studies a correlation between the liquid phase conductance from soil-to-leaf (*Ks–l*; per leaf area), and g_s or *E* (Sperry *et al.*, 2000; Meinzer and Grantz, 1990; Meinzer *et al.*, 1995; Saliendra *et al.*, 1995) has been observed. This correlation results from an active response of stomata to *Ks–l* because g_s changed almost immediately when *Ks–l* was experimentally modified. When *Ks–l* per leaf area was increased by partial defoliation or shading, g_s of the untreated foliage increased. When *Ks–l* was decreased by stem or root pruning, g_s decreased (Meinzer and Grantz, 1990; Sperry *et al.*, 1993; Whitehead *et al.*, 1996; Pataki *et al.*, 1998). Indeed, the interaction between hydraulics and g_s becomes more important under water stress conditions, as *Ks–l* declines when Ψ drops. The interaction between *Kh* and Ψ sets the limit to the plant's hydraulic transport capacity and exerts a significant influence on stomatal regulation of water use during drought. Observation suggests that plants tend to be somewhat conservative in terms of decreased water potential from root to leaf. For this reason it is not surprising that hydraulic (xylem) and diffusive (stomatal) resistances (or conductances) have been found to be well correlated among tree species (Whitehead *et al.*, 1984; Meinzer, 2002).

Given the fact that the stomatal conductance of leaves closely correlates with assimilation rates both within individuals and generally across C3 plants (Wong *et al.*, 1979), some kind of relationship between hydraulic conductance and assimilation rate is expected. However, only a few studies have dealt directly with the question of how xylem hydraulics relate to the photosynthetic rate. The first study illustrated a linear relationship between stem hydraulic conductivity and photosynthetic quantum yield in a range of temperate rainforest species (Brodribb and Feild, 2000). This relationship was supported by a later study showing that artificial depression of Kh_{plant} led to a proportional decrease in the foliar assimilation rate of ponderosa pine seedlings (Hubbard *et al.*, 2001). Considering the large proportion of whole plant hydraulic resistance that resides in leaves, it is likely that the efficiency of water delivery to evaporating cells should correspond closely with photosynthetic rate. This was one of the main conclusions of a recent study showing that a decline in leaf hydraulic conductivity around midday in a tropical tree species was linked to midday

depression of stomatal conductance and photosynthesis (Brodribb and Holbrook, 2004a).

Regardless of the mechanism several studies on grapevines have shown that they strongly respond to changes in plant water status through hydraulic tensions caused in the xylem and thereby affecting the leaf turgor. Indeed, positive correlations between pre-dawn water potential and maximum g_s have generally been found in grapevines subjected to varying water deficits (Correia *et al.*, 1995; Flexas *et al.*, 1998; Rodrigues *et al.*, 2008). As in other species, a decrease in shoot hydraulic conductivity has been shown to occur in water-stressed grapevines (Schultz and Matthews, 1988; Lovisolo and Schubert, 1998; Lovisolo *et al.*, 2002) and is linearly correlated with g_s under mild stress levels (Lovisolo and Schubert, 1998). Some experiments involving water stress, partial root drying or others (Lovisolo *et al.*, 2002; Loveys and Kriedemann, 1974, Rodrigues *et al.*, 2008) presented strong evidence that it was leaf abscisic acid (ABA) and not whole-plant hydraulic conductivity that determines g_s in grapevines. It was shown that a decline in leaf water potential might enhance stomatal sensitivity to ABA, which either was synthesized in roots in response to water stress and transported via the xylem to the leaves (Correia *et al.*, 1995; Lovisolo *et al.*, 2002, Pou *et al.*, 2008) or locally synthesized in buds and leaves (Soar *et al.*, 2004, 2006). This interactive effect can explain the decrease in g_s observed at midday in grapevines growing under field conditions, including well-watered ones, in spite of constant diurnal [ABA] in the xylem stream (Correia *et al.*, 1995; Rodrigues *et al.*, 2008).

Further works in grapevine showed that whole-plant hydraulic conductivity often correlates well with g_s during drought imposition (Winkel and Rambal, 1993; Schultz, 2003; Pou *et al.*, 2008), and much better during recovery after water stress (Lovisolo *et al.*, 2008; Pou *et al.*, 2008), suggesting that g_s may be regulated by hydraulic signals during water stress. However, additional experiments are required to understand the role of hydraulics on stomatal regulation in grapevines.

1.4.3. The role of aquaporins as a mechanism involved in hydraulic conductance

1.4.3.1. Plant aquaporins

Aquaporins or water channels are membrane intrinsic proteins that facilitate the movement of water and other small neutral solutes across cellular membranes.

Plant aquaporins belong to a large family of highly conserved proteins called the Membrane Intrinsic Protein (MIP) superfamily members of which are found in almost all living organisms (Agre *et al.*, 1998). Phylogenetic analyses (based on DNA sequence similarities) reveals the MIP superfamily can be divided into four different subfamilies, which to some extent reflect distinct subcellular localizations: the Plasma Membrane Intrinsic Proteins (PIPs), Tonoplast Intrinsic Proteins (TIPs), Nodulin-like Intrinsic Proteins (NIPs) and the Small Intrinsic Proteins (SIPs) (Chaumont *et al.*, 2001; Johanson *et al.*, 2001; Zardoya and Villalba, 2001; Sakurai *et al.*, 2005; Danielson and Johanson, 2008).

AQPs may function as a water selective (aquaporins) or non-selective channels for water and other small non-electrolytes (aquaglyceroporins). Members of the MIP superfamily have been shown to have a broad range of transport selectivity including the permeability to water (Biela *et al.*, 1999; Santoni *et al.*, 2000; Tyerman *et al.*, 2002), glycerol (Biela *et al.*, 1999; Weig and Jakob, 2000; Moshelion *et al.*, 2002), H_2O_2 (Bienert *et al.*, 2007), CO_2 (Uehlein *et al.*, 2003), urea (Gerbeau *et al.*, 1999; Liu *et al.*, 2003), NH_3 (Jahn *et al.*, 2004; Loque *et al.*, 2005) and silicon (Ma *et al.*, 2006). Nowadays, the introduction of aquaporins (Terashima and Ono, 2002; Uehlein *et al.*, 2003; Flexas *et al.*, 2006b) and carbonic anhydrase (Badger and Price, 1994; Gillon and Yakir, 2000) as facilitators of CO_2 diffusion has opened up a new window for g_m regulation on the cellular level.

Because of their abundance, PIP`s and TIP`s represent important pathways for transcellular and intracellular water transport (Holm *et al.*, 2005; Jahn *et al.*, 2004; Loque *et al.*, 2005). The PIP`s are further divided into two subclasses, PIP1 and PIP2 (Chaumont *et al.*, 2001). In general PIP1 proteins facilitate the diffusion of small neutral solutes (Dean *et al.*, 1999; Biela *et al.*, 1999; Gaspar *et al.*, 2003), however they often exhibit a low or no activity for water transport when expressed in *Xenopus laevis* oocytes. By contrast, all analyzed PIP2 proteins showed a high water transport

activity, increasing the water permeability of oocyte membranes about 10- to 20-fold (Chaumont *et al.*, 2000). The reason for this difference among PIP1 and PIP2 remains still unclear. Moreover, it has recently been shown that the presence of one aquaporin in a membrane can synergistically influence the activity of other aquaporins (Fetter *et al.*, 2004). Thus, it has been demonstrated that the coexpression of PIP1 and PIP2 aquaporins increases the water permeability of oocytes, supporting the notion of such a cooperative effect (Moshelion *et al.*, 2002; Fetter *et al.*, 2004).

In the Vitis genome 28 aquaporin genes have been identified (Fouquet *et al.*, 2008). In fact, eight cDNA's encoding putative aquaporins were identified in the Vitis rootstock Richter-110 by screening a leaf cDNA library with homologous probes (Baiges *et al.*, 2001). Using reverse Northern-blots, different tissue specific expression patterns were obtained for each of the putative aquaporins. Moreover, two plasma membrane aquaporins (PIPs) from ripening grape berry of *Vitis vinifera* cv Pinot Noir have been shown to transport glycerol (Picaud *et al.*, 2003).

In fact, several studies on grapevine aquaporin expression in roots or shoots have been carried out during the last years (Perrone *et al.*, 2006; Reid *et al.*, 2006; Galmés *et al.*, 2007b; Fouquet *et al.*, 2008; Glissant *et al.*, 2008; Schlosser *et al.*, 2008; Shelden *et al.*, 2009 and Vandeleur *et al.*, 2009).

1.4.3.2. The role of aquaporins in response to water stress

Although water transport across membrane pores had been proposed in the past (Dainty and Ginzburg, 1963; House, 1974; Finkelstein, 1987), the discovery of the molecular structure of AQP`s and detailed studies of their function revolutionized research on plant water relations, at least those involving membranes (Steudle and Henzler, 1995; Maurel, 1997; Kjellbom *et al.*, 1999; Tyerman *et al.*, 1999; Steudle, 2001). There is evidence that AQPs play an important role in stomatal regulation and plant hydraulics related to the cell, tissue, organ, and whole plant level (Tyerman *et al.*, 1999; Johansson *et al.*, 2000). They facilitate the rapid, passive exchange of water across cell membranes and are responsible for up to 95% of the water permeability of plasma membranes (Henzler *et al.*, 2004).

Nowadays, many studies deal with molecular responses to water shortage (Kreps *et al.*, 2002; Salekdeh *et al.*, 2002; Seki *et al.*, 2002; Xiong and Zhu, 2002; Bray, 2004; Kawaguchi *et al.*, 2004; Vera-Estrella *et al.*, 2004; Hajheidari *et al.*, 2005), while however, aquaporins are strongly regulated in response to various environmental signals, including both biotic and abiotic factors (Maurel *et al.*, 2002, Tyerman *et al.*, 2002). Abiotic factors include drought, salt and cold stress, while biotic factors include symbiotic and pathogen interactions as well as hormonal factors. The regulation of aquaporins in response to water stress, including drought and salinity has been documented to include transcriptional and post-transcriptional modifications, as well as post-translational modification of the aquaporin proteins. A number of aquaporin genes have been found to be down-regulated or up-regulated or to do not change under conditions of water stress (Tyerman *et al.*, 2002), depending on the time course and the intensity of water stress (Malz and Sauter, 1999; Barrieu *et al.*, 1998; Jang *et al.*, 2004; Alexandersson *et al.*, 2005; Galmés *et al.*, 2007b). The different responses of AQP expression (up/down-regulation or no change) to water stress suggest that AQP isoforms can be divided into different functional groups, which contribute differently to water transport and its regulation (Hachez *et al.*, 2006).

Aquaporin mediated water transport can be inhibited by mercurial sulfhydril reagents, which are known as non-specific water channels inhibitors, thus allowing the estimation of the proportion of water transported by mercury-sensitive aquaporins in a whole-root system under water-sufficient (Maggio and Joly, 1995; Wan and Zwiazek, 1999; Barrowclough *et al.*, 2000) and water-deficient (North and Nobel, 2000; Martre *et al.*, 2001) soil conditions. Such studies indicate that aquaporins can account for 35% to 80% of root hydraulic conductance under wet conditions, and for 60% to 80% of root hydraulic conductance in drying or rewetted soil. However, although $HgCl_2$ rapidly depolarizes the plasma membrane of cells and may have other effects in addition to the direct inhibition of aquaporin activity (Zhang and Tyerman, 1999), they still allow an estimation of aquaporin activity. Moreover, the mercury sensitivity of the water-transport activities differs among aquaporin isoforms. In that sense, Suga and Maeshima (2004) measured aquaporin activity

using the membrane vesicles of yeast cells expressing radish plasma membrane aquaporins. They demonstrated that PIP2s generally have a much higher water transport activity than PIP1`s. As the vesicles were incubated with 5mM $HgCl_2$, the activities of PIP2`s were severely inhibited while those of PIP1`s were relatively insensitive to the mercury treatments.

In studies on grapevine with the metabolic and water transport inhibitor mercuric chloride the presence of an active mechanism involving the contribution of living cells can be assumed (Lovisolo and Schubert, 2006), as well as a possible contribution of aquaporins too. Furthermore, in such experiments, using mercury as an inhibitor of the activity of some aquaporins, it has been suggested that aquaporins are involved in the recovery after water stress of shoot (Lovisolo and Schubert, 2006) and root hydraulic conductivity (Lovisolo *et al.*, 2008b). However, the role of AQP in regulating plant water status in grapevine remains a complex issue because different subfamilies and subclasses may be up- or down-regulated or remain unchanged depending on the degree of water deficit and/or the time during the stress period (Galmés *et al.*, 2007b).

1.5. THE REGULATION OF CARBON FLOW UNDER WATER STRESS AND DURING RE-WATERING

1.5.1. Photosynthesis limitations

Carbon assimilation rates are associated with either the diffusion of carbon dioxide to the intracellular leaf space, controlled by stomatal functioning, or biochemical processes within the leaf. Common nomenclature for this separation is stomatal limitation versus non-stomatal limitation, and quantitative methods have been derived to assess these relative limitations based on gas exchange data (Jones 1985, Assmann 1988, Jones 1998).

Quantitative limitation analyses can be obtained directly from the response of assimilation (A_N) to the intercellular carbon dioxide concentration (C_i) (A_N/C_i curves). Leaf photosynthetic responses to changes in incident light or in chamber CO_2 concentration can be done following different approaches (Farquhar and Sharkey, 1982; Jones, 1985), in order to estimate the relative contribution of stomatal and

mesophyll characteristics. As verified by von Caemmerer and Farquhar (1981), the initial slope of the CO_2 response curve is correlated with Rubisco activity and, similarly, at saturating irradiance, maximum photosynthesis (A_{maxCO_2}) is correlated with ribulose-1,5-bisphosphate availability in the mesophyll (i.e. maximum carboxylation rate of Rubisco; $V_{c,max}$) and with RuBP regeneration capacity mediated by maximum electron transport (J_{max}).

Rubisco, a chloroplast bifunctional enzyme that catalyzes the transfer of CO_2 to ribulose 1,5-biphosphate in the Calvin cycle, requires a certain CO_2 threshold concentration to be efficient, and the CO_2 concentration in the vicinity of the enzyme is therefore the limiting factor for photosynthesis. The diffusion of CO_2 from the atmosphere into the substomatal cavity via the somata and to sites of carboxylation via the mesophyll is the main factor controlling CO_2 availability for Rubisco. Consequently, mesophyll conductance (Harley *et al.*, 1992; Loreto *et al.*, 1992) is a measure of the transfer capacity of CO_2 between the leaf internal airspaces and the site of carboxylation in the chloroplast stroma and is a fundamental property of leaves that may influence photosynthetic capacity (Epron *et al.*, 1995; Evans and Loreto, 2000). It is regulated by the diffusion barriers for CO_2 formed by the intercellular space, apoplastic liquid phase, plasma membrane, cytosol, chloroplast membranes, and stroma.

Hence, we can describe the rate of net CO_2 assimilation by:

$$A_N = g_s(C_a-C_i) = g_m(C_i-C_c);$$

where C_a, C_i and C_c are the CO_2 concentrations (μmol mol^{-1} air) in the atmosphere, the sub-stomatal cavity and the chloroplast stroma, respectively, with g_s and g_m being the stomata and mesophyll conductances, respectively (Long and Bernacchi, 2003).

1.5.2. Drought-induced limitations to CO_2 flux: stomatal conductance and mesophyll limitations to photosynthesis

Drought might influence plant growth directly through inhibiting cell growth, or more commonly through a reduction of stomatal conductance and metabolic activity, reducing photosynthesis and the availability of carbohydrates (Dreyer, 1997; Escós *et al.*, 2000). Under progressive water stress, a parallel decline in photosynthesis and stomatal conductance has been reported previously (Constable and Rawson, 1980; Medrano *et al.*, 1997). Nowadays it is generally assumed that drought-induced decreases in photosynthesis are due primarily to stomatal closure, which decreases CO_2 availability in the mesophyll, rather than to the direct effect on the capacity of the photosynthetic apparatus (Jones 1973; Genty *et al.*, 1987; Sharkey, 1990; Chaves, 1991; Cornic and Massacci, 1996; Cornic, 1994). However there is evidence that also non-stomatal limitations inhibit CO_2 metabolism (Escalona *et al.*, 1999; Flexas *et al.*, 2002; Lawlor and Cornic, 2002; Maroco *et al.*, 2002). The relative importance of diffusive (Cornic, 2000) and metabolic (Tezara *et al.*, 1999) factors to the overall control of photosynthesis even under mild environmental stress are still the matter of current debates (Flexas and Medrano, 2002).

As reported by Tezara and Lawlor (1995), stomatal control of photosynthetic rate becomes progressively less effective as water stress intensifies. To date, a widely accepted theory states that, under mild to moderate drought conditions diffusive resistances predominately limit photosynthesis, whereas under severe drought, when stomatal conductance (g_s) drops below 0.1-0.05 mol H_2O m^{-2} s^{-1}, metabolic limitations become dominant (Flexas *et al.*, 2004, 2006a). To study these patterns in various species under water stress may be useful to reveal how differential limitation of stomatal and non-stomatal components of photosynthesis is related to drought tolerance. In that sense, Luo (1991), Brodribb (1996) and more recently Flexas *et al.* (2002), proposed the use of g_s as an indicator to assess the inflexion point between stomatal and non-stomatal limitations to photosynthesis under drought. Consequently, Flexas and Medrano (2002) have analyzed the threshold values of g_s among various species –and thus at different severity of drought– when photosynthetic metabolic processes were impaired.

A number of detailed studies have defined the severity of water stress based on sequential response of photosynthesis processes (Stewart *et al.*, 1995, Flexas and Medrano, 2002). Keeping leaf water potential above critical values is considered an important sign of drought adaptation and resistance (Lüttge and Scarano, 2004), but such a response comes at a cost of reduced photosynthesis, and maintenance of the activity of Calvin Cycle enzymes and of the maximum rates of carboxylation ($V_{c,max}$) and electron transport (J_{max}) has generally been observed (Souza *et al.*, 2005). Decreases of leaf water potential resulting from more severely drying soils has often been linked to additional physiological responses (Hinckley *et al.*, 1978; Procházka *et al.*, 1998) such as increases in the production of proline and other amino acids (Hare and Cress, 1997). However, further decreases result in more general and dominant non-stomatal limitations to photosynthesis, especially under conditions where water stress is accompanied by very high temperature and irradiance. In such cases, severe water stress (i.e when g_s drops below 50 mmolH_2O m^{-2} s^{-1}) may affect mesophyll metabolism and reduce photosynthetic capacity as a consequence of decreased ribulose bisphosphate (RuBP) synthesis (Giménez *et al.*, 1992), as well as of decreases in Rubisco activity, in carboxylation efficiency (Martin and Ruiz-Torres, 1992) or both (Plaut and Federman, 1991; Faver *et al.*, 1996). Reductions in pigment content, as well as functional and/or structural modifications to the photosystem II (PSII) are likewise believed to be drought-induced responses (Baker, 1991; Manes *et al.*, 2001). The photochemistry of PSII has been extensively studied using chlorophyll fluorescence (Colom and Vazzana, 2003), which can provide information on the response of plant`s photosynthetic machinery to environmental stress. Fluorescence can also provide an insight into the amount of damage to the photosynthetic apparatus induced by environmental stress (Maxwell and Johnson, 2000).

The overall effect of water stress on vine leaf photosynthesis has also been extensively studied under different environmental conditions and with different grapevine varieties (Liu *et al.*, 1978; Kliewer *et al.*, 1983; Delgado *et al.*, 1995; Schultz, 1996). Reductions of net photosynthesis induced by water stress have usually been related to stomatal closure (g_s) (Chaves and Rodrigues, 1987; Rodrigues

et al., 1993; Delgado *et al.*, 1995). Even though non-stomatal effects, such as changes in mesophyll resistance and photochemical capacity, could also be present (Flexas *et al.*, 1998; Correia *et al.*, 1990).

1.5.3. Photosynthesis regulation after re-watering: CO_2 diffusion

1.5.3.1. Recovery of photosynthesis after water stress

Despite rapid rehydration of leaves following irrigation, stomata of many species commonly fail to reach their maximum opening after a period of water stress (Meidner and Mansfield, 1968). This 'stomatal after effect' may be associated with elevated levels of ABA (Sullivan and Eastin, 1974; Lovisolo *et al.*, 2008a) or CO_2 in leaves (Meidner, 1962). Furthermore, the deficiency of a substance which promotes stomatal opening (Livne and vaadia, 1972), or the slow turnover of carboxylative enzymes (Loveys and Kriedemann, 1973) may also affect the delayed response of stomata. Nowadays, there is great interest in studying the recovery period after a water stress event since, under field conditions, many crops experience fluctuating water availability, due to the alternation of rainfall and drought periods or as a result of the irrigation frequency. In the last few years many studies have assessed the recovery after a water stress event for a wide range of annual (Anyia and Herzog, 2004; Souza *et al.*, 2004) and perennial crops (Dichio *et al.*, 2006; Gallé *et al.*, 2007; Ortuño *et al.*, 2005; Torrecillas *et al.*, 1999), including also *Vitis* plants (Gómez-del-Campo *et al.*, 2007; Pou *et al.*, 2008; Santesteban *et al.*, 2009; Flexas *et al.*, 2006a, 2009).

Stomata are known to respond to stress alleviation by reopening and re-establishing the gaseous exchanges between leaf and air. Photosynthesis may therefore recover and even attain pre-stress rates if it was only dependent on CO_2 concentration present in the leaf. In general, plants subjected to severe water stress recover only 40-60% of the maximum photosynthesis rate during the day after re-watering, and recovery continues during the next days, but maximum photosynthesis rates are not always reached (Kirschbaum, 1987, 1988; Sofo *et al.*, 2004). Moreover, there are some indications, suggesting that previous water stress intensity is a crucial factor affecting both the velocity and the extent of recovery after re-watering. Thus,

the carbon balance of a plant during a period of water stress and recovery may depend as much on the velocity and degree of photosynthetic recovery as on the degree and velocity of photosynthesis decline during water depletion (Flexas *et al.*, 2006a).

Up until now there have been only a few studies on recovery from drought stress, which provid evidence for a dependency of the rate of recovery on the previously experienced stress (Kirschbaum, 1988; Souza *et al.*, 2004; Cai *et al.*, 2005; Miyashita *et al.*, 2005). This has been suggested for *Vitis vinifera* as well (Gómez-del-Campo *et al.*, 2007; Flexas *et al.*, 2009). In fact, when Flexas *et al.* (2002) subjected Tempranillo grapevines to moderate (i.e. maximum stomatal conductance among 0.1-0.15 mol H_2O m^{-2} s^{-1}) and severe water stress (i.e. maximum stomatal conductance of 0.05 mol H_2O m^{-2} s^{-1}), the moderately water stressed plants recovered completely overnight after re-watering, while plants of the same cultivar subjected to sever water stress recovered slowly during the next week, and did not reach the maximum photosynthesis rates reached before water stress.

In addition to the severity of stress, differences associated with the cultivar and the environmental conditions may also affect recovery. For instance, g_s and A_N recovered completely but slowly (3-5 days to complete recovery) after a moderate water stress in the cultivars Airén and Chardonnay. Grapevines with the rootstock Richter-110 recovered slowly (2 weeks for complete recovery) even after a mild water stress (Pou *et al.*, 2008; Flexas *et al.*, 2009). In contrast, after severe water stress several cultivars and rootstocks including Cabernet Sauvignon (Guan *et al.*, 2004) and *V. labruscana* (Liu *et al.*, 1978) recovered almost completely after 2 days of re-watering.

Furthermore, enhanced protection against oxidative stress to prevent irreversible damage seems to be also relevant for withstanding severe stresses and for a rapid and complete recovery (Reddy *et al.*, 2004). Consequently, apart from the understanding of how plants respond to increased drought in the field and how this effects productivity and growth, the capacity and rate of recovery from drought stress are equally important aspects in terms of growth and survival in a rapidly changing climate (‘global warming, extreme events’). As drought events are predicted to occur

more frequently in the coming decades, further investigations are needed to elucidate the mechanisms of recovery from drought in the field. This research could also improve predictions of ecosystem productivity or better irrigation systems.

1.5.3.2. Recovery of g_m after water stress

In line with the idea of adaptation or tolerance to drought, the recovery phase after relief of stress becomes another important part of the overall plant physiological response to a water stress period. Moreover, processes that limit recovery of photosynthesis seem to be key issues to understand what makes some plants withstand and survive drought better than others.

A way to asses photosynthetic limitation processes during water stress and recovery has been proposed by Grassi and Magnani (2005), who divided the total limitation in a stomatal, a mesophyll diffusion and a biochemical (i.e., carboxylation activity) component. From the results of their quantitative limitation analysis on ash and oak trees growing in the field they could explain that the high non-stomatal limitation during summer drought was mainly due to restrictions of CO_2 diffusion within the mesophyll. Other short-term water stress-experiments have also shown a decrease of g_m (Ennahli and Earl, 2005; Galmes *et al.*, 2007a; Flexas *et al.*, 2008), indicating a general trend of decline in g_m under water stress and a remaining high resistance during re-watering (due to a delayed or lacking restoration). Generally, it has been shown that g_m, Rubisco and ETR recovered quickly (1-3 days) after re-watering, while g_s remained lower, becoming the most limiting factor for photosynthesis recovery (Flexas *et al.*, 2009). However, the behaviour of g_m differed depending on the impact of additional environmental factors (Gallé *et al.*, 2009). These indoor and outdoor experiments with tobacco plants (*Nicotiana sylvestris* L.) revealed that g_m strongly declined with water stress in outdoor plants, but it recovered faster (1-2 days) after re-watering in spring then in summer (6-7 days) experiments. In indoor plants (growth chamber plants) g_m initially declined with water stress, but then recovered to control values already during the stress-acclimation period (before the start of re-watering). These differences were reflected in different velocities of

recovery of A_N after re-watering, being the slowest in outdoor summer plants and the fastest in indoor plants.

1.6. THE STUDY OF GRAPEVINE RESPONSES TO WATER STRESS AND RE-WATERING

As already explained above, drought is the main environmental factor limiting plant photosynthesis, growth and yield. Drought may even affect plants that are well adapted to arid conditions, such as grapevine (*Vitis vinifera*) (Chaves, 1991; Lawlor, 1995; Cornic and Massacci, 1996) among others, and has been shown to limit photosynthesis through stomatal closure (Hsiao, 1973; Sharkey, 1990; Chaves, 1991; Cornic, 1994), and also by metabolic impairment (Boyer, 1976; Lawlor, 1995; Tezara *et al.*, 1999; Flexas and Medrano, 2002; Lawlor and Cornic, 2002). In that sense, the main area of research of our group focuses on leaf transpiration and photosynthesis under water stress (Flexas, 2000; Esclaona, 2003; Bota, 2004). In most of the studies, stomatal conductance has been widely documented to be one of the most sensitive and earliest responses of leaf photosynthesis to water stress. However, as stated above, not only stomatal barriers affect the CO_2 availability for carboxylation, but also the presence of leaf mesophyll barriers limits the rate of photosynthesis. Furthermore, in addition to stomatal closure mediated by chemical signaling like ABA (Loveys and Kriedemann, 1974; Lovisolo *et al.*, 2002) or by variations in xylem pH (Rodrigues *et al.*, 2008), losses of hydraulic conductivity of xylem vessels have been suggested to induce a down-regulation of g_s in grapevines under water stress, but especially during recovery after water stress. Recent data on leaf hydraulic conductance (Schultz, 2003; Sack and Holbrook, 2006) point out that this parameter may serve as a key process to control transpiration fluxes in grapevines. Therefore, analyzing those parameters under drought and during re-watering would help to clarify the importance of diffusion and metabolic impairments of photosynthesis in grapevines. In addition, there is increasing evidence that both CO_2 and H_2O fluxes could be linked to a fine and rapid regulation of expression and/or activity of plasma membrane aquaporins. In grapevine leaves, all these aquaporin genes are down-

regulated during water stress and up-regulated after re-watering (Galmés *et al.*, 2007b).

Thus, understanding the physiological and molecular bases of grapevine responses to mild to moderate water deficits and their capacity of recovery from drought stress is fundamental to optimize deficit irrigation management and identify the most suitable varieties for those conditions. In this context, the main focus of the present work is to study the regulation of water and carbon flow during water stress and re-watering, and to assess the implications of this control on the water use efficiency in grapevine. Therefore the following objectives are proclaimed.

Chapter 2

OBJECTIVES AND OUTLINE

2.1 GENERAL OBJECTIVES

Various studies on the responses to water stress in grapevines, both at the agronomic and physiological level, have confirmed the importance and relevance of this subject. As a consequence, the regulation of plant growth, leaf carbon and water flow, photosynthesis and stomatal regulation under water deficit are widely documented.

However, under field conditions, plants often suffer repeated cycles of drought and re-watering rather than a single/prolonged drought event. Despite of this fact, our current knowledge on the responses to drought and to subsequent re-watering, as well as their physiological mechanisms is relatively scarce. After re-watering, the recovery of plant growth and photosynthesis may consist of complex processes. The rate of recovery may be affected by pre-drought intensity, its duration and the genotype analysed. Moreover, there may be a lag in recovery, indicating that the response of physiological variables after the relief of stress may be very different from the driving variables and those caused by the perturbation during stress, showing some hysteresis in the particular response.

In the current view on grapevine responses to water stress it is often assumed that ABA-mediated stomatal closure plays a major role in stomatal control, leading to decreased water loss and conservation of leaf water status, and thereby having some penalty on photosynthesis and thus on the optimization of WUE. The work of the present thesis has focused mainly on the physiological responses to prolonged water stress (i.e. acclimation conditions) and recovery following re-watering. The working hypothesis was that, apart from ABA-mediated stomatal regulation under those conditions complex regulations of plant hydraulics and CO_2 diffusion in the leaf mesophyll exert a major role during water stress and recovery after subsequent re-watering.

This general hypothesis is split into the following main objectives: (1) To understand how the particular regulation of transpiration and photosynthesis can affect the leaf water use efficiency; (2) to discern how grapevine hydraulics respond to episodic drought acclimation and re-watering; and (3) to study whether water and CO_2 diffusion share common diffusion pathways in the mesophyll, including the

transcellular pathway, in which the transport of both water and CO_2 is facilitated by aquaporins.

2.2. SPECIFIC OBJECTIVES

The specific objectives of this Thesis are:

1. To analyze how water relations, transpiration and stomatal conductance, are regulated under water stress and re-watering and how this regulation is mediated by plant and leaf hydraulics and how it affects leaf WUE.

2. To analyze the interdependence of the two CO_2 diffusion limitations (g_s and g_m) on photosynthesis under acclimation to water stress and recovery.

3. To characterise the interconnection between diffusion pathways of water and CO_2 through the mesophyll (g_m), and to assess whether the isotopic analysis is a viable method of monitoring changes in the lamina hydraulic conductivity (Kh_{lamina}).

4. To understand the relative contributions of leaf xylem and extra-xylem (the latter involving aquaporins) hydraulic resistances to the leaf water flow, and assess to what extent Kh_{leaf} is related to aquaporin expression.

2.3. OUTLINE OF THIS THESIS (PUBLICATIONS)

To achieve the previous objectives we conducted different experiments with potted plants grown outside on the experimental field site of the UIB Campus, and under greenhouse conditions in Adelaide University (Australia). The presented results have been published, submitted or have been prepared for publication as follows, referenced in order of appearance in this Thesis:

Objective 1.

1- **Pou A.**, Flexas J., Alsina M.M., Bota J., Carambula C., Herralde F., Galmés J., Lovisolo C., Jiménez M., Ribas-Carbó M., Rusjan D., Secchi F., Tomàs M., Zsófi Z., Medrano H. (2008). Adjustments of water-use efficiency by stomatal regulation during drought and recovery in the drought-adapted Vitis hybrid Richter-110 (*V. berlandieri x V. rupestris*). *Physiologia Plantarum* 2, 313-323.

2- **Pou A.**, Flexas J., Martorell S., Tomàs M., Medrano H., (2011). Water use efficiency during drought and recovery in grapevines: differential behaviour of three cultivars. *Acta Horticulturae* (submitted).

3- **Pou A.**, Medrano H., Tomàs M., Martorell S., Ribas-Carbó M., Flexas J. (2011). Anisohydric behaviour in grapevines results in better performance under moderate water stress and recovery than isohydric bahaviour. *Plant & Soil* (submitted).

Objective 2.

4- Flexas J., Baron M., Bota J., Ducruet J.M., Galle A., Galmés J., Jimenez M., **Pou A.**, Ribas-Carbo M., Sajnani C., Tomas M., Medrano H. (2009). Photosynthesis limitations during water stress acclimation and recovery in the drought-adapted Vitis hybrid Richter-110 (*V. berlandieri x V. rupestris*). *Journal of Experimental Botany* 8, 2361-2377.

Objective 3.

5- Ferrio J.P*, **Pou A.***, Flores-Sarasa I., Gessler A., Kodama N., Ribas-Carbó M., Flexas J., Medrano H. (2011). Oxygen isotope enrichment in leaf water reflects changes with drought progression in leaf hydraulic conductivity and mesophyll conductance for CO_2 in grapevine (*JPF and AP contributed equally to this work) *Plant physiology* (submitted).

Objective 4.

6- Galmés J., **Pou A.**, Alsina M.M., Tomàs M., Medrano H., Flexas J. (2007). Aquaporin expression in response to different water stress intensities, acclimation and recovery in Richter-110 (*Vitis sp.*): relationship with ecophysiological status. *Planta* 3, 671-681.

7- **Pou A.**, Medrano H., Flexas J., Tyerman S.D. (2011). Hydraulic conductivity dynamics in Chardonnay under water stress and re-watering and the relationship of aquaporin expression (in preparation).

In chapter 5 a general discussion is presented to clarify the main aspects covered in this work including the attempt to relate and summarize all the presented results of each specific experiment. Finally, chapter 6 presents a list of the main conclusions drawn from this Thesis covering the objectives set out in Chapter 2.

Chapter 3

MATERIAL AND METHODS

An overview of the used methods and techniques is presented in this section. However, each article in the results section presents a description of the methodology referred to each specific experiment.

3.1. Plant material and treatments

The experiments were carried out with different grapevine cultivars (*Vitis vinifera* L) original from different climates (Fig. 3.1): Grenache, from Mediterranean origin; Syrah, from mesic origin; and Chardonnay, from the humid zone of Burgundy (France), as well as with the hybrid Richter-110 (*Vitis berlandieri* × *Vitis rupestris*), that has the reputation of being a genotype strongly adapted to drought (Galet 1988).

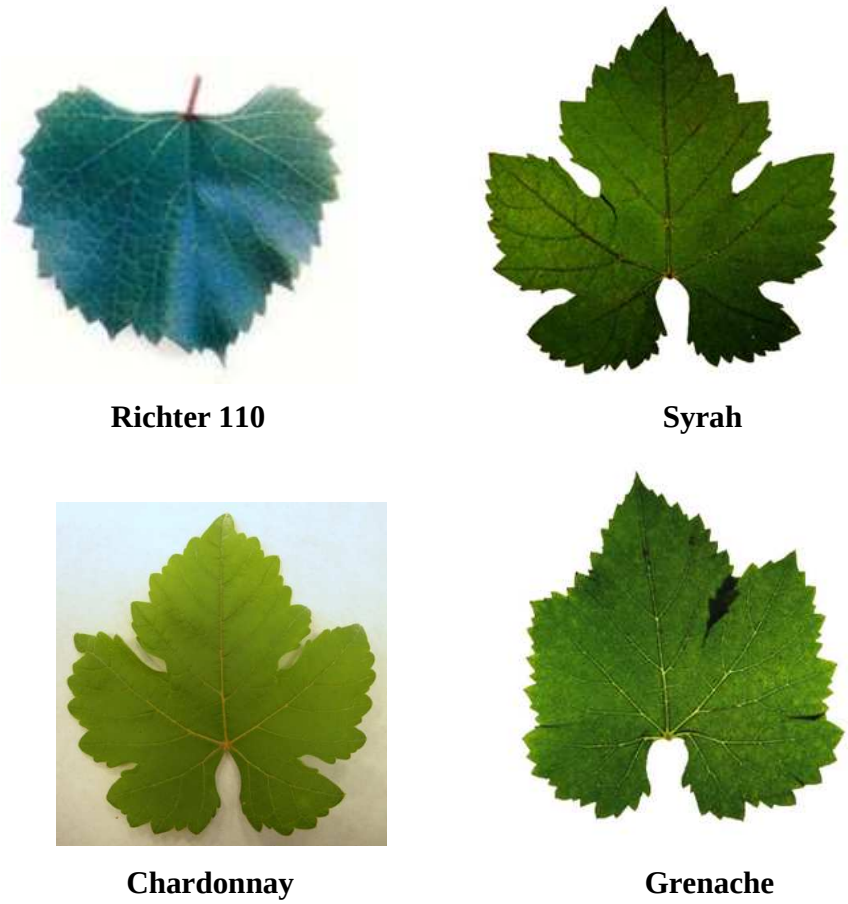

Figure 3.1. Grapevine cultivars used in this thesis.

Plants were grown outdoors (in the University of Balearic Islands; Mallorca, Spain) or in a temperature controlled greenhouse (in the University of Adelaide;

Adelaide, Australia) in 30, 15 or 7.5 L pots containing a mixture of soil and organic substrate. Plants were developed with two vertical shoots and all lateral shoots and fruit were removed during development. A superficial layer of perlite was added in each pot in order to avoid direct evaporation from soil.

Plants were watered to field capacity every one or two days and supplemented weekly with 50% Hoagland's solution (Hoagland and Arnon, 1950) until water stress treatments were started.

In Mallorca, the experiments were in summer time outdoors. Typical Mediterranean climate were observed with monthly mean maximum and minimum temperatures of 32 and 19ºC respectively and an average annual rainfall of about 575mm (data from the last 17 years) usually concentrated from September to April. Drought period usually lasts from May to September and its length is highly variable from year to year.

In Adelaide, plants were placed in a temperature controlled greenhouse were night/day temperatures were controlled at approximately 19/24ºC.

3.1.1. Water stress and recovery treatment

All genotypes were subjected to water withholding followed by re-watering, and one or two levels of water stress were established, defined by the leaf maximum daily stomatal conductance (g_s), as suggested by Flexas *et al.* (2002): moderate drought (g_s about 150 mmol H_2O m^{-2} s^{-1}) and severe drought (g_s about 50 mmol H_2O m^{-2} s^{-1}) (Fig. 3.2).

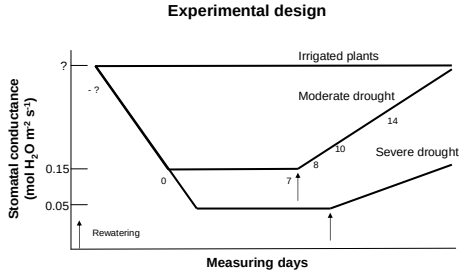

Figure 3.2. Experimental design, showing the expected time courses and the targeted levels of stomatal conductance for each treatment. Numbers indicate sampling days (only those for moderately stressed plants are showed for clarity) in Richter-110, but they are changing depending of the experiment. Arrows indicate the onset of re-watering.

Because of the diversity of the treatments used in this thesis, Chapter IV presents a detailed description of the used experimental design specifically performed in each experiment.

3.1.2. Xylem sap perfusion solution

Two nearby leaves from three different Chardonnay plants per treatment were harvested every 30-40 minutes under water between 10 AM and 13 PM, and immediately exposed to different filtered (0.45 μm), degassed solutions (15 mM KNO_3, 100 μM $HgCl_2$) by immersing the petiole inside 5 ml containers and placed under light for 3 hours allowing leaf transpiration. A superficial layer of parafilm was added in each pot wrapping petioles to avoid evaporation. Light was provided by lamps delivering approximately 500 μmol $m^{-2}s^{-1}$ at leaf level as measured by a Delta T AP4 porometer (Delta T Devices; Cambridge UK).

3.1.3. Leaf vein severing treatment

Before water withholding was applied, two leaves per plant were subjected to *in vivo* severing treatments. The treatment consisted in cutting the leaf lamina across the midrib, 1 cm from the petiole-lamina junction, and severing secondary and tertiary veins by using a scalpel while supporting the leaf on a cardboard, i.e. fully eliminating water transport through the xylem (Sack *et al.*, 2003a; 2004). Abaxial and adaxial cuts of the leaf were taped over with a 1.9mm transparent waterproof tape. Every treated leaf was paired with a nearby control leaf (uncut leaf) on the same branch matched approximately in same size and light exposure. Plants were protected from direct sunlight until wounds healed (approximately after 3-4 days), then returned to their position and left to re-acclimate for 1-2 days. Approximately one fourth of the severed leaves died before being included in the experiment. Only healthy leaves were selected, discarding those with clear symptoms of desiccation or providing negative assimilation rates.

3.2. Plant water relations

3.2.1. Leaf water potential

Pre-dawn (Ψ_{PD}) and midday leaf water potential (Ψ_{MD}) were determined with a Scholander pressure chamber (Soilmoisture Equipment Corp., Santa Barbara, CA, USA) (Fig. 3.3) as described by Scholander *et al.* (1965). Measurements were performed on young, fully expanded and apical leaves, usually on four to five replicates per treatment.

Figure 3.3. Scholander chamber.

Before excision, the water column in the xylem is under tension. When the water column is broken by excision of the organ (leaf), water is pulled rapidly from the xylem into the surrounding living cells by osmosis. Consequently, the cut surface appear dull and dry until the distribution of water between the living cells and the xylem conduits returns to its initial pre-excision-state. This can be detected visually in the cut surface by observing the returned water to the open ends of the xylem conduits, which becomes wet and shiny when the balance pressure is attained.

3.2.2. Leaf relative water content

Leaf relative water content (RWC) stated by Slatyer in 1967, express in percentage the water content at a given time and tissue as related to the water content at full turgor, was determined as follows by Slavik (1974) and Turner (1981):

$$RWC\ (\%) = [(\text{fresh weight} - \text{dry weight}) \div (\text{turgid weight} - \text{dry weight})] \times 100$$

Turgid weight was determined by placing samples in distilled water and maintaining them at 4°C in darkness until they reached a constant weight 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.

3.2.3. Leaf P-V Curves

Pressure-volume curves were carried out (Tyree and Richter 1981; Alsina *et al.*, 2007) on randomly selected mature leaves mid canopy and sun exposed. Leaves were excised, immediately sealed in plastic bags containing water, and transported to the laboratory. Petioles were re-cut under water, set in water filled beakers, and then the beaker was enclosed in a plastic bag to rehydrate the leaf for 24 h at 4ºC 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 leaf water potential (Ψ). Data for initial saturated weight, intermediate fresh weight corresponding to values for Ψ, and final dry weight were used to calculate the relative water content (RWC). The RWC and the corresponding Ψ were plotted as a "Type II" ($\Psi^{-1} \times$ RWC) transformation (Tyree and Richter 1981, 1982). Osmotic potential at full turgor ($\Psi_{\pi 100}$), osmotic potential at zero turgor ($\Psi_{\pi 0}$), water potential at turgor loss point (Ψ_{tlp}) and leaf bulk modulus of elasticity (ε) were obtained from the pressure-volume curves (PV) (Turner, 1988).

3.3. Water availability in the substrate and whole-plant water use

3.3.1. Amount of water available in the substrate

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.

All plants were weighted every evening, before irrigation. Daily water lost was obtained from the weight differences between two consecutive days.

3.3.2. Whole-plant water use efficiency

Whole plant biomass was determined at the end of the experiment in Richter-110. At this time, eight plants per treatment were harvested, and for each one of them, leaf, stems, main and fine roots were separated and dried in an oven at 60ºC 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 due to the effects of treatments on plant production during the experiment. Therefore, while we cannot provide absolute estimates of whole-plant water use efficiency, estimations of the relative change in whole-plant water use efficiency caused by the different water stress treatments with respect to controls can be attained.

Relative whole-plant water use efficiency 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:

Relative WUE = (Dry weight Treatment / Dry weight Control) / (Water losses Treatment / Water losses Control).

In remaining varieties (Syrah, Grenache and Chardonnay), whole plant biomass was determined at the beginning and at the end of the experiments. At those times, five plants per treatment and variety were harvested, and for each one of them, leaf, stems, main and fine roots were separated and dried in an oven at 60ºC to obtain dry weight. The sum of all fractions was the total plant dry weight.

At the end of the experiment, estimations of the total leaf area index (LAI; cm^2) were determined using images of all the leaves from each plant, processed in Matlab Softare (Matlab 7.0, MathWorks, Inc.).

3.4. Hydraulic conductance

3.4.1. Whole-plant

Whole plant hydraulic conductance (Kh_{plant}) was calculated by the Ohm's law analogy for the soil-plant-atmosphere continuum (Sperry and Pockman, 1993; Lovisolo *et al.*, 2002):

$$E = Kh_{plant} \times (\Psi_{MD} - \Psi_{soil}),$$

where E, Kh_{plant}, Ψ_{leaf}, and Ψ_{soil} represent transpiration rate, whole-plant hydraulic conductivity, leaf water potential and soil water potential, respectively. Ψ_{PD} was taken as a proxy for Ψ_{soil} and Ψ_{MD} was taken as $\Psi_{leaf.}$

Ψ_{PD} and Ψ_{MD} were determined with an Scholander pressure chamber (Soilmoisture Equipment Corp., Santa Barbara, CA, USA) (Scholander *et al.*, 1965) as an average of five different young, fully expanded and exposed apical leaves. E was measured at mid-morning with an open gas exchange system (Li-6400; Li-Cor, Inc., Lincoln, NE, USA) (see section 8.1.1. for details).

3.4.2 Roots and Shoots

Hydraulic conductivity was measured in roots and shoots in three replicates per treatment and sampling day by using Sperry *et al.* (1988) method. Briefly, roots and shoots (with no largest diameters than 0.3cm) were detached from plants and the cut surfaces were rapidly sealed with vaseline to prevent embolisms caused by air entering into the cut vessels. Prior measurements, the bark and the cambium from the cut internode segment were removed. The initial hydraulic conductance (K_i) of each root or twig segment was measured gravimetrically connecting the xylem segment to a low-pressure water source (approximately 0.005 MPa) and registering the weight of the flowing water every second on a balance, until a steady state was reached. Then existing embolisms were flushed out using a 0.15 MPa water flow, coming from a compressed air tank during at least 10 minutes, and the hydraulic conductance (K) was measured again. This process was repeated until k no longer increased after flushing, and this point was taken to be the maximum conductance (k_{max}).

Xylem hydraulic conductivity (*Kh*) was obtained from measured k and the length of the segment (*L*, m^2):

$$Kh = k \times L$$

3.4.3 Leaf and Lamina

Leaf hydraulic conductance on a surface area basis (Kh_{leaf}, mmol m^{-2} s^{-1} MPa^{-1}) was measured with the XYL'EM apparatus (Instrutec Company, H. Cochard and T. Ameglio, INRA-PIAF Laboratory, Clermont-Ferrand, France) or by using a Hydraulic Conductance Flow Meter (HCFM-XP; Dynamax Inc. Houston TX, USA) (Fig. 3.4), described in detail by Tyree *et al.* (1995).

Figure 3.4. Hydraulic Conductance Flow Meter (HCFM-XP).

The principle was to measure the water flow (F; mmol s^{-1}) entering the petiole of a cut leaf when exposed to positive pressure (P; MPa). Upon steady state (typically, after 0.5h, when flow reached a stable value), the hydraulic conductance of a leaf (Kh_{leaf}) was computed as:

$$Kh_{leaf} = \left(\frac{F}{P_{in} - P_{out}} \right)$$

The hydraulic conductance of a leaf (Kh_{leaf}) is defined as the water mass flow (F) per hydrostatic pressure drop ($P_{in} - P_{out}$) across the sample. Kh_{leaf} is typically normalized by leaf area (LA, m^2). The units are in mmol m^{-2} s^{-1} MPa^{-1}. During measurements, leaves were submerged in an inner compartment filled with tap water. The temperature in this compartment was adjusted to 20ºC with a regulated bath

(Ministat; Huber) or maintained between 20ºC and 25ºC by monitoring with a thermocouple and adding hot or cold water uniformly over the leaf blade. The leaf hydraulic conductance was then calculated standardized to the viscosity of water at 22 ºC. At the end of the experiment, the projected leaf area (LA; m^2) were determined using scanning images from each leaf processed in Matlab Softare (Matlab 7.0, MathWorks, Inc.) or by using a leaf area meter (AM-100 Area Meter, Analytical Development Co. Hoddesdon, UK).

XYL'EM apparatus is equipped with a pressure transducer and two flow meters (Liquiflow; Bronkhorst; 5 and 50g h^{-1} ranges) that measure the thermal mass flow rate. The XYL'EM was interfaced with a computer to log different data automatically. Detached sample leaves were excised under water allowed to reach a transpirational steady-state while attached to a flow meter through the petiole using compression fittings and connected to the XYL'EM apparatus. The flow entering each leaf was measured by using high pressure flow meter (HP), typically employed to determine the hydraulic conductance of highly resistive plant material such has leaf blades. Leaves were supplied with degassed and filtered (0.1µm) 15 mmol KCl solution from the XYL'EM 2-L captive air tank.

When using the HCFM-XP apparatus, quasi-steady state flow meter measurements were done on leaves, because flow can be approximately constant with constant applied pressure. The resistances are reported rather than conductance, because one of the common aims with quasi-steady state measurements is to measure the resistance of the whole shoot and its components, e.g., leaf blades, petioles, small stems, large stems etc. In our experiments, detached sample leaves were equally excised under water and allowed to reach a transpirational steady-state while attached to a flow meter through the petiole using compression fittings. 15mM KCl solution filtered at 0.1µm was forced into the leaves at a pressure (*P*; MPa) up to 0.4 MPa, while measuring the instantaneous flow (*F*; mmol s^{-1}) every 8 s. Corresponding hydraulic conductances (*Kh*; mmol s^{-1} MPa) were computed as $Kh = F/P$.

After measurement of Kh_{leaf}, the leaf lamina was excised with a razor blade and the hydraulic conductance of the petiole (*KP*) recorded. Petiole hydraulic

conductivity ($Kh_{petiole}$) was calculated as *KP* multiplied by petiole length (Sack et al. 2002).

Hydraulic conductance of the leaf lamina (Kh_{lamina}) was calculated as: $Kh_{lamina} = 1/(1/Kh_{leaf} - 1/Kh_{petiole})$ (Sack *et al.*, 2002). Kh_{lamina} was then normalized on a leaf area basis by dividing Klamina by leaf area (Sack *et al.*, 2002). The units are in mmol m^{-2} s^{-1} MPa^{-1}.

3.4.4. Percentage loss of hydraulic conductance (PLC)

The percentage loss of hydraulic conductance (PLC) was calculated for measuring the degree of xylem embolism of small segments (few cm) of roots and shoots as:

$$PCL = 100 \times \left(\frac{Kh_{\max} - Kh_i}{Kh_{\max}} \right)$$

where Kh_i and Kh_{max} represent the initial and maximum xylem hydraulic conductance, respectively (see 4.2 for details). The reference 'hydraulic' method was introduced by Sperry *et al.* in 1988 and then substantiated by Cochard *et al.* (2000). The method consists in estimating the initial hydraulic conductance (Kh_i) of a sample and then re-saturating this sample by successive flushings under pressure with degassed water to dissolves the air contained in the embolised xylem vessels. This perfusion leads to obtain the maximum conductance (Kh_{max}). The initial conductance/full saturated conductance ratio gives a quantitative value of embolism level.

3.5. Molecular analysis

3.5.1. RNA extraction and quantification

Leaves and roots from Richter-110 were harvested on sampling days at 10 a.m., and immediately frozen in liquid nitrogen and then stored at –80ºC until total RNA was extracted. Total nucleic acids were extracted with CTAB (hexadecyltrimethylammonium bromide) buffer (Doyle and Doyle, 1987) coupled with reusable tissue homogenization systems such as a mortar and pestle. Pestles,

mortars and all glassware used in the isolation of total RNA from plant material were autoclaved at 121° C for 20 min before use, whereas solutions used in RNA extractions were treated with 0.1% (v/v) diethyl pyrocarbonate (DEPC) and autoclaved to inactivate RNases. Rnase Zap (Ambion) was used to clean gel electrophoresis equipment.

For the CTAB-based procedure, 500µL of extraction buffer and 1µL of β-mercaptoethanol added just before used, were added to each sample (± 200 mg) previously powdered in liquid nitrogen. Tubes were then incubated at 60°C for 30 min. The total RNA was isolated by a conventional chloroform/isoamyl alcohol isolation procedure and precipitated with LiCl. Briefly, an equal volume of chloroform:isoamyl alcohol (24:1 v/v) was added and the tube was inverted vigorously for 10-15 min and centrifuged at 10,000g for 40 min at room temperature. The supernatant was transferred to new microcentrifuge tube and the RNA was precipitated with 0.7 vols of cold isopropanol. RNA was selectively pelleted after centrifugation at 10,000g for 5 min at 4°C. The pellet was washed 2 times with ethanol (76%), dried (for 20 min) and resuspended in 100 µL of TE buffer (10 mM Tris–HCl pH 8.0, 1mM EDTA). DEPC-water was added up to 500 µL. The mixture was then precipitated with 0.33 vols of 8M LiCl and incubated o/n at 4ºC. RNA was selectively pelleted after centrifugation at 13,000g for 30 min at 4°C. The pellet was 3 times washed with 100 µL ethanol (70%), dried and resuspended in 50 µL DEPC-water.

RNA purity and concentration were assessed by determining the spectrophotometric absorbance of the samples at λ=230, 260 and 280 nm and ratios of A260:A280 and A260:A230. RNA integrity was evaluated from the 28S and 18S rRNA bands on a 1% agarose gel after electrophoresis, staining with 0.5 µg ml^{-1} of ethidium bromide. Samples were loaded in sample buffer and electrophoresed at ~ 80 V.

In order to remove contaminant DNA from the RNA samples, the nucleic acid extract was treated with RNase-free DNaseI (Roche Diagnostic GmbH; Mannheim, Germany), according to the manufacturer's instructions.

Another way to complete total RNA extraction from powdered leaves (150–250 mg leaf tissue) was carried out in Chardonnay by using Spectrum Plant Total RNA extraction Kit (Sigma-aldrich) including a special step for genomic DNA degradation. In this case, quantification of total RNA in the processed samples was done by spectrophotometrical measurements at wavelengths of 230, 260 and 280 nm by using a NanoDrop Spectrophotometer ND-1000 (Bio-lab Ltd, Australia) and the integrity of the RNA was verified by visual inspection of rRNA banding, following 2% agarose gel electrophoresis.

3.5.2. RNA Analysis

Retrotranscription (RT): In Richter-110, cDNA was synthesized by SuperScript TM II RNase H–Reverse Transcriptase (Invitrogen) according to manufacturer instructions, using Oligo $(dT)_{15}$ as a primer. The RT reaction mix containing 3 μg of total RNA, 1μl of Oligo $(dT)_{15}$ and 1μl of 10 mM dNTP mix was carried to a total volume of 12 μl with sterile, distilled water and heated at 65º for 5 min. Once the mixture was kept in ice, 4 μL of 5X First Standard Buffer (Invitrogen) and 2 μl of 0.1 M DTT were added to each sample and then incubated at 42ºC for 2 min. Finally 1 μL of reverse transcriptase Superscript II (M-MLV RT, *moloney murine leukemia virus reverse transcriptase*) was added to the mixture. The reactions were incubated at 42ºC for 30 min, 50ºC for 40 min and 95ºC for 5 min.

In Chardonnay, single stranded cDNA was synthesized from 1 μg of total RNA by reverse transcription using iScript cDNA synthesis Kit (Bio-Rad, CA, USA) according to the manufacturer's instructions. The RT reaction-mix contained 4 μl of 5X iScript Reaction Mix, 1 μl of iScript reverse transcriptase (purified M-MLV RNase H^+), RNA template (1μg) and nuclease free water up to 20 μl. The reactions were incubated at 25 ºC for 5 min, 42ºC for 30 min, and 85ºC for 5 min.

Real-Time quantitative RT-PCR: In Richter-110 expression analysis of aquaporin isoforms was done by real-time PCR amplification by using LightCycler 2.0 system (Roche) and SYBR Green I master mix (Roche) as a fluorescent dye. Transcript abundance of AQPs PIP1.1, PIP1.2, PIP1.3, PIP2.1, PIP2.2, TIP1 and TIP2 was analyzed using specific primers (Baiges et al. 2001). To test the suitability

of these primers, the specificity and identity of the reverse transcription (RT)-PCR products was monitored after each PCR by a melting curve analysis of the reaction products, which can distinguish the gene-specific PCR products from the non-specific PCR products. The temperature of PCR products was elevated from 55 to 99ºC at a rate of 1ºC/5 s, and the resulting data were analyzed by using the LightCycler software. Only one single band with a characteristic melting point was observed for each sample, indicating that the RT-PCR reaction produced a product specific to the primers used for the reaction. To further confirm that the primer sets produced only the target genes, the RT-PCR products were separated by electrophoresis and visualized in a 1% agarose gel.

Each 8 μL reaction contained 2 μL of diluted cDNA (1/10), 0.8 μL of each primer, 0.75 μL SYBR Green I master mix (Roche) and 0.6 μL $MgCl_2$. The cDNA was amplified by 35 cycles of denaturation (94ºC for 36 s), annealing (56–60ºC depending on each primer for 90 s), and elongation (72ºC for 90 s) followed by a final extension step of 72ºC for 6 min.

Values for the threshold cycle (Ct) were determined in duplicate using the LightCycler software. Relative gene expression numbers were calculated as a percentage of control plants, using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) with malic enzyme gene as a reference. To further detect changes in transcript abundance, relative gene expression values were also normalized using two other reference genes: ubiquitin and elongation factor 1. Negative controls without cDNA were used in all PCR reactions.

In Chardonnay, sequences of used primers for the amplifications were designed by Vandeleur et al. (2009) based on published sequences of aquaporins found in grapevine. Real-time polymerase chain reaction (PCR) was performed in a 20 μL mixture containing 1 μL of diluted cDNA (1/10), 10 μL of SYBR Green Reaction-Mix (Bio-Rad), and 0.6 μM of each primer with an iCycler iQ system (Bio-Rad). The PCR cycle profile was as follows: one cycle of 30 s at 95ºC followed by 40 cycles of 20 s at 95ºC, 20 s at 59ºC, and 20 s at 72ºC. Amplification data were collected during the extension step (72ºC). Melt curve analyses were made by elevating the temperature from 57ºC to 95ºC at a rate of 0.5ºC s^{-1}. Only a single band

with a characteristic melting point was observed for each sample, indicating that the product was specific to the primers. Products were routinely checked by 2% (w/v) agarose gel electrophoresis. The fluorescence threshold value (Ct) was calculated using the iCycle iQ system software (Bio-Rad). Overall, a mean Ct value was calculated from three independent biological replicates, each with three PCR replicates and differential fold expression was calculated using the method described by Muller et al. (2002), provided by the software package Q-Gene which transform means of raw data CT values and the related standard errors (SEs) into means of normalized expression levels and their respective SEs. In these quantitative PCR experiments, standard curves using known amounts of cDNA were used to quantify the starting amounts of cDNA for each gene. The final value of relative gene expression was the ratio of the starting quantity of the gene of interest to the starting quantity of VvUBQ, the reference gene, to account for differences in the original RNA concentration and the efficiency of cDNA transcription.

3.6. Estimation of Symplastic/Apoplastic flow ratio

Relative changes in symplastic/apoplastic water flux were estimated in leaves from irrigated and water stressed Chardonnay plants using two different fluorescent tracer dyes, trisodium 3-hydroxy-5,8,10- pyrenetrisulphonate (HPTS) frequently used as an apoplastic marker (Peterson *et al.*, 1981; Hanson, Sucoff and Markhart, 1985; Moon *et al.*, 1986; Wright *et al.*, 1996; Kamaluddin and Zwiazek, 2001) and 8-acetoxypyrene-l,3,6, trisulphonic acid, trisodium salt (HPTS-acetate) retained within the symplast (Wright *et al.*, 1996). Two detached leaves (from the same branch and matched approximately in the same size and position) were cut under water and immediately placed with their petioles ends in vials containing 3 mL of 1 of the 2 dye solutions chosen to trace the transpiration for a previously tested time of 2.5-3 h. Both fluorocroms were used as a solution of 5mg ml^{-1} dissolved in 15mM KCl. To explode either apoplastic or symplistic pathways from the leaf surface inwards, HPTS and Acetate-HPTS concentrations were measured respectively, with a Molecular Imager-ProPLus (Bio-Rad) fluorescence spectrophotmetry (fluorimeter) using an excitation wavelength of 405 nm and an emission wavelength of 515 nm. The

recorded counts were normalized to the perfusion time and leaf area as: counts m^{-2} h^{-1}.

The proportion of the symplastic/apoplastic flow was estimated by dividing the Acetate-HPTS concentration in the symplastic and transcellular pathways in one leaf by the HPTS concentration in the xylem sap of the adjacent leaf. Between 2 and 5 leaves per treatment (irrigation and water stress) and dye solution, were used to complete the experiment, and from each leaf two images were took, one from the upper-side and one from under-side

3.7. Chlorophyll fluorescence measurements

Chlorophyll *a* fluorescence was measured with the integrated fluorescence chamber head (Li-6400-40; Li-Cor Inc., Nebraska, USA) of the open gas exchange system (Licor-6400).

3.7.1. Principles

Fluorescence occurs when a compound, in this case chlorophyll, absorbs a specific wavelength of light and subsequently emits light at another wavelength. Light energy that is absorbed by chlorophyll in a leaf can undergo three fates: a) driving photosynthesis (photochemistry), b) dissipation of excess energy to heat and c) emission of chlorophyll fluorescence (Fig. 3.5).

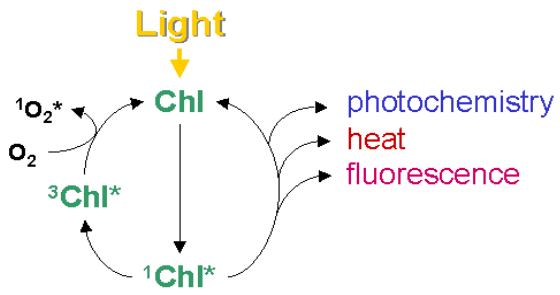

Figure 3.5. Possible fates of excited chlorophyll.

As these three processes are in competition, where a decrease in one will result in an increase in the yield of the other two, information about changes in the

efficiency of photochemistry and heat dissipation can be derived by measuring chlorophyll fluorescence (Maxwell and Johnson, 2000). This is easy to measure, due to the fact that the spectrum of fluorescence differs from that of absorbed light, with a peak of emission of longer wavelength (>710 nm) than that of absorption (<680 nm). Therefore, fluorescence yield can be quantified by exposing a leaf to light of defined wavelength and measuring the amount of light reemitted at longer wavelength. An important modification for the application of chlorophyll fluorescence has been undertaken by using a modulated measuring system, the so-called quenching analysis of modulated fluorescence by the saturation pulse method, in which the light source for fluorescence measurement is switched on and off at high frequency and the detector is tuned to detect only the emitted fluorescence from this signal even in the presence of a strong background ambient light.

Observations of chlorophyll fluorescence were first reported by Kautsky *et al.* (1960). They acclimated plant cells to darkness for several minutes, clearing all the excited electrons from the electron transport chain and emptying the acceptor pools. Then they exposed the cells to a brief pulse of high intensity photosynthetically active light and monitored the rise and fall of the ensuing fluorescence emission with a sensitive photometer. In a "Kautsky curve" (Fig. 3.6), emissions rise to a point, F_o, which represents fluorescence where all reaction centers are open and the fraction of open PSII reaction centers (*qP*) is maximal and is produced after switching on the measuring light. Then, there is a sharp rise to a point of maximum fluorescence (F_m) after applying a saturating flash (> 4000 μmol photons m^{-2} s^{-1}). This measurement allows the determination of the maximum quantum efficiency of photosystem II (PSII) primary photochemistry, given as *Fv/Fm*.

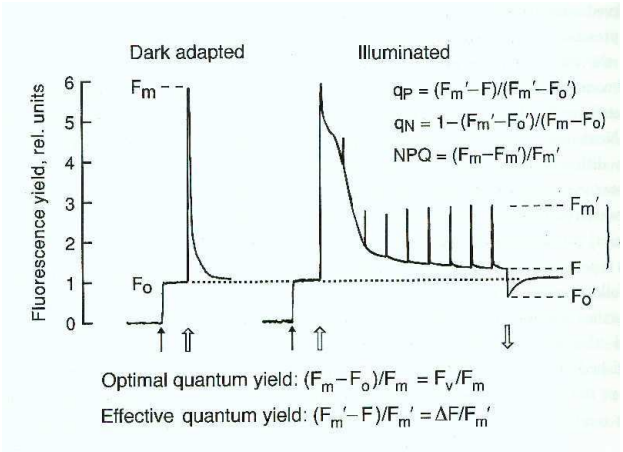

Figure 3.6. Measurement of chlorophyll fluorescence by the saturation pulse method (from Schreiber *et al.* 1998). *Fo* is the fluorescence emanating from the light harvesting complex. Fm is maximum fluorescence. *Fv*, variable fluorescence = *Fm – Fo*. The thin arrows indicate the switching on of modulated light. The thick arrows indicate switching on and switching off actinic light.

Upon subsequent application of constant illumination, a transient rise in fluorescent yield is observed. Thereafter, upon the onset of photochemical and heat dissipation processes, the fluorescence efficiency is quenched and reaches a steady state value (F_s). This effect was first observed by Kautsky and Hisrch in 1931.

The application of a saturating flash in the presence of actinic light allows the determination of the maximum fluorescence in the light-adapted state (F_m'). A decrease in F_m' as compared to Fm indicates the presence of non-photochemical quenching (NPQ).

3.7.2. The fluorescence parameters

$\Delta F/F_m'$ (ϕ_{PSII})

One of the most useful fluorescence parameter is the so-called Genty parameter, wich it is determined by measuring steady-state fluorescence in the light (here PFD 1500 μmol m^{-2} s^{-1}) (F_s) and maximum fluorescence during a light-saturating pulse of ca. 8000 μmol m^{-2} s^{-1} (F_m') following the procedures of Genty *et al.* (1989):

$$\phi_{PSII} = (F_m'-F_s)/F_m'$$

Since $\Delta F/F_m'$ is theoretically proportional to the operating quantum efficiency of PSII photochemistry, it is a measure of the proportion of the light absorbed by PSII that is used in photochemistry. The PSII quantum efficiency is affected by the level of electron acceptors, usually NADP+, available at the acceptor side of PSI. Consequently, ϕ_{PSII} decreases in situations with limiting consumption of NADPH, for example, at low internal CO2 concentration.

ETR (J_{flu})

As Φ_{PSII} represents the number of electrons transferred per photon absorbed by PSII, the electron transport rate (J_{flu}) can be calculated as:

$$J_{flu}\ (\mu mol\ e^-\ m^{-2}\ s^{-1}) = \phi_{PSII} \cdot PPFD \cdot \alpha \cdot \beta$$

where PPFD is the photosynthetically active photon flux density (in μmol quanta m^{-2} s^{-1}), α is leaf absorptance and β reflects the partitioning of absorbed quanta between photosystems II and I. The product $\alpha \cdot \beta$ was determined for each treatment, following Valentini *et al.* (1995), from the slope of the relationship between ϕ_{PSII} and ϕ_{CO2} (i.e. the quantum efficiency of gross CO_2 fixation) obtained by varying either light intensity under non-photorespiratory conditions in an atmosphere containing <1% O_2. For α and β general assumptions can be made, as for a mature non-succulent leaf α is usually around 0.84 and β is 0.5 under steady state photosynthesis (Laisk and Loreto 1996).

NPQ

Excess of excitation energy can be de-excited by thermal dissipation processes. Non-photochemical quenching of chlorophyll fluorescence is an indicative of the level of non-radioactive energy dissipation in the light-harvesting antenna of PSII. The importance of the non-photochemical quenching results from the fact that it shows that the level of excitation energy in the PSII antenna can be regulated. This is thought to prevent over-reduction of the electron transfer chain and, therefore, provides protection from photodamage.

The most used way to quantify non-photochemical quenching is by measuring the fluorescence parameter NPQ, which is calculated as: (NPQ = (F_m-F_m')/F_m'). Non-photochemical quenching is induced under conditions where the photosynthetic apparatus cannot use the total absorbed light energy for photochemistry. Stress conditions such as high light intensity and low internal CO_2 concentration markedly promote non-photochemical quenching. Therefore, the amount of non-photochemical quenching is an indicator of the stress severity.

F_v/F_m

The maximum quantum efficiency of PSII (F_v/F_m) is determined in a dark-adapted leaf as F_v/F_m = (F_m-F_o)/F_m, where F_o is the fluorescence signal after switching on the measuring light and F_m is the maximal fluorescence (see above) after applying a saturating flash (> 4000 μmol photons m^{-2} s^{-1}). Under non-stressful conditions F_v/F_m ranges around 0.8, with some species-specific variations. In principle, a decrease in F_v/F_m results from an increase in thermal dissipation (non-photochemical quenching) at the expense of photochemical activity. Thus, a lowered F_v/F_m is good indicator for sustained impaired photochemistry ("photoinhibition"), when measure after an appropriate period (usually 10-30 min) of dark adaptation.

3.8. Leaf gas exchange measurements

3.8.1. Instantaneous gas exchange measurements

3.8.1.1. System overview

Gas-exchange measurements were performed between noon and 1 P.M., using an open infrared gas-exchange system (Li-6400; Li-Cor, Inc., Lincoln, NE, USA) (Fig. 3.7).

Figure 3.7. The LI-6400 Portable Photosynthesis System.

As depicted in Figure 3.8, the major parts of this system consist of (1) an air supply unit, (2) a precision flow-meter, (3) a transparent enclosure or leaf chamber and (4) IRGA(s).

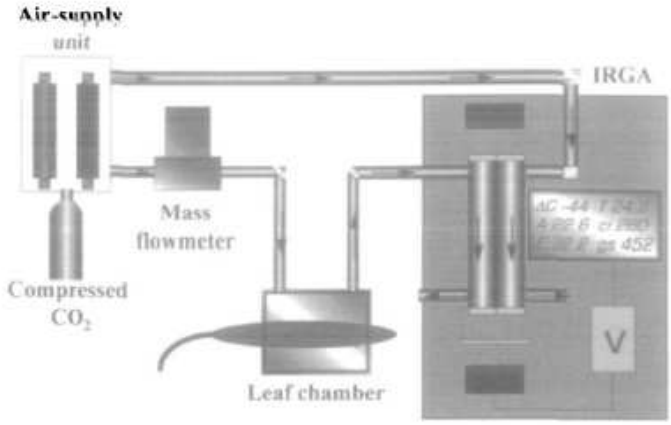

Figure 3.8. Schematic layout of an open gas exchange system. Air from the outside is pumped into the air-supply unit and CO2 and water vapour are removed by absorbents and then replaced at a controlled level. Before entering the leaf chamber, the air stream is split into a reference and an analyse stream, passing via a thermal mass flowmeter. Finally, both streams pass a dual beam IRGA and the air is released into the atmosphere again. Comparing reference and analysis beam provides information about changes in CO2 and water vapour, hence leaf gas exchange rates can be determined (A_N, g_s, etc.). From Gallé and Flexas, 2010.

The two gas analyzers in the sensor heat allow obtaining the absolute CO_2 and H_2O values as well from the reference as for the sample (intact attached leaves). The IRGA system is based in the measure of the CO_2 concentration decrease in the air stream which flows through a cuvette with photosynthetic tissue. For any given decrease, air flow rate and leaf surface, the CO_2 assimilation rate (A_N) can be calculated and expressed in μmol CO_2 m^{-2} s^{-1}. In an analogous way, measuring the

increase in water content of the same air stream, the transpiration rate (*E*) can be determined, and expressed in mmol H_2O m^{-2} s^{-1}.

$$Photo = \frac{Flow \times \Delta CO_2}{Area} \qquad Trans = \frac{Flow \times \Delta H_2O}{Area}$$

The basic principle is based in the fact that both CO_2 and vapour H_2O are strong absorbents of infrared radiation at different wavelengths. A thermocouple inside the leaf chamber allows the measurement of the temperature of the attached leaf and another thermocouple within the cuvette determines the air temperature. This system has the possibility to fix all desired parameters, and so the user can control the environmental conditions in the leaf chamber, such as light, temperature, humidity and CO_2 concentration. The humidity control in Li-6400 is done by a combination of two mechanisms, manually by adjusting the incoming air in the leaf chamber which is routed through desiccant, and controlling the flow rate of air through the chamber. In order to provide a certain concentration of CO_2 this can be firstly removed from the air by using soda lime ($C_a(OH)_2$ and NaOH granulate) and Drierite ($CaSO_4$), and then disposable compressed CO_2 gas cylinders can be used to provide a controlled rate of CO_2 added back to the CO_2-free air. Thus, 6400-01 CO_2 Mixer allows the control of the CO_2 concentration inside the leaf chamber, which is the basis of the photosynthetic CO_2 response curves (Escalona *et al.*, 1999; Gulías *et al.*, 2002) (see above section 8.2.1).

3.8.1.2. Day-time measurements

According Farquhar *et al.* (1980) and Farquhar and Sharkey (1982), from these measurements, other photosynthetic parameters are derived, i.e. stomatal conductance for water vapour (g_s), expressed in mol H_2O m^{-2} s^{-1}, and internal CO_2 conectration (C_i), expressed in μmol CO_2 mol air^{-1}. From *E*, g_s can be deducted, implementing leaf temperature and air pressure for the estimation of leaf water vapour ("total conductance"), and subtracting the boundary layer conductance. Inter-conversion of g_s for water vapour to g_s for CO_2 can be easily done by dividing with

the factor 1.6, which is the ratio of H_2O/CO_2 diffusivities in air. The internal or sub-stomatal CO_2 concentration (C_i) can be derived from simultaneous measurements of A_N and g_s, according to Fick`s first law of diffusion:

$$C_i = C_a - \left(\frac{A_N}{g_s} \right)$$

where C_a is the CO_2 concentration around the leaf. However, C_i values can be overestimated due to two main problems described particularly under water stress: an increasing importance of the cuticular conductance to vapour pressure as stomata get closed (Boyer *et al.*, 1997) and heterogeneous ('patchy') stomata closure (Laisk, 1983; Buckley *et al.*, 1997). We calculated leaf cuticular conductance and tested for patchy stomatal closure as detailed below.

In our experiments, measurements were performed on young, fully expanded apical leaves at 1500 μmol photon m^{-2} s^{-1} (provided by the light source of the Li-6400 with 10% blue light) to ensure light saturation. The CO_2 concentration in the Li-6400 leaf chamber (C_a) was set to 400 μmol CO_2 mol^{-1} air and the relative humidity of the incoming air ranged between 40 and 60%. Temperature and vapour pressure deficit were not controlled. From instantaneous measurements, net CO_2 assimilation (A_N), stomatal conductance (g_s) and the sub-stomatal CO_2 concentration (C_i) were recorded.

3.8.1.3. Dark respiration

Respiration in the night (R_n) or dark respiration was measured as the net CO_2-exchange rate at 25ºC and at a C_a of 400 μmol CO_2 mol air^{-1} after maintaining plants for 3 hours in darkness with the Li-6400.

3.8.2. Other gas exchange measurements

3.8.2.1. A_N-C_i curves

The CO_2 response curves of photosynthesis (A_N-C_i) were started at a cuvette CO_2 concentration (C_a) of 400 μmol CO_2 mol air^{-1}. After inducing photosynthesis under these conditions and once steady-state was reached (i.e. A_N showed no systematic decreases or increases higher than ± 2%), photosynthesis response curves

were performed varying CO_2 concentration around leaves (C_a) stepwise in the range of 50 to 400 μmol CO_2 mol air^{-1}. In some case C_a was increased stepwise from 400 to 1500 μmol CO_2 mol air^{-1}.

3.8.2.2. Respiration in the light and apparent CO_2 photocompensation point

Respiration in the light or 'day' respiration (R_d) and the apparent CO_2 photocompensation point (C_i*) were determined according to the method of Laisk (1977) as described in Von Caemmerer (2000). A_N-C_i curves (see above) were measured at three different light intensities (i.e. 50, 200 and 500 μmol m^{-2} s^{-1}), at six different CO_2 levels ranging from 400 to 50 μmol CO_2 mol^{-1} air. The intersection point of the three A_N-C_i curves was used to determine C_i* (x-axis) and R_d (y-axis). C_i* was used as a proxy for the chloroplastic CO_2 photocompensation point (Γ*) according to Warren and Dreyer (2006). Photosynthetic compensation point (Γ*) is the CO_2 concentration of the photosynthetic compensation point, at which the photorespiratory CO_2 efflux equals photosynthetic CO_2 uptake. According to Galmés *et al*. (2006), only C_i* values for irrigated plants were considered, which averaged 42.0 ± 0.9 μmol CO_2 mol^{-1} air at a leaf temperature of 30ºC, i.e. a Γ* of 43.1 μmol CO_2 mol^{-1} ($\Gamma^* = C_i^* + R_d/g_m$), corresponding to a Rubisco specificity factor of 90. Considering published Γ* response functions to temperature for several species (reviewed by Warren and Dreyer, 2006), this would correspond to a Rubisco specificity factor of about 100 at 25ºC, i.e., totally coincident with the actually determined value for *Vitis* (Bota *et al*., 2002).

3.8.3. Evaluation of potential errors and correction factors

3.8.3.1. Leaks

When conducting gas exchange measurements a number of precautions have to be taken to minimize or eliminate possible errors. Firstly, a common problem during gas-exchange measurements is a leakage of CO_2 through the foam gaskets of the leaf chamber, which has its greatest effects during a run of an A_N-C_i curve when CO_2 differences between inside and outside the chamber become large (Flexas *et al.*,

2007; Rodeghiero *et al.*, 2007). Leakage of CO_2 into and out the leaf cuvette was determined with photosynthetically inactive leaves (obtained by heating the leaves until no variable chlorophyll fluorescence was observed) enclosed in the leaf chamber (Flexas *et al.*, 2007) and recalculation of the in vivo data sets.

3.8.3.2. Cuticular conductance

Measurements of g_s with an IRGA system are the sum of stomatal and cuticular conductance (g_c), the latter being usually low under standard conditions (relative high g_s) and therefore negligible. However, under stressful conditions such as drought g_s may become very low and thus g_c contributes to a larger extent to the measured g_s. To account for this possible overestimation of g_s due to g_c, the following relatively easy-to-conduct methods can be applied. First, in hypostomatal plants the lower side of the leaf, which commonly presents most or all stomata, can be sealed with Teflon or other water-impermeable lubricant and then the conductance of the sealed leaf is measured. The recorded conductance represents the upper-side leaf conductance (without stomata), in particular the g_c (Boyer *et al.*, 1997). Another way to determine g_c is to measure g_s continuously on a detached leaf until the decline of g_s flattens out and values become stable. This end point represents the remaining conductance (g_c) after stomata have been completely closed.

Vitis are hypostomatous species, and hence cuticular conductance was estimated in Richter-110 in three different ways: (1) measuring it with the IRGA on leaves with the abaxial surface covered with silicone grease and a polyethylene filter to prevent stomatal gas exchange (Boyer *et al*., 1997); (2) measuring gas exchange of leaves by night (Kerstiens, 1996), although there is now evidence that this could overestimate cuticular conductance due to incomplete stomatal closure at night (Kerstiens, 2006); and (3) by determining cuticular transpiration after turgor loss during the measurements of pressure-volume curves (Burghardt and Riederer, 2003). The three methods yielded similar values, of 0.007 ± 0.001, 0.006 ± 0.001 and 0.008 ± 0.001 mol H_2O m^{-2} s^{-1}, respectively, which were similar to those found by Boyer *et al*. (1997) for another *Vitis* species but, contrary to Boyer *et al.* (1997) without any significant difference between treatments. Therefore, a value of 0.007 mol H_2O m^{-2} s^{-}

[1] was used to re-calculate g_s and C_i as described previously (Boyer *et al*., 1997; Flexas *et al*., 2002).

3.8.3.3. Heterogeneous stomatal closure (Patchines)

Heterogeneous stomatal closure or patchy stomatal response is also a common phenomenon in leaves. However, it can be neglected when transpiration and g_s are high (non-stressed conditions) or when gas exchange measurements are carried out on a relatively large leaf area, covering a high number of stomata. In particular under drought stress, when stomata close und hence g_s decreases, the stomatal patchiness may impair the correct determination of *Ci*, as demonstrated in grapevines under certain conditions (Downton *et al.*, 1988a,b) but not in others (Flexas *et al.*, 2002, 2009). In order to detect symptoms of stomatal patchiness, two different checks were performed in Richter-110. In the first, chlorophyll fluorescence was measured in different areas of the leaf blade (Flexas *et al.*, 2002). Five to six patches were measured over each leaf, and the differences in fluorescence parameters were usually lower than 10%, except over leaf veins. In the second, the initial slope of several phototosynthetic response curves to intercellular CO_2 concentration (A_N-C_i curves) on the same leaf was determined under conditions of increasing vapor pressure deficit (VPD) and decreasing stomatal conductance, following Grassi and Magnani (2005). When g_s was above 0.06 mol CO_2 m^{-2} s^{-1} all three curves looked identical, which was taken as evidence for the absence of patchiness. For g_s values ranging between 0.01 and 0.06 mol CO_2 m^{-2} s^{-1} some deviation was observed, but still very minor to consider it causing a significant bias in the value of C_i. Only when g_s dropped below 0.01 mol CO_2 m^{-2} s^{-1} there was clear evidence of impairment of the calculation of C_i. Since these low g_s values were never averaged by any treatment during the experiment, no correction to account for patchiness was done in the calculation of C_i.

3.8.4. Calculations from chlorophyll fluorescence and gas exchange measurements

3.8.4.1. *Cc* and g_m

Combining gas exchange and chlorophyll fluorescence analysis has become very powerful tool to investigate the relationship between CO_2 fixation, light use efficiency and photoinhibition. As CO_2 fixation and electron transport can be measured simultaneously, it is possible to estimate the extent of photorespiration in vivo. The photorespiration rate (P_r) is calculated according to Valentini *et al*. (1995). In their model, they assumed that all the reducing power generated by the electron transport chain is used for photosynthesis and photorespiration, and that chlorophyll fluorescence gives a reliable estimate of the quantum yield of electron transport. Thus, P_r can be solved from data of A_N, R_L and J_{flu}, and from the known stochiometries of electron use in photorespiration, as follows (Valentini *et al*., 1995):

$$P_r = 1/12\ [J_{flu} - 4\ (A_N + R_L)]$$

Moreover, leaf intrinsic diffusion components (g_m, C_c) can be derived from these simultaneous measurements according to Fick`s law of diffusion ($A_N = g_m\ (C_i - C_c)$) (Flexas *et al.*, 2008; Niinemets *et al.*, 2009). g_m can be derived from fluorescence and gas exchange data, using the so called constant J or variable J method when photosynthesis is limited by the regeneration of RuBP or by Rubisco (Bernacchi *et al.*, 2002; Epron *et al.*, 1995; Harley *et al.*, 1992). In this Thesis, the variable J method according to Harley *et al*. (1992) was applied and g_m calculated as:

$$g_m = A_N / (C_i - (\Gamma^* \cdot (J_{flu} + 8 \cdot (A_N + R_d)) / (J_{flu} - 4 \cdot (A_N + R_d))))$$

were A_N and C_i were obtained from gas exchange measurements at saturating light. C_i^* was used as a proxy for Γ^* following (Warren and Dreyer 2006). Γ^* and R_d were determined according to the 'Laisk-method' (Laisk, 1977).

Determination of g_m can then be used to calculate C_c. Using C_c instead of C_i has been shown to be a powerful tool for the analysis of A_N-C_i curves (the response of

A_N to varying C_a) and thus, leading to more reliable results of photosynthetic activity *in vivo*. Thus, in this work, calculated values of g_m were used to convert A_N-C_i curves into A_N-C_c curves according to the following equation:

$$C_c = C_i - (A_N / g_m)$$

3.8.4.2. Farquhar model

A_N-C_i curves were converted to A_N-C_c curves as described above. Consequently, the model of Farquhar *et al.* (1980) has been adapted to account for a finite mesophyll conductance.

The model of Farquhar *et al.* (1980) has provided a tried and tested means to partition quantitatively biochemical and stomatal limitations on photosynthesis, from the response of CO_2 uptake to intercellular mole fraction of CO_2. Simultaneous measurements of chlorophyll fluorescence now extends this analysis, providing a means to determine the partitioning of energy between photosynthesis and photorespiration, and therefore to convenrt C_i into C_c, as explained above. From A_N-C_c curves, the maximum carboxylation activity of Rubisco ($V_{c,max}$) and the maximum capacity for electron transport rate driving regeneration of RuBP (J_{max}) were calculated using the temperature dependence of kinetic parameters of Rubisco described on a C_c basis by Bernacchi *et al.* (2002), whereby net assimilation rate is given as:

$$A_N = \min \{A_c, A_q\} - R_d$$

With:

$$A_c = V_{c,max} \frac{C_c - \Gamma*}{C_c + K_c[1 + (o_i / K_o)]}$$

$$A_q = \frac{J(C_c - \Gamma*)}{4(C_c + 2\Gamma*)}$$

where A_c and A_q represent photosynthesis limited by carboxylation and RuBP regeneration, respectively, K_c and K_o are the Rubisco Michaelis-Menten constants for

carboxylation and oxygenation, respectively, and o_i is the leaf internal oxygen concentration (assumed equal to the external).

3.8.4.3. Quantitative photosynthetic limitation analysis

By using simultaneous gas exchange and fluorescence measurements, a quantitative limitation analysis of photosynthesis can be conducted (Flexas *et al.*, 2009; Grassi and Magnani, 2005; Wilson *et al.*, 2000). This approach, which requires the measurement of A_N, g_s, g_m and $V_{c,max}$, makes it possible to divided photosynthesis limitations into components related to: stomatal conductance (g_s, S_L), mesophyll conductance (g_m, MC_L) and leaf biochemical characteristics ($V_{c,max}$ or J_{max}, B_L), assuming that a reference maximum assimilation rate can be defined as a standard. Such limitation analysis can provide a powerful tool for studying the photosynthetic limitations under different stress situations, as well as during the photosynthetic recovery from stress (Flexas *et al.*, 2009).

The maximum assimilation rate, concomitantly with g_s, g_m and $V_{c,max}$, was generally reached under well-watered conditions, therefore the control treatment was used as a reference. However, since A_N of irrigated plants declined during the experiment, presumably due to leaf ageing, the values for irrigated plants for each day were considered as the reference for the moderate and stressed plants determined during the same day. In doing so, photosynthesis limitations due to leaf ageing in irrigated plants were assessed by comparing the values along the experiment with the maximum values observed. On the other hand, 'pure' water stress limitations (i.e., without interaction with leaf ageing) were obtained for moderate and severely stressed plants. Whenever one of the involved parameters (g_s, g_m and $V_{c,max}$) was higher in stressed than in irrigated plants, its corresponding limitation was set to zero, and the other limitations re-calculated accordingly.

Finally, non-stomatal limitations were defined as the sum of the contributions of mesophyll conductance and leaf biochemistry ($NS_L = MC_L + B_L$), while diffusive limitations were the sum of stomatal and mesophyll conductance components ($D_L = S_L + MC_L$).

3.9. Water use efficiency

3.9.1. A_N/E and A_N/g_s

At the leaf level, the instantaneous net CO_2 assimilation (A_N), the transpiration (E) rates, as well as the determination of stomatal conductance (g_s) obtained by an open infrared gas-exchange system (Li-6400; Li-Cor, Inc., Lincoln, NE, USA) (see section 8.1.1) permits the use of the ratio A_N/E as the "instantaneous water-use-efficiency (WUE_{inst})", and the ratio A_N/g_s as the "intrinsic water-use-efficiency (WUE_i)" (Fischer and Turner, 1978).

3.9.2. Carbon-isotope composition in leaf dry matter

The carbon isotope ratio in leaf dry matter ($\delta^{13}C$) has been proposed as an integrative indicator of leaf WUE over the long term (Farquhar and Richards, 1984, Condon *et al.*, 2004). Most recently developed and initially marked leaves from each treatment were sampled for carbon-isotope analysis. The samples were dried for 48 h at 70ºC, ground into powder and sub-sampled for C-isotope ratio analysis. Samples were combusted in an elemental analyser (Thermo, Bremen, Germany); CO_2 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 every six samples. The calculation of $\delta^{13}C$ was done as $\delta^{13}C_{sample}\ (‰) = (R_{sample}/R_{standard} - 1) \times 1000$ (Farquhar and Richards, 1984), where $R_{sample}/R_{standard}$ were referred to a PDB standard.

3.10. Other measurements

3.10.1. Abscisic acid concentration

Xylem abscisic acid (ABA) concentration was determined in young fully expanded and sun exposed leaves. Leaves xylem exudation was collected after applying sufficient pressure with a leaf pressure chamber (Soilmoisture Equipment). After discarding the first exudation, sap was collected and immediately submerged in liquid nitrogen and kept at –80 ºC. The ABA concentrations in xylem exudation were measured with the Phytodetek ABA enzyme immunoassay test kit (Agdia Inc.; Elkhart, IN, USA) as according to the manufacturer's instructions. This kit uses the

competitive antibody binding method to measure concentrations of ABA in plant extracts.

3.10.2. Thermoluminiscence measurements

Thermoluminiscence glow curves of *Vitis* R-110 leaf discs was measured using home-built apparatus (SBE-INRA/CEA-Saclay, France), as described in detail by Sajnani *et al*. (2007). Data acquisition and signal analysis were performed using dedicated software according to Ducruet (2003).

3.10.3. Determination of ascorbate

Ascorbate (Asc) and dehydroascorbate (DHAsc) were determined using modified bipyridyl methods of Okamura (1980) and Knörzer *et al*. (1996).

3.10.4. Determination of the effective pathlength (*L*) for leaf water transport

During each sampling/measuring round, atmospheric water vapour was collected by cryogenic condensation (Roden and Ehleringer, 1999). Petiole and leaf lamina water were extracted by cryogenic vacuum distillation (Ehleringer and Dawson, 1992). For both, water vapour and distilled water, cryogenically trapped water was transferred immediately into sealed 2 ml crimp cap vials (Infochroma, Zug, Switzerland) and kept cooled until isotope analysis. An aliquot of 0.6 µl of each water sample was then injected in a High Temperature Combustion Elemental Analyzer (TC/EA, Thermo Finnigan, Bremen, Germany), pyrolized at 1450°C on glassy carbon to CO, the oxygen isotope ratio of which was determined by isotope ratio mass spectrometry (Delta plus XP, Thermo Finnigan, Bremen) and the values expressed as deviations in per mil (‰) from the international standard VSMOW ($\delta^{18}O$). Overall precision was less than 0.2‰.

Isotopic enrichment of mean lamina leaf water above source water (Δ_L, in ‰) was calculated as $\Delta_L = (\delta^{18}O_L - \delta_S)/ (1+\delta_S)$, where $\delta^{18}O_L$ and δ_S stand for the isotopic composition of leaf lamina (after removing main veins) and source water, respectively. Petiole water ($\delta^{18}O_P$) was considered to be representative for source

water. Steady-state isotopic enrichment at the site of evaporation (Δ_e) was modeled according to Craig and Gordon (1965) after Dongmann *et al.* (1974):

$$\Delta_e = \varepsilon^+ + \varepsilon_k + \left(\Delta_v - \varepsilon_k\right)\frac{e_a}{e_i} \qquad (1)$$

where ε^+ is the equilibrium fractionation between liquid water and vapour (Majoube 1971); ε_k is the kinetic fractionation of vapour diffusion from the leaf to the atmosphere (Farquhar *et al.*, 1989), Δ_v is the isotopic enrichment of atmospheric water vapour, and e_a/e_i is the ratio of ambient to intercellular vapour pressures.

The steady-state isotopic enrichment of mean lamina mesophyll water (Δ_{Lss}) was calculated by correcting for the gradient from xylem source water to enriched water at the evaporating sites, the so-called *Péclet* effect (Farquhar and Lloyd, 1993):

$$\Delta_{Lss} = \Delta_e \frac{1 - e^{-\wp}}{\wp} \quad \text{with } \wp = \frac{E \cdot L}{C \cdot D} \qquad (2)$$

where $\wp$ is the *Péclet* number, E the leaf transpiration rate (mol m^{-2} s^{-1}), L the scaled effective path length (m) for water movement from the veins to the site of evaporation, C the molar concentration of water (55.56 10^3 mol m^{-3}), and D the tracer-diffusivity (m^2 s^{-1}) of the heavy water isotopologue ($H_2{}^{18}O$) in 'normal' water. The effective pathlength L under the steady state assumption (L_{ss}) was determined by fitting Eq. 2 to measured Δ_L. L_{ss} values were fitted independently for each leaf.

Non-steady-state effects in lamina mesophyll water enrichment (Δ_{Lnss}) were tested using the simplified non-steady-state *Péclet* description (Farquhar and Cernusak, 2005):

$$\Delta_{Lnss} = \Delta_{Lss} - \frac{\alpha^+\alpha_k}{g_t w_i}\frac{1 - e^{-\wp}}{\wp}\frac{d\left(V_m \Delta_{Lnss}\right)}{dt} \qquad (3)$$

where $\alpha = 1+\varepsilon$, (α^+ and α_k are corresponding to ε^+ and ε_k, respectively), V_m is lamina leaf water molar concentration (mol m^{-2}), t is time (s), g_t is the total conductance for water vapour of stomata and boundary layer (mol m^{-2} s^{-1}), and w_i is the mole fraction of water vapour in the leaf intercellular air spaces (mol mol^{-1}). The term $d\left(V_m \Delta_{Lnss}\right)/dt$ stands for the rate of change in "isostorage" ($V_m \Delta_{Lnss}$) between a given measuring time-point (t_0) and a previous measurement, used as reference (t_{-1}),

and is applied to estimate the "net isoflux" during transpiration (Farquhar and Cernusak, 2005). According to previous studies on Grenache grapevines (Lovisolo *et al.*, 2010; Schultz, 2003a; Soar *et al.*, 2006; Vandeleur *et al.*, 2009), leaves were considered to behave isohydrically, and no changes in V_m were included in the non-steady state model. Indeed, any significant effect of the treatments for this parameter was found, so it was considered as a constant throughout the experiment (V_m=10.9 ±0.02 mol H_2O m^{-2}). To prevent artefacts due to differences between the individual leaves sampled at each time-point, Δ_{Lnss} was calculated using average values for each time-point and treatment (N=3). *Sensu stricto*, the model could only be applied to the values from intact leaves in the afternoon, using morning data as reference values (t_{-1}). Nevertheless, the non-steady state model to severed leaves and to morning values in intact leaves was also applied. In these cases, in which measured values for t_{-1} were not available, the rate of changes in Δ_L was estimated from the average and the 95% confidence interval for the differences in Δ_L between morning and afternoon values (2.60 ‰ and 1.66-3.53 ‰, respectively), and the average dt (8324 s). Although the non-steady state model under these assumptions may have lost its predictive value, the comparison between L_{ss} and L_{nss}, as well as the sensitivity of L_{nss} to Δ_L changes, provided a qualitative test of the potential risks associated to assuming steady-state conditions.

Equilibrium fractionation ε^+ was calculated after (Majoube, 1971), and kinetic fractionation ε_k was calculated after (Farquhar *et al.*, 1989) with the diffusional fractionation factors of (Cappa *et al.*, 2003). Tracer-diffusivity D as depending on temperature was estimated using a Vogel-Tamman-Fulcher relationship (Cuntz *et al.*, 2007):

$$D = a_D a_1 \exp\left(-\frac{a_2}{T - a_3}\right) \qquad (4)$$

with $a_1 = 100 \cdot 10^{-9}$, $a_2 = 577$, $a_3 = 145$ and $a_D = 1/1.026$ for $H_2{}^{18}O$.

Effective pathlength L under non-steady state conditions (L_{nss}) was calculated by fitting iteratively Eq. 3 until Δ_{LnP} values at both sides of equation differed by less than 0.01 ‰.

3.11. Statistical analysis

Regression coefficients and correlations between pairs of variables were assessed by means of linear regressions and simple correlation analyses with the 8.0 Sigma Plot software package (SPSS; Chicago, IL, USA), indicating determination coefficients and *P*-values as main statistics. Differences between means were assessed by Duncan analyses ($P<0.05$), performed with the SPSS 17.0 software package. Unless otherwise stated, means are reported together with the standard errors of the mean.

The treatment effect on physiological variables and isotopes was first assessed by an ANOVA including water status (control, drought) and leaf treatment (non-severed, severed) as fixed factors. Additionally, the effect of day and measuring time were considered by adding them as fixed factors to the model, but due to the lack of replicates this was only possible for non-severed plants. All the ANOVAs were performed using standard SAS–STAT procedures (SAS 1988).

Chapter 4

RESULTS AND DISCUSSION

4.1. ADJUSTMENTS OF WATER-USE EFFICIENCY BY STOMATAL REGULATION DURING DROUGHT AND RECOVERY IN THE DROUGHT-ADAPTED VITIS HYBRID RICHTER-110 (*V. berlandieri* x *V. rupestris*)

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ABSTRACT

The hybrid Richter-110 (*Vitis berlandieri* × *Vitis rupestris*) 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 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. Stomatal conductance (g_s) during water stress was mainly related to the content of abscisic acid (ABA) in the xylem, and partly related to plant hydraulic conductivity, but not to leaf water potential. By contrast, low g_s 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, g_s and leaf transpiration (E) were strongly affected by leaf-to-air vapour 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.

KEYWORDS

Stomatal conductance, water use efficiency, water stress, drought, water potential, water relations, plant hydraulics, abscisic acid, vapour pressure deficit

Abbreviations

ABA, abscisic acid; A_N, net photosynthesis; A_N/E, instantaneous leaf water use efficiency; A_N/g_s, intrinsic leaf water use efficiency; AWA, amount of water available in the substrate; $\delta^{13}C$, carbon isotope ratio; E, rate of transpiration; ε, bulk modulus of elasticity; g_s, stomatal conductance; Kh_{plant}, whole-plant hydraulic conductivity; PV, pressure-volume curves; R-110, Richter-110 (an hybrid of *Vitis berlandieri* × *Vitis rupestris*); RWC_{PD}, leaf relative water content at pre-dawn; RWC_{MD}, leaf relative water content at midday; WUE, water use efficiency; Ψ_{PD}, pre-dawn leaf water potential; Ψ_{MD}, midday leaf water potential; $\Psi_{\pi 100}$, osmotic potential at full turgor; $\Psi_{\pi 0}$, osmotic potential at turgor loss point; Ψ_{tlp}, water potential at turgor loss point.

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 due to 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 are needed.

Besides agronomic approaches (Gregory 2004) to enhance crop water-use efficiency (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 (RFLPs) or quantitative trait loci (QTL) (Martin et al. 1989, Handley et al. 1994, Saranga et al. 2004, Masle et al. 2005). 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, since WUE depends on complex arrangements and interactions of physiological mechanisms such as stomatal behaviour, 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 abscisic acid (ABA) is considered the most important (Wilkinson 2004, Christmann et al. 2005), hydraulic signals and cavitation of xylem vessels (Christmann et al. 2007, Cochard et al. 2002, Brodribb and Holbrook 2003), 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 is 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 since 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*) is well adapted to water stress conditions (Galet 1988). Growing as a complete plant, R-110 shows a completely isohydric behaviour 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 in drought-adapted R-110 variety how stomatal conductance 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 multi-level phenomenon, hence not correlated with a single signaling factor (ABA, Ψ 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.

MATERIAL AND METHODS

Plant material and water stress treatments

Plants of Richter-110 (*Vitis berlandieri* × *Vitis rupestris*) 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 28th (D.O.Y. 209), twenty 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 stomatal conductance (g_s), as suggested by Flexas et al. (2002): moderate water stress (g_s about 0.15 mol H_2O m^{-2} s^{-1}) and severe water stress (g_s about 0.05 mol H_2O m^{-2} s^{-1}). These values of stomatal conductance corresponded to approximately 55% and 20% that of control plants for moderate and severe water stress, respectively. After the first level of g_s was achieved (D.O.Y. 213), 20 plants were maintained at similar g_s of ~0.15 mol H_2O 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 g_s was ~0.05 mol H_2O m^{-2} s^{-1} (D.O.Y. 217), and sustained for a week at that level of stress as previously described. After one 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 stomatal conductance was first achieved (day 0), seven 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, that is days 8, 10 and 14, respectively.

Gas-exchange measurements

Gas-exchange measurements were performed on 10-12 leaves from different plants per treatment, between noon and 1 P.M., using an open gas-exchange system (Li-6400; Li-Cor, Inc., Lincoln, NE, USA). All measurements were performed at 1500 μmol m^{-2} s^{-1} to ensure light saturation, with a CO_2 concentration in the cuvette of 400 μmol CO_2 mol^{-1} air. Temperature and vapour pressure deficit were not controlled.

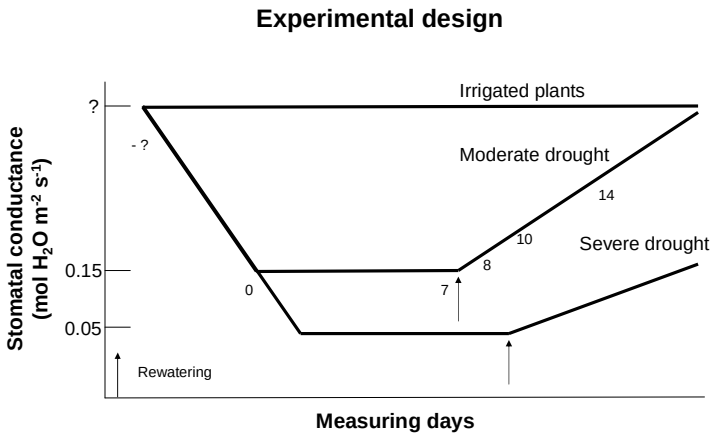

Figure 1. Experimental design, showing the expected time courses and the targeted levels of stomatal conductance for each treatment. Numbers indicate sampling days (only those for moderately stressed plants are showed for clarity). Arrows indicate the onset of re-watering.

Leaf water status

Pre-dawn (Ψ_{PD}) and midday leaf water potential (Ψ_{MD}) were determined with a Scholander pressure chamber (Soilmoisture Equipment Corp., Santa Barbara, CA, USA). Five replicates per treatment were obtained from five different plants.

Leaf relative water content at pre-dawn (RWC_{PD}) and midday (RWC_{MD}) 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 days 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, 4 leaves per cultivar were collected to develop pressure-volume curves (PV curves) (Tyree

and Richter 1981; Alsina et al. 2007). Leaves were excised, immediately sealed in plastic bags containing water, and transported to the laboratory. Petioles were re-cut 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 8ºC 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 leaf water potential (Ψ). Data for initial saturated weight, intermediate fresh weight corresponding to values for Ψ, and final dry weight were used to calculate the relative water content (RWC). The RWC and the corresponding Ψ were plotted as a "Type II" (Ψ^{-1} x RWC) transformation (Tyree and Richter 1981, 1982). Osmotic potential at full turgor ($\Psi_{\pi 100}$), osmotic potential at turgor loss point ($\Psi_{\pi 0}$), water potential at turgor loss point (Ψ_{tlp}) and leaf bulk modulus of elasticity (ε) were obtained from the pressure-volume curves (PV) (Turner 1988).

Hydraulic conductivity

Whole plant hydraulic conductivity (Kh_{plant}) was calculated considering Kh_{plant} from the Ohm's law analogy for the soil-plant-atmosphere continuum (Lovisolo et al. 2002):

$$E = Kh_{plant} \times (\Psi_{substrate} - \Psi_{MD}),$$

where E, Kh, Ψ_{leaf}, and $\Psi_{substrate}$ represent transpiration rate, whole-plant hydraulic conductivity, leaf water potential and substrate water potential, respectively. Ψ_{PD} was taken as a proxy for $\Psi_{substrate.}$

Concentration of abscisic acid in the xylem sap

The concentration of abscisic acid (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). After discarding the first droplet, sap was collected and immediately submerged in liquid nitrogen and kept at -80ºC. ABA concentrations in the xylem sap were measured with the

Phytodetek ABA enzyme immunoassay test kit (Agdia Inc.; Elkhart, IN, USA), following manufacturer 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 seven days, just before re-watering. Samples were dried for 48 h at 70ºC, ground into powder and sub-sampled for C-isotope ratio analysis. Samples were combusted in an elemental analyser (Thermo, Bremen, Germany); CO_2 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 every six samples. The calculation of $\delta^{13}C$ was done as $\delta^{13}C_{sample}\ (‰) = (R_{sample}/R_{standard} - 1) \times 1000$ (Farquhar and Richards 1984), where $R_{sample}/R_{standard}$ were referred to a 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 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 in order 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, main and fine roots were separated and dried in an oven at 60ºC 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 due to the effects of treatments on plant production during the experiment. Hence, while we cannot provide absolute estimates of whole-plant water use efficiency, estimations of the relative change in whole-plant water use efficiency caused by the different water stress treatments with respect to controls can be attained.

Relative whole-plant water use efficiency 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:

Relative WUE = (Dry weight Treatment / Dry weight Control) / (Water losses Treatment / Water losses Control).

Statistical analysis

Standard errors were calculated directly from crude data or, in the case of Kh_{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 Sigma Plot software package (SPSS; Chicago, IL, USA). 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-August 2005) were those typical for Mediterranean regions, with mean day-time temperatures above 25ºC (Fig. 2A), night temperatures above 10ºC (not shown) and daily irradiance frequently reaching 24.5 MJ m^{-2} d^{-1} (Fig. 2B). Peak PPFD at midday was usually 1500-1700 μmol m^{-2} s^{-1} (not shown). Vapour-pressure deficits were moderate, due to the proximity of the sea, with most days ranging between 1.5 and 2.5 kPa (Fig. 2C).

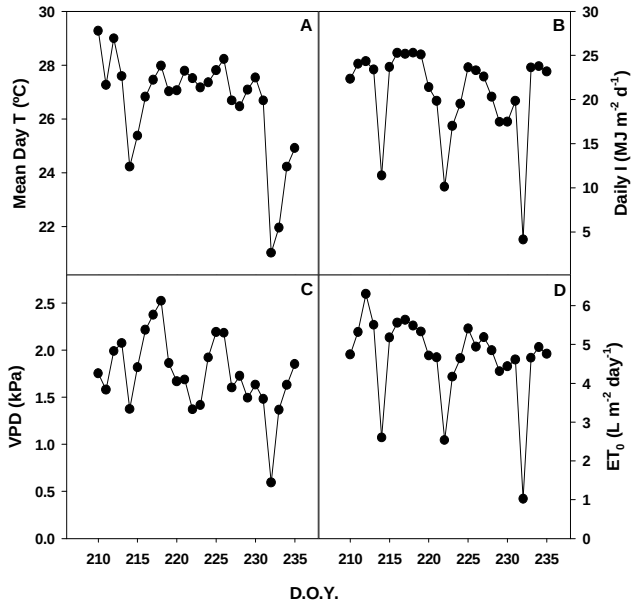

Figure 2. Climatic variables during the experimental period (July 28th 2005 to August 18th 2005). Measuring days are expressed as Day of Year (D.O.Y.). The displayed values are (A) daily mean air temperature (T), (B) total daily irradiance (I), (C))daily mean vapour pressure deficit (VPD), and (D) total daily potential evapotranspiration (ET_0). Data were averaged (T, VPD) or integrated (I, ET_0) over the light period (from 7:30 to 21:00 h).

Potential evapotranspiration (ET_0) 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 ETo (Fig. 2). A small amount of rain on August 4th (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 which were, on the other hand, 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 due to 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, Ψ_{PD} of moderately and severely stressed plants was not significantly different than in control plants, although severely stressed plants showed lower values two of the days, notably after re-watering (Fig. 3B). Despite non-significant differences in leaf water potential, 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 than in control plants for up to the first 6 days in severely stressed plants, and for up to 10 days in moderately stressed ones. 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 leaf intrinsic water-use efficiency (A_N/g_s) even after re-watering (Fig. 3D). Ψ_{MD} remained within a narrow range regardless of treatments (Fig. 3E), but whole plant hydraulic conductivity (Kh_{plant}) was substantially reduced by water stress, and part of the reduction persisted upon re-watering (Fig. 3F).

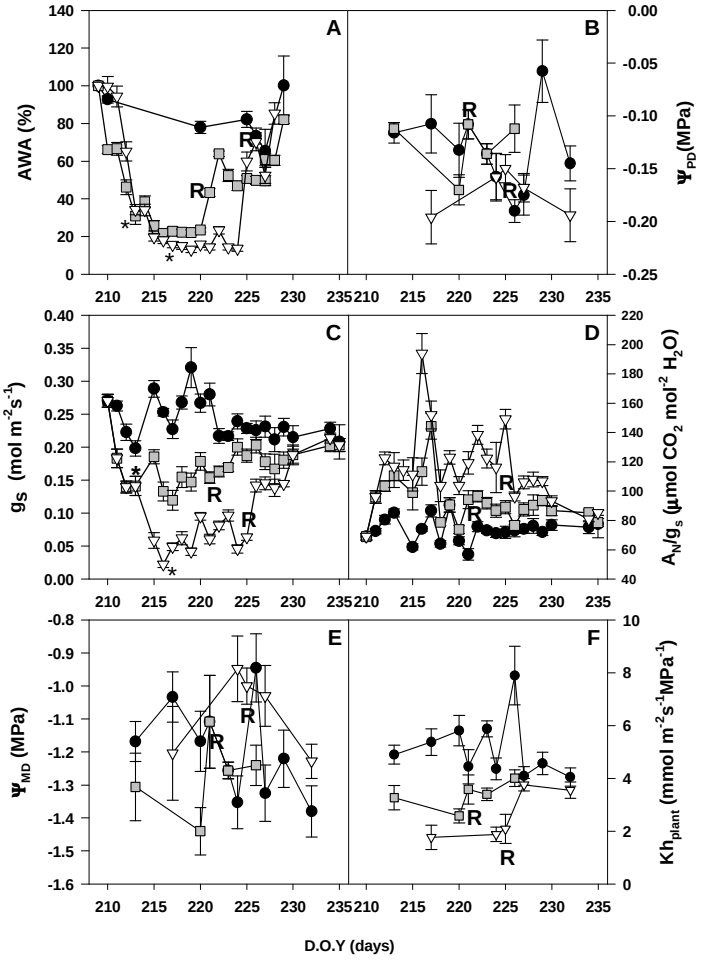

Figure 3. Changes from July 28th 2005 to August 18th 2005 in: (A) amount of water available in the substrate (AWA), (B) pre-dawn leaf water potential (Ψ_{PD}), (C) stomatal conductance (g_s), (D) intrinsic water-use-efficiency (A_N/g_s), (E) midday leaf water potential (Ψ_{MD}), and (F) whole-plant hydraulic conductivity (Kh_{plant}). Measuring days are expressed as Day of Year (D.O.Y.). Black circles represent control plants, grey squares represent moderately stressed plants, and white triangles represent severely stressed plants. (*) in (A) and (C) indicate the day the targeted treatments were reached (i.e. the first day the desired g_s was achieved) and (R) indicates the onset of re-watering of each treatment. Values for AWA, Ψ_{PD} and Ψ_{MD} are means ± S.E. of 2-5 replicates, while values for g_s and A_N/g_s are means ± S.E. of 10-12 replicates. Values of Kh_{plant} are means ± S.E. calculated from average and error values of E, Ψ_{PD} and Ψ_{MD} following Talylor (1982).

No differences were observed between treatments in PV curves when they were performed (data not shown). Among treatments and days, water potential at turgor loss point (Ψ_{tlp}) averaged -1.98 ± 0.13 MPa, π100 averaged -1.52 ± 0.05 MPa, and the bulk modulus of elasticity (ε) averaged 9.76 ± 0.70 MPa. The fact that Ψ_{tlp} was ca. -2 MPa, while Ψ_{MD} never dropped below -1.5 MPa means that the Ψ_{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 and 14 h (local time) in any of the treatments (data not shown).

Dependency of g_s on physiological variables

Although Ψ_{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 Ψ_{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).

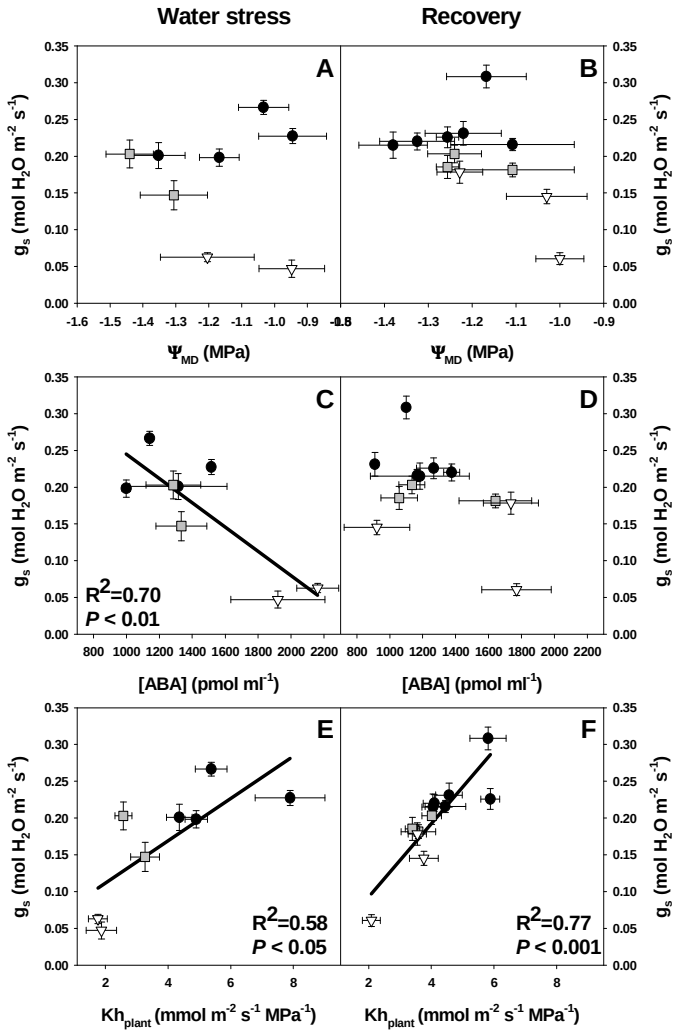

Figure 4. The relationships between stomatal conductance (g_s) and (A, B) midday leaf water potential (Ψ_{MD}), (C, D) xylem sap ABA concentration ([ABA]), and (E, F) whole-plant hydraulic conductivity (Kh_{plant}). Data for water stress (A, C, E) and recovery (B, D, F) periods are plotted separately. For control plants, only values obtained during the same days are plotted in each type of panels to avoid biasing the regressions. Black circles represent control plants, grey squares represent moderately stressed plants, and white triangles represent severely stressed plants. Values for Ψ_{MD} and [ABA] are means ± S.E. of 3-5 replicates (except for control plants, 1-3 replicates per day), while values for g_s are means ± S.E. of 10-12 replicates. Values of Kh_{plant} are means ± S.E. calculated from average and error values of E, Ψ_{PD} and Ψ_{MD} following Talylor (1982). In panels B, C and E, regression lines are displayed with their R^2 and level of significance. Relationships in the other three plots all have an $R^2 <$ 0.2, none of them being significant.

The correlation between g_s and Kh_{plant} 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 A_N and g_s when combining all data from this experiment (data not shown). Consequently, leaf intrinsic WUE (A_N/g_s) increased from 60 to 150 μmol CO_2 mol^{-1} H_2O when g_s decreased as water stress intensified (Fig. 3D). Leaf-level instantaneous WUE (A_N/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 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, leaf 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).

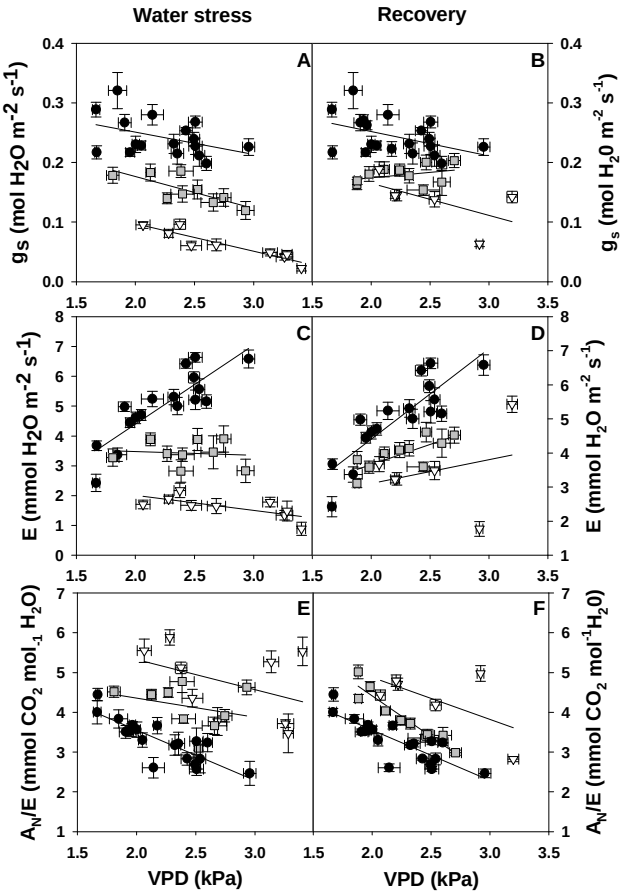

Figure 5. The relationship between vapour pressure deficit (VPD) and (A, B) stomatal conductance (g_s), (C, D) transpiration (E) and (E, F) instantaneous water-use-efficiency (A_N/E). Data for plants during water stress (A, C, E) and recovery (B, D, F) are plotted separately. For control plants, all values are plotted in the four panels. Data are average ± SE of 10-12 replicates. Black circles represent control plants, grey squares represent moderately stressed plants, and white triangles represent severely stressed plants.

Leaf-level instantaneous WUE (A_N/E) not only increased with increasing water stress, but it also became more independent of VPD (Fig. 5E). Hence, while in control plants A_N/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 A_N/E remained higher in previously stressed than in control plants. Integrating A_N/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‰) than in stressed plants, with the highest values (-24.7‰) for severely stressed plants and intermediate values for moderately stressed plants (-25.1‰). 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 Ψ 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 behaviour, strongly reducing g_s in response to water stress without a significant change in leaf Ψ (Fig. 3). When AWA was reduced by 70%, g_s was almost halved, without changing Ψ_{PD} or Ψ_{MD}, and when soil water content was reduced by ca. 80%, g_s decreased below 0.05 mol H_2O m^{-2} s^{-1} with only a slight decrease in Ψ_{PD}, but not in Ψ_{MD} (Fig. 3). Since leaf Ψ_{PD} is expected to be in equilibrium with substrate Ψ, 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 as to keep Ψ_{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 Ψ. Similarly, Medrano et al. (2002) showed in grapevines that g_s at any given Ψ_{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 to changes in leaf water potential (Ψ_{MD}), xylem acid abscisic ([ABA]) and hydraulic conductivity (Kh_{plant}). The dependency of g_s on any of these parameters was not general (Fig. 4), possibly indicating a multi-scale regulation of g_s. First, g_s did not correlate with Ψ_{MD}, since 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, Stoll et al. 2000; Lovisolo et al. 2002) 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 Kh_{plant} under water stress. During recovery, the correlation between g_s and Kh_{plant} was stronger and highly significant. Therefore, stomatal regulation during water stress in Richter-110 is complex, differing during water stress and recovery. Regulation of g_s 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 which persist long time after re-watering (Gallé and Feller 2007).

Table 1. Water use efficiency of control, moderately stressed and severely stressed plants. A_N/E values were averaged from mid-morning values for the entire experimental period and expressed in g L^{-1}. Data are average ± S.E. of 10-12 replicates. $\delta^{13}C$ 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 (1st May 2005 to 31st August 2005). Whole-plant relative WUE was calculated as described in Material and Methods. All data are average ± S.E. of 6 replicates. Different letters denote significant differences within each treatments at $P<0.05$ by Duncan's analysis. Values without letters denote no statistical differences within treatments at $P<0.05$ by Duncan's.

	Control Plants	**Moderately stressed plants**	**Severely stressed plants**
A_N/E (g L^{-1})	$3.2^a \pm 0.1$	$3.97^b \pm 0.1$	$4.56^c \pm 0.2$
Leaf $\delta^{13}C$ (‰)	$-26.9^a \pm 0.1$	$-25.1^b \pm 0.2$	$-24.7^c \pm 0.1$
Total Biomass (g)	316.1 ± 22. 6	274.7 ± 32. 5	253.4 ± 28.7
Average daily water expense (L)	$2.26^b \pm 0.04$	$2.16^b \pm 0.04$	$2.00^a \pm 0.05$
Relative whole-plant WUE	1	0.91	1.04

Besides the complex physiological regulation of stomatal closure, the important daily variation in VPD clearly affected g_s 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 g_s-VPD relationship. Contrarily, in R-110 the relationships between g_s and VPD for the three treatments presented similar slopes, which allowed a greater adjustment of g_s and E to substrate water availability and atmospheric demand. For instance, in control plants g_s decreased about 15% per each kPa of increased VPD, similar to olive and pines, which decreased g_s by 12% and 18% per kPa, respectively at a similar range of VPDs (Moriana et al. 2002, Maseyk et al. 2008). However, in moderately stressed olive and pines, the g_s reduction was only 8-10% per kPa, while in severely stressed plants it was almost 0% (Moriana et al. 2002, Maseyk et al. 2008). By contrast, in R-110 g_s decreased up to 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 semi-arid 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, A_N/g_s rapidly increased and was kept above control values during the entire experiment, even ca. two weeks after re-watering. The values achieved (up to 150-200 $\mu mol\ mol^{-1}$) are among the highest found for any species. In drought-adapted crops, such as olives or

grapevines, maximum reported values of A_N/g_s 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 A_N/g_s, R-110 seems capable of maintaining high A_N/E at elevated VPD (Fig. 5). Consistent with the optimisation theory (Cowan 1977), A_N/E is expected to decline with increasing VPD which has been experimentally confirmed in numerous species, irrespective of water stress (Farquhar et al. 1980, Moriana et al. 2002, Maseyk et al. 2008). However, when subjected to water stress, R-110 displayed a much lower dependency of A_N/E on VPD, keeping A_N/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 intrinsic leaf-level WUE 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 $\delta^{13}C$ on the dry matter of leaves expanded during the experimental period (Farqhuar and Richards 1984, Condon et al. 2004). Plant dry weight showed a declining, although non-significant tendency from control to severely stressed plants of ca. 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 as 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 $\delta^{13}C$ 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, Rytter 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 leaf-level WUE under 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 multi-level 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 semi-arid regions.

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4.2. WATER USE EFFICIENCY DURING DROUGHT AND RECOVERY IN GRAPEVINES: DIFFERENTIAL BEHAVIOUR OF THREE CULTIVARS

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Keywords: *Vitis vinifera*, water stress, photosyntesis, stomatal conductance, whole plant hydraulic conductivity.

ABSTRACT

A comparative study on water-use-efficiency (WUE) under severe water deficit and recovery was conducted on potted grapevines of Grenache, Syrah and Chardonnay cultivars grown in Mallorca (Spain). Deficit irrigation was established according to the leaf maximum daily g_s to achieve severe water deficit conditions in one week treatment.

The goal was to analyze how g_s is regulated under water stress and recovery, as well as how water stress affects the adjustments of WUE at leaf and whole plant level. Soil water content, climatic conditions, leaf photosynthesis, transpiration, g_s and mesophyll conductance (g_m) were recorded daily throughout the experiment. Water relations and plant hydraulic conductivity (Kh_{Plant}), were performed on five specific sampling days: the day the desired stomatal conductance (50 $mmolm^{-2}s^{-1}$) was first achieved (day 0), seven days after sustaining the plants at constant soil moisture, just before re-watering (day 7), and then 1, 2 and 7 days after re-watering.

The relative contribution of g_s and g_m limitations during acclimation to water stress, changes from predominant g_s early during water stress to similar g_s and g_m after acclimation. Nonetheless during re-watering photosynthesis recovery was mostly limited by g_s, since stomatal closure recovered much more slowly than g_m. Water stress induced an increase in WUE, which interestingly persisted many days

after re-watering. Kh_{Plant} variations during drought and recovery were in accordance with g_s values. However, the relationship with g_m variations was lower.

Chardonay showed the largest differences between water stress and irrigation plants and the lowest leaf water potential. Under drought and recovery this cultivar maintained lower g_s and leaf photosynthesis (A_N) but their WUE values were not the highest.

INTRODUCTION

The overwhelming limitation on plant productivity is leaf water supply (Kramer & Boyer 1995), and hence factors influencing water supply play a central role in the adaptation of plants to their terrestrial environment.

Grapevine cultivars have been shown to differ in their responses to water deficit due to differences in stomatal sensitivity to increasing water deficit, thus affecting, photosyntesis, transpiration and water-use efficiency rates (Bota et al. 2001; Medrano et al. 2003; Schultz, 2003). Plants strategies to cope with drought normally involve a mixture of stress avoidance and tolerance that depend on genotypes (Bota et al 2001). The pattern of Ψ regulation – whether it is isohydric or anisohydric (Shultz 2003), and the particular thresholds of Ψ that are controlled – have to be tuned to the soil moisture regime and the hydraulic capability of the plant's root system and xylem.

Besides acclimation, the carbon balance of a plant during a period of water stress and recovery may depend as much on the velocity and degree of photosynthetic recovery as it depends on the degree and velocity of photosynthesis decline during water depletion (Flexas et al., 2006). In general, plants subjected to severe water stress recover only 40–60% of the maximum photosynthesis rate during the day after re-watering, and recovery continues during the next days, but maximum photosynthesis rates are not always recovered (Kirschbaum, 1988; Gallé et al. 2007). The strong influence of previous water stress severity in the velocity and extent of photosynthesis recovery has been illustrated in kidney bean by Miyashita et al. (2005) and has been suggested for Vitis vinifera as well (Gómez-del-Campo et al., 2007; Flexas et al. 2009). However, the factors limiting photosynthesis recovery after water

stress seems to be multiple, and strongly dependent on the species and conditions analyses (Ennahli and Earl 2005; Galmés et al. 2007). This important aspect of plant physiology was the subject of an experiment within this study. In the present work we compare the effect of the acclimation of water stress and re-watering on three different grapevine cultivars (Syrah, Grenache and Chardonnay) and some interesting patterns were obtained during the experiment.

MATERIAL AND METHODS

Two experiments were carried out in two subsequent years: 2008 and 2009. In June 2008 forty two-year-old-grapevines (*Vitis vinifera* 'Grenache' and 'Syrah', both grafted on *Vitis berlandieri* × *Vitis rupestris*) were grown outdoors in 15L pots filled with a mixture of soil and organic substrate in Mallorca (Spain). When the experiments were started 10 plants per cultivar were kept as controls, while irrigation was stopped for the remaining 20 plants. Water stress was established by the leaf maximum daily stomatal conductance (g_s), as suggested by Flexas et al. (2002) (g_s about 0.05 mol H_2O m^{-2} s^{-1}). After the stress level of g_s was achieved, plants were maintained at similar g_s for a week by daily replacing the amount of water consumed, as determined by weighting of pots every evening. After one week at the established soil water deficit, all plants were irrigated to field capacity and recovery was followed for several days.

Gas-exchange measurements were taken daily, while the rest of physiological measurements (pre-dawn and midday leaf water potential), were performed on five specific sampling days per each treatment: the day the desired stomatal conductance was first achieved (day 0), seven days after sustaining the plants at constant soil moisture, just before re-watering (day 7), and then 1, 2 and 7 days after re-watering, that is days 8, 9 and 14, respectively.

In mid-July of 2009, 20 plants 2-year-old Chardonnay were used, and the same growth conditions of 2008 were applied. The same experimental design and same measurements of 2008 were performed for Chardonnay as well.

Instantaneous gas-exchange and chlorophyll fluorescence measurements were taken daily throughout the experiment, between 10 to 1 P.M., on 10-12 leaves from

different plants per treatment, using an open gas-exchange system (Li-6400; Li-Cor, Inc., Lincoln, NE, USA) with an integrated leaf chamber fluorometer (Li-6400-40; Li-Cor Inc., Nebraska, USA). All measurements were performed on young, fully expanded leaves, at 1500 μmol m^{-2} s^{-1} to ensure light saturation, with a CO_2 concentration in the cuvette of 400 μmol CO_2 mol^{-1} air. From instantaneous measurements, net CO_2 assimilation (A_N), stomatal conductance (g_s) and the sub-stomatal CO_2 concentration (C_i) were recorded.

The method by Harley *et al*. (1992) was used to make estimations of g_m as:

$$g_m = A_N / (C_i - (\Gamma^* \cdot (J_{flu} + 8 \cdot (A_N + R_d)) / (J_{flu} - 4 \cdot (A_N + R_d))))$$

were A_N and C_i are taken from gas exchange measurements at saturating light and Γ^* and R_d were estimated using the Laisk (1977) method.

Pre-dawn (Ψ_{PD}) and midday leaf water potential (Ψ_{MD}) were determined with a Scholander pressure chamber (Soilmoisture Equipment Corp., Santa Barbara, CA, USA). Measurements were performed on five fully expanded leaves per treatment and sampling day from five different plants.

Whole plant hydraulic conductivity (Kh_{plant}) was calculated considering Kh_{plant} from the Ohm's law analogy for the soil-plant-atmosphere continuum (Lovisolo et al. 2002):

$$E = Kh_{plant} \times (\Psi_{MD} - \Psi_{substrate}),$$

where E, Kh, Ψ_{leaf}, and $\Psi_{substrate}$ represent transpiration rate, whole-plant hydraulic conductivity, leaf water potential and substrate water potential, respectively. Ψ_{PD} was taken as a proxy for $\Psi_{substrate.}$

The amount of water available in the substrate (AWA) or soil-water status was calculated as: (Pot Weight – Minimum Pot Weight) / (Maximum Pot Weight – Minimum Pot Weight) × 100. Minimum pot weight was considered at the wilting point. Maximum pot weight was considered as the pot weight at field capacity. Their weight difference between their daily weight and the weight of the day before was considered as the daily pot weight.

Regression coefficients and correlations were calculated with the 8.0 Sigma Plot software package (SPSS; Chicago, IL, USA). Differences between means were

assessed by Duncan analyses ($P < 0.05$), performed with the SPSS 17.0 software package (SPSS).

RESULTS AND DISCUSSION

Experimental conditions, soil water content and plant water status

In general, climate conditions during the experimental period were typical for Mediterranean conditions and there weren't big climatic differences between both periods (June 2008 and July 2009). In that sense we can corroborate that variability between cultivars are not due to climatic conditions.

Treatments produced clear differences in AWA (Table 1), which was kept stable along the deficit irrigation. The rate of desiccation was different for each of the cultivars, being faster for Chardonnay (using just 3 days to reach the required AWA value) and slower for Grenache (with 7 required days). These different desiccation patterns could be explained by different stomatal conductances values at the initial of the experiment, with higher values for Chardonnay (Fig. 1A), so, in this cultivar, higher evaporation rates where obtained (data not shown), which caused higher water losses. In fact, in that case, fewer days were necessary to reach the required AWA, and different responses to water depletion were obtained.

The quick recovery of AWA was also reflected in Ψ_{PD} (data not shown) and Ψ_{MD} (Table 1). All three cultivars were shown water potential (WP) acclimation during drought adaptation, showing anisohydric stomatal behaviour in all cases. After re-watering, all values were mostly recovered within 2 days period.

Table 1. The time course of soil water availability and midday-water potential. Values within a row followed by an asterisk are significantly different (P<0.05).

	SYRAH		**GRENACH**		**CHARDONNAY**	
	WP_{MD} (MPa)	AWA (%)	WP_{MD} (MPa)	AWA (%)	WP_{MD} (MPa)	AWA (%)
CONTROL	-0.59±0.04	83.4±1.3	-0.54±0.02	86.3±1.1	-0.68±0.08	76.6±1.2
WS	-0.84±0.02*	23.7±0.9	-0.80±0.03	19.4±1.5	-1.32±0.06*	17.7±1.1
ACCLIMAT	-1.0±0.06	21.7±1.2	-0.86±0.05*	15.6±1.5	-1.32±0.18*	14.4±1.1
RECOV 1	-0.64±0.02*	62.7±1.8	-0.53±0.03	59.8±2.9	-0.48±0.02	67.5±1.2
RECOV 2	-0.59±0.03	78.4±1.9	-0.63±0.02	76.2±2.6	-0.69±0.04	66.4±2.2
RECOV 3	-0.63±0.04*	81.1±1.3	-0.54±0.02*	76.4±2.0	-0.89±0.14*	76.0±1.9

Chardonnay sowed bigger differences in WP between stressed and control plants. This would suggest a higher response in terms of WP to water stress. However, the same pattern is not in agreement with all others parameters that had been measured, as at lower water potentials, Chardonnay showed higher g_s than Syrah and Grenache. In that cultivar osmotic adjustments may contribute to the maintenance of open stomata at lower water potentials, by enabling an improved turgor in response to imposed water depletion. Moreover, various experiments have shown that stomatal responses are often more closely linked to soil moisture content than to leaf water status, and this could suggest that stomata are mostly responding to chemicals signals (e.g. ABA) produced by dehydrating roots (Gowing et al. 1990; Stoll et al. 2000).

Grenache and Syrah also maintained lower and constant values of Ψ_{MD} during water stress and acclimation period; however decreases of Ψ_{MD} in treated plants in respect to control plants, are lowest in Grenache than in Syrah and Chardonnay. After irrigation, same values (-0.5 MPa) of Ψ_{MD} were reached for all cultivars.

The experimental course of Kh_{plant} at different days during water stress and recovery confirmed one clear pattern for grapevine plants, with a significant drop during water stress, and a subsequent and slow recovery during re-watering (Fig. 1). In irrigated conditions, Chardonnay had the highest Kh_{plant}. Maximization of gas exchange rates, which is adaptive in environments with high resource availability (Tyree et al. 1998; Nardini and Tyree 1999), could be achieved most efficiently by this cultivar by increasing Kh_{plant}.

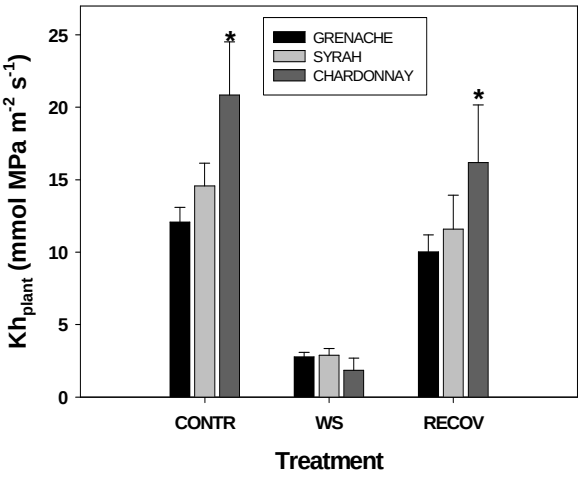

Figure 1. Plant hydraulic conductance, as a function of irrigation treatment. Data shown the mean of ± S.E 8-10 replicates per cultivar. Significant differences (*) were reflected between cultivars.

Plant dynamics during water stress and recovery

As well as Ψ_{MD}, stomatal conductance (g_s; Fig. 2A) strongly responded to water withholding, and the desired level of severe stress was reached in 2 to 4 days after stopping irrigation. Water stress imposition resulted also in larger reductions in Net photosynthesis (A_N) of stressed plants (Fig. 2B). During acclimation to water stress, A_N wasn't kept within a stable range, particularly for Syrah and Grenache, that even increased A_N values among 7 days of acclimation. Upon re-watering, recovery of A_N was almost completed in about 3-4 days in all cultivars (Fig. 2B). So here, reduces of g_s resulted in a higher leaf intrinsic water-use efficiency (A_N/g_s) even after re-watering.

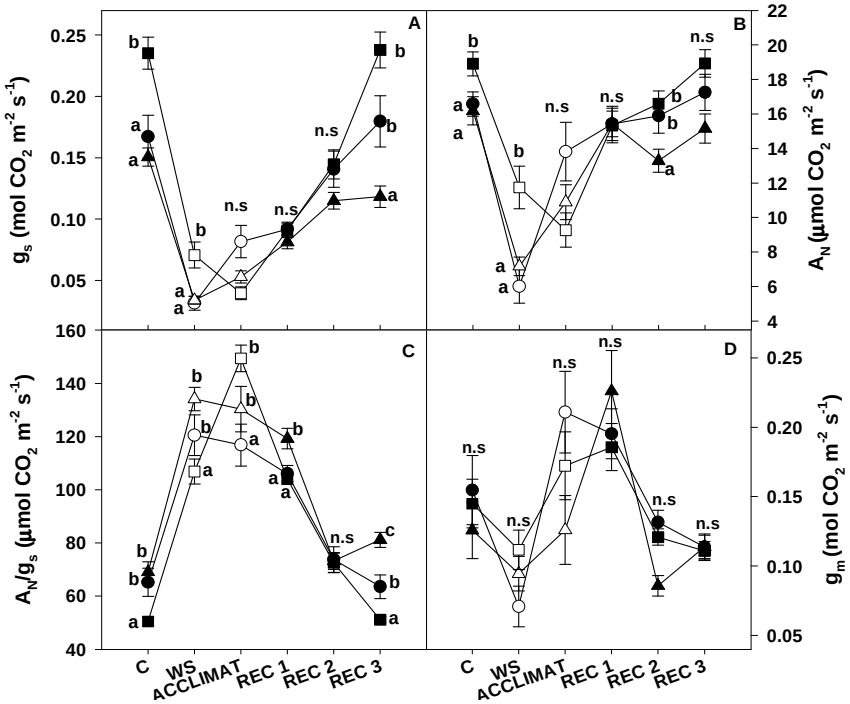

Figure 2. Evolution of net leaf photosynthesis (A), stomatal conductance (B), intrinsic water-use-efficiency (C) and mesophyll conductance (D). Abreviations: C, is the average of the all mean values from control plants along the experimental period (i.e. ± S.E. of 60 replicates); WS, first day under the correspondent treatment; ACCLIMAT is acclimation after 7 days under the correspondent treatment; REC 1, REC 2, and REC 3 are 1, 2 and 7 days after re-watering, respectively. g_s, A_N and A_N/g_s are mean values ± S.E from 10 replicates, while Kh_{plant} are mean values ± S.E. from 4-5 replicates.

The Mesophyll conductance to CO_2 (g_m) dynamics showed interesting differences in respect to g_s both under water stress and recovery. For stressed plants, g_m was strongly reduced during the first days of acclimation to water withholding, but interestingly, it was totally restored to control values by almost the fourth day of water stress (Fig. 2D).

In Syrah and Grenache, increases of photosynthesis rates prior to re-watering, suggest an acclimation to water stress by an increase of gm. However, a limitation of photosynthetic recovery by g_m after re-watering was of minor importance, as g_m of previously stressed plants, were similar than control values. Delayed stomatal response was the main limitation here, resulting also in slightly higher intrinsic WUE (Fig. 2B, C).

During recovery, gas-exchange parameters increased earlier in Chardonnay than in Syrah and Grenache, implying a more efficient stomatal response to drought-recovery for this cultivar. In general, stomatal limitations appeared to be the most important in delaying photosynthesis recovery after re-watering. This is in agreement with previous reports by Gallé and Feller (2007) and Flexas et al. (2009), who showed a sustained reduction of g_s lasting for weeks after re-watering in Beech and a grapevine rootstock, respectively.

Dependency of g_s and g_m on Khplant

In grapevine (and for other several species) a co-ordination between liquid flow conductivity and the vapour phase conductance, limited either by the stomata has been already shown (Lovisolo y Schubert, 1998; Schultz, 2003) and has been extended to demonstrate a link between hydraulic supply and photosynthesis (Lovisolo y Schubert, 1998; Escalona et al., 2002; Lovisolo et al., 2002; Schultz, 2003). In this study, a high significant correlation (r^2= 0.83; P<0.001) between Kh_{plant} and g_s was observed when all the values from different cultivars and different treatments were plotted (Fig. 3A).

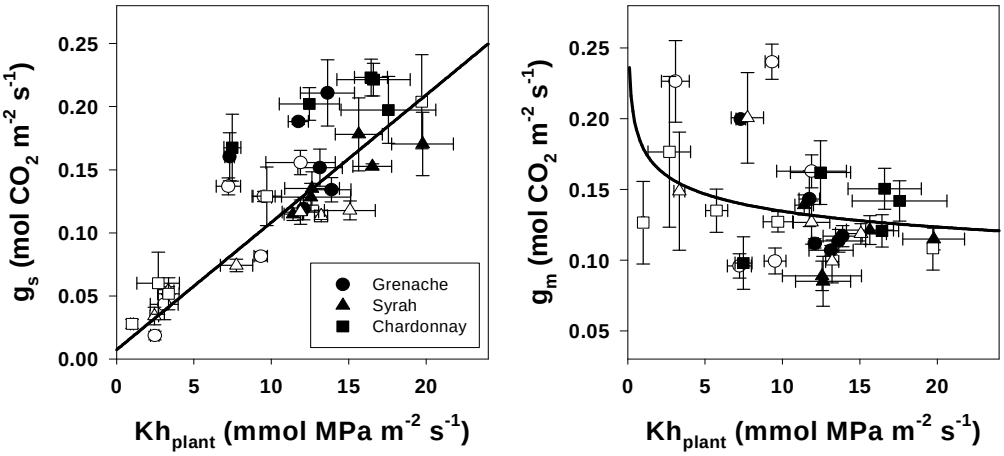

Figure 3. Relationship between Kh_{plant} and stomatal conductance. Values are mean of ± S.E 4-5 replicates per cultivar, treatment and sampling day. White colour means values during water stress and acclimation. A black colour means control and recovery. g_s (r^2=0.83 P<0.01); g_m (r^2=0.1 n.s.).

Contrarily, a relationship between Kh_{plant} and g_m was not observed (r^2 of 0.1; ns.) (Fig. 3B). It is already described that the diffusion of CO_2 through the leaf internal structure, or mesophyll conductance to CO_2 (g_m), decreases concomitantly with g_s (Flexas et al. 2002), but the relationship between both conductances is curvilinear, so that the higher the intensity of water stress, the higher g_m is compared with g_s (Flexas et al. 2009). As the decrease in g_m was of a smaller magnitude compared with changes in g_s, and the g_m was recovered to control values during the acclimation period, a relation similar to that, between g_s and Kh_{plant}, was not observed for g_m.

CONCLUSION

In Grenache and Syrah at higher midday-water potentials, lower values of A_N were obtained, however, when water stress was imposed, higher A_N/g_s ratios indicated that photosynthetic CO_2 fixation in these cultivars was favourably affected by water stress, suggesting that species adaptability to a given site may be influenced by several factors. Moreover, Grenache experimented higher decreases in Kh_{plant} during water stress and recovery, so increasing the sensitivity of the stomatal response to drought, may promote Grenache as the most drought tolerant cultivar, showing isohydric response.

Stomata of Chardonnay remained comparatively open, even at lower water potentials; this could suggest that Chardonnay sustain transpirational flow through less sensitive stomates (i.e. lower control over stomatal aperture under water stress) which implies a higher water transport capacity in the soil-plant-atmosphere continuum, and the possibility to reduce sensitivity to xylem embolism.

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4.3. ANISOHYDRIC BEHAVIOUR IN GRAPEVINES RESULTS IN BETTER PERFORMANCE UNDER MODERATE WATER STRESS AND RECOVERY THAN ISOHYDRIC BAHAVIOUR

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Running Title: Differential responding behaviours in grapevine under moderate water stress and recovery.

Key words: hydraulic conductivity, photosynthesis, stomatal conductance, *Vitis vinifera*, water stress

ABSTRACT

Aims: Increasing evidences of differential responding behaviours in grapevines were contrasted when comparing three grapevine varieties original from different climates under water deficit and recovery after re-watering: Grenache, from Mediterranean origin; Syrah, from mesic origin; and Chardonnay, from the humid zone of Burgundy (France). The aim of this experiment was to analyze how transpiration, photosynthesis, stomatal and mesophyll conductances were regulated under water stress and recovery, and how this regulation affected water-use-efficiency, as well as to characterize how this regulation was affected by the environmental conditions and the variations among different grapevine varieties.

Methods: To assess these issues, changes of photosynthetic CO_2 assimilation (A_N), factors affecting photosynthesis, and their possible interactions with other environmental conditions were followed during prolonged water stress and subsequent re-watering in Chardonnay, Grenache and Syrah.

Results: In this study, Grenache confirmed its reputation as isohydric and Chardonnay as anisohydric, but Syrah, a variety often considered as anisohydric, showed here near-isohydric behaviour. Chardonnay displayed higher hydraulic conductivities during both irrigation and water stress and a faster recovery after water stress as compared to the two isohydric-behaving varieties.

The results do not support the common assumption that isohydric behaviour results in a better performance under water stress conditions. Indeed, under moderate water stress Chardonnay showed some advantages over the two varieties displaying near-isohydric behaviour. By delaying its adjustment of lamina hydraulic conductivity (Kh_{lamina}) in response to water stress, Chardonnay attained lower decreases in stomatal conductance, which in turn resulted in the maintenance of higher photosynthesis and photosynthetic capacity, favoring faster recovery upon re-watering. Moreover, although intrinsic WUE (WUE_i) was highest in Grenache, the instantaneous WUE (WUE_{inst}) was highest in Chardonnay because this variety maintained lower leaf-to-air vapor pressure deficits (LAVPD), hence reducing transpiration (E) at a given stomatal conductance (g_s).

Conclusions: In consequence, spanned over a period including water stress imposition, acclimation and recovery Chardonnay displayed higher CO_2 assimilation than Grenache and Syrah, which implies a higher yield potential under these conditions.

Abbreviations

ABA; abscisic acid; AWA, water available in the substrate; ACCL, acclimation; A_N, light-saturated net photosynthesis; C, control; C_a, atmospheric CO_2 concentration; C_c, chloroplast CO_2 concentration; C_i, sub-stomatal CO_2 concentration; C_i*, C_i at the CO_2 compensation point in the absence of mitochondrial respiration; E, transpiration; ET_0, potential evapotranspiration; Φ_{PSII}, photochemical efficiency of photosystem II; g_m, CO_2 mesophyll conductance to CO_2; g_s, stomatal conductance; J_{flu}, electron transport rate determined by chlorophyll fluorescence; J_{max}, maximum rate of electron transport; K_{lamina}, lamina hydraulic conductivity; K_{leaf}, leaf hydraulic conductivity; LAVPD, leaf-to-air vapor pressure deficit; 1103P, 1103 Paulsen (an hybrid of *Vitis berlandieri* × *Vitis rupestris)*; PPFD, photosynthetically active photon flux density; REC, recovery; R-110, Richter-110 (an hybrid of *Vitis berlandieri* × *Vitis rupestris*); R_D, leaf respiration in the dark; Γ*, C_c at the CO_2 compensation point in the absence of mitochondrial respiration; $V_{c,max}$, maximum rate of carboxylation; VPD, vapor pressure deficit; WS, water stress; WUE, water use efficiency; WUE_i instrinsic WUE; WUE_{inst}, instantanea WUE.

INTRODUCTION

There is an overwhelming limitation on plant productivity by leaf water supply (Kramer and Boyer 1995) and hence, factors influencing water supply play a central role in the adaptation of plants to their terrestrial environment. Thus, survival and distribution of sessile organisms such as plants depend strongly on their ability to adjust to environmental variations.

Stomatal control of water losses has been identified as an early event in plant response to water deficit (Chaves, 1991; Cornic and Massacci, 1996), leading to the prevention of a critical decrease in water potential (Ψ) (Cochard *et al.* 2002; Sperry *et al.* 2002), though this goes at the expense of reduced CO_2 assimilation (Tyree and Ewers 1991; Cochard *et al.* 1996, 2002; Breda *et al.* 2006). Regulation of plant water potential (Ψ) by stomatal control and leaf area adjustment may be necessary to maximize water uptake on the one hand, while avoiding loss of hydraulic contact with soil water on the other. Not all species, however, behave similarly in their stomatal control over Ψ. Different genotypes may rely to different extents on avoidance and tolerance strategies to cope with different degrees of acclimation to drought stress. Besides acclimation, the carbon balance of a plant during a period of water stress and recovery may depend as much on the velocity and degree of photosynthetic recovery as it depends on the degree and velocity of photosynthesis decline during water depletion (Flexas *et al.* 2006a). In general, plants subjected to severe water stress recover only 40–60% of the maximum photosynthesis rate during the day after re-watering, and recovery continues during the following days, although maximum photosynthesis rates are not always recovered (Kirschbaum 1987, 1988; Sofo *et al.* 2004; Gallé *et al.* 2007). The strong influence of the severity previous water stress in the velocity and extent of photosynthesis recovery has been illustrated in kidney bean by Miyashita *et al.* (2005) and has been suggested for *Vitis vinifera* as well (Gómez-del-Campo *et al.* 2007; Pou *et al.* 2008; Flexas *et al.* 2009).

In grapevines, there is strong evidence for hormonal control of stomatal closure mainly mediated by abscisic acid (ABA) (Correia *et al.* 1995; Lovisolo *et al.* 2002; Pou *et al.* 2008). However, there is also strong evidence for hydraulic control, which seems dominant under persistent stomatal closure, such as under long-term

water stress and recovery after re-watering (Lovisolo *et al.* 2008a; Pou *et al.* 2008). Plant hydraulics can be regulated by – among other mechanisms – cavitation events, which depend on soil-leaf Ψ gradient which in turn is partly regulated by stomatal movements. Therefore, there is a strong interdependence of plant hydraulics and stomatal responses (Salleo *et al*. 2000). Depending on their Ψ responses to soil water stress plants are described as isohydric or anisohydric (Stocker 1956; Tardieu and Simonneau 1998). Isohydric plants maintain relatively high plant water potential (Ψ) under water stress, because g_s is finely regulated to prevent a drop of xylem water potential to levels that would provoke excessive loss of conductivity (Vogt 2001). Consequently, isohydric plants have a minimum midday water potential that remains more or less constant as soil dries. Contrarily, anisohydric plants exhibit substantial depression of Ψ during drought without lasting impairment, i.e. they show some degree of tolerance. In these plants, transpiration is not as tightly regulated by stomatal clousure. According to Bucci et al. (2005), isohydric plants transpiration exhibits a linear dependence on leaf hydraulic conductivity (Kh_{leaf}), while in anisohydric plants, transpiration increases asymptotically with Kh_{leaf}, causing minimum leaf water potential to vary with Kh_{leaf}.

The grapevine is an interesting model for woody horticultural crops, with a wide range of physiological responses between genotypes, thus affecting their drought tolerance (Smart and Coombe 1983; Carbonneau 1985; Bota *et al.* 2001; Schultz 2003). Grapevine varieties have been shown to differ in their responses to water deficit due to differences in stomatal sensitivity to increasing deficit, thus affecting photosynthesis, transpiration and water-use efficiency (Bota *et al.* 2001; Medrano *et al.* 2003; Schultz 2003). Grenache, for example, of Mediterranean origin, was classified as near-isohydric, while Syrah and Chardonnay, of mesic origin, displayed anisohydric characteristics (Schultz 1996, 2003; Soar *et al.* 2006; Vandeleur *et al.* 2009). When compared to anisohydric genotypes, isohydric genotypes have been shown to have several differential characteristics like (i) more pronounced increase of intrinsic water use efficiency (WUE_i) in response to water stress (Poni *et al.* 2007), (ii) stronger stomatal sensitivity to increased vapor pressure deficit (Soar *et al.* 2006), (iii) lower hydraulic conductance and lower susceptibility

to cavitation (Schultz 2003; Vandeleur *et al.* 2009), and (iv) similar velocity of recovery of leaf gas exchange rates after water stress as anisohydric plants (Schultz 2003). All together, these characteristics have led to consider isohydric varieties to be more adapted to drought environments than anisohydric varieties (Schultz 2003; Soar *et al.* 2004, 2006; Vandeleur *et al.* 2009).

However, this assumption is far from being proved. In fact, although grapevine varieties of Mediterranean origin – such a Grenache – display near-isohydric behaviour, typically anisohydric varieties – such as Syrah and Chardonnay – are commonly grown and used for wine making in Mediterranean semi-arid regions (Jones 2006). In addition, combining data from many iso- and anisohydric varieties, Lovisolo *et al.* (2010) showed that, on average, both types of genotypes display similar gas exchange rates and WUEi under both irrigation and drought. Moreover, contradictory reports appeared in the literature showing that the same variety could behave as iso- or anisohydric depending on experimental conditions (Medrano *et al.* 2003; Chaves *et al.* 2010; Lovisolo *et al.* 2010).

So, it is likely that the iso- and anisohydric behaviour of a given variety and its adaptability to water deficits depends on complex interactions between the characteristics of the variety itself and those of the rootstock, the climate conditions (VPD and temperature), or the intensity and duration of water deficits. The purpose of our research was to evaluate how grapevine genotypes displaying different strategies and often classified as iso- and anisohydric respond to a moderate drought such as those frequently endured under Mediterranean field conditions, as well to a period of recovery subsequent to re-watering.

In this study we evaluated how grapevine genotypes reputed as iso- (Grenache) and anisohydric (Syrah, Chardonnay) responded to the imposition of a week-long moderate drought followed by a period of recovery subsequent to re-watering. The general aim was to search for evidences that an isohydric behaviour results in better adaptation to drought environments than anisohydric behaviour, as usually assumed (Schultz 2003; Soar et al. 2004, 2006; Vandeleur et al. 2009). Several specific questions were addressed: (i) is isohydric versus anisohydric behaviour related to differences in leaf hydraulics?; (ii) is stomatal conductance of

isohydric plants more sensitive to water stress and LAVPD than that of anisohydric plants?; (iii) do isohydric plants present higher WUE than anisohydric in response to water stress?; (iv) if so, is it related to a stronger stomatal closure or to the maintenance of higher photosynthetic capacity?.

MATERIAL AND METHODS

Plant material and water stress treatments

The experiment was carried out in the 2009 growing season (July-August) at the University of Balearic Islands (Mallorca, Spain), were the climate is typically Mediterranean, with hot and dry summers and mild winters. In July 30 two-year-old-grapevines (*Vitis vinifera* L.) var. Chardonnay, grafted on 1103 Paulsen (1103P; *Vitis berlandieri* × *Vitis rupestris*), were grown outdoors in 15L pots filled with a mixture of soil and organic substrate (3:1). In August, 30 two-year-old-grapevines of each of the varieties Grenache and Syrah, both grafted on Richter-110 (R-110; *Vitis berlandieri* × *Vitis rupestris*) were used under the same growing conditions. All plants were daily irrigated until the experiment started and supplemented once a month with 100% Hoagland's solution.

Ten plants per variety were kept as controls, while the remaining 20 plants were subjected to moderate water stress. Moderate water stress was established as a drop in the amount of water available in the substrate (AWA) from a maximum to 20%. This level was determined by previous calibration of the response of stomatal conductance (g_s) to AWA, to ensure g_s values higher than 0.05 mol H_2O m^{-2} s^{-1}, which would correspond to severe water stress according to Flexas *et al.* (2002). Irrigation was stopped until the desired AWA was achieved, and then plants were maintained at constant AWA for a week by daily replacing the amount of water consumed, as determined by weighting of pots every evening. After one week at the established soil water deficit, all plants were irrigated to field capacity and recovery was followed for several days.

Measurements of gas-exchange and chlorophyll fluorescence, pre-dawn and midday leaf water potential, and plant and leaf hydraulic conductivity were performed on five specific sampling days per each treatment (Fig. 1): the day the

desired AWA was first achieved (day 0), seven days after sustaining the plants at constant soil moisture, just before re-watering (day 7), and then 1, 2 and 7 days after re-watering, that is days 8, 9 and 14, respectively.

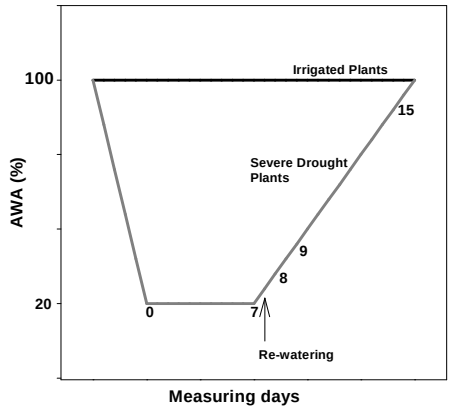

Figure 1. Experimental design, showing the expected time courses and the targeted levels of the amount of water available in the substrate (AWA) for sever stressed (grey line) and irrigated (black line) plants. Numbers indicate sampling days.

Meteorological data (Table 1) was provided from an automated meteorological station (Davies 7450, USA) installed inside the experimental field in the Universitat de les Illes Balears (39º38'N, 2º38'E).

Amount of water available in the substrate

The amount of water available in the substrate (AWA) or soil-water status was calculated as: (Pot Weight – Minimum Pot Weight / Saturated Pot Weight – Minimum Pot Weight) × 100. Minimum pot weight was considered at the wilting point. For its measurement, four plants per treatment were non-irrigated until a constant weight value was achieved. Saturated pot weight was considered as the pot weight at field capacity.

All plants were weighted every evening, before irrigation. Daily water lost was obtained from the weight differences between two consecutive days.

Biomass production and Leaf area

Whole plant biomass was determined at the beginning and at the end of the experiments. At those times, five plants per treatment and variety were harvested, and for each one of them, leaf, stems, main and fine roots were separated and dried in an oven at 60ºC to obtain dry weight. The sum of all fractions was the total plant dry weight at the end of the experiment. Therefore, differences in dry weight at the end and at the beginning of the experiment are due to the effects of treatments on plant production during the experiment.

At the end of the experiment, estimations of the total leaf area (LA; cm^2) were determined in the same previous plants, using images of all the leaves from each plant, processed in Matlab Softare (Matlab 7.0, MathWorks, Inc.).

Instantaneous gas exchange and chlorophyll fluorescence measurements

Instantaneous gas-exchange and chlorophyll fluorescence measurements were taken daily throughout the experiment, between 10 A.M. and 1 P.M., on 10-12 leaves from different plants per genotype and treatment, using an open gas-exchange system (Li-6400; Li-Cor, Inc., Lincoln, NE, USA) with an integrated leaf chamber fluorometer (Li-6400-40; Li-Cor Inc., Nebraska, USA). All measurements were performed alternated between treatments on young, fully expanded leaves, at 1500 μmol m^{-2} s^{-1} to ensure light saturation, with a CO_2 concentration in the cuvette of 400 μmol CO_2 mol^{-1} air. From instantaneous measurements, net CO_2 assimilation (A_N), stomatal conductance (g_s) and the sub-stomatal CO_2 concentration (C_i) were recorded.

Concerning chlorophyll fluorescence, the actual photochemical efficiency of photosystem II (ϕ_{PSII}) was determined by measuring steady-state fluorescence (Fs) and maximum fluorescence during a light-saturating pulse of ca. 8000 μmol m^{-2} s^{-1} (Fm') following the procedures of Genty *et al*. (1989):

$$\phi_{PSII} = (Fm'-Fs)/Fm' \qquad [1]$$

The electron transport rate (J_{flu}) was then calculated as:

$$J_{flu} = \phi_{PSII} \cdot PPFD \cdot \alpha \cdot \beta \qquad [2]$$

where PPFD is the photosynthetically active photon flux density, α is leaf absorptance and β reflects the partitioning of absorbed quanta between photosystems II and I. The product $\alpha \cdot \beta$ was determined, following Valentini *et al*. (1995), from the relationship between ϕ_{PSII} and ϕ_{CO2} obtained by varying either light intensity under non-photorespiratory conditions in an atmosphere containing less than 1% O_2.

Respiration in the light, apparent CO_2 photocompensation point and mesophyll conductance to CO_2

Under well-watered conditions (Control plants), respiration in the light or 'day' respiration (R_d) and the apparent CO_2 photocompensation point (C_i*) were determined according to Laisk (1977). Briefly, A_N-C_i curves were measured at three different PPFDs (50, 150, and 750 μmol m^{-2} s^{-1}) at six different CO_2 levels ranging from 400 to 50 μmol CO_2 mol^{-1} air, using the 2 cm^2 leaf chamber. The intersection point of the three A_N-C_i curves was used to determine C_i* (x-axis) and R_d (y-axis). C_i* was used as a proxy for the chloroplastic CO_2 photocompensation point (Γ*), according to Warren and Dreyer (2006). According to Galmés *et al*. (2006), only C_i* values for irrigated plants were considered, which averaged 42.0 ± 0.9 μmol CO_2 mol^{-1} air at a leaf temperature of 30ºC, i.e. a Γ* of 43.1 μmol CO_2 mol^{-1} ($\Gamma^* = C_i^* + R_d/g_m$), corresponding to a Rubisco specificity factor of 90. Considering published Γ* response functions to temperature for several species (reviewed by Warren and Dreyer 2006), this would correspond to a Rubisco specificity factor of about 100 at 25ºC, i.e., totally coincident with the actually determined value for *Vitis* (Bota *et al*. 2002).

The estimated Γ* and R_d, together with instantaneous measurements of A_N, C_i and J_{flu}, were used to estimate mesophyll conductance to CO2 (g_m) using the method by Harley *et al*. (1992) as:

$$g_m = A_N / (C_i - (\Gamma^* \cdot (J_{flu} + 8 \cdot (A_N + R_d)) / (J_{flu} - 4 \cdot (A_N + R_d)))) \qquad [3]$$

Leaf hydraulic conductivities

Maximum leaf hydraulic conductivity on a surface area basis (Kh_{leaf}; mmol m^{-2} s^{-1} MPa^{-1}) was determined using a high-pressure flow meter (HPFM; Dynamax Inc.Houston TX, USA), described in detail by Tyree et al. (1995). Detached sample leaves were excised under water allowed to reach a transpirational steady-state while attached to a flow meter via the petiole using compression fitting. 15mM KCl solution filtered at 0.1μm was forced into the leaves at a constant pressure (P; MP) up to 0.4 MPa, while measuring instantaneous flow (F; Kg s^{-1}) every 8 s. Corresponding hydraulic conductances (K) were computed as K = F/P. Quasi Steady-State mode was used for each measurement. K decreased during the early phases of measurements as the likely effect of progressive infiltration of leaf air spaces, and reached stable values after 25-30 minutes. After K was recorded, leaf blades were removed using a fresh razor blade. The hydraulic conductance of the petiole ($K_{petiole}$) was similarly measured and the lamina hydraulic conductance (K_{lamina}) (K_{lamina}=1/R) was calculated as:

$$1/ K_{lamina} = (1/ K_{leaf}) – (1/ K_{petiole})$$

During measurements, leaf temperature was monitored by a thermocouple and maintained between 20ºC and 25ºC by adding water uniformly across the leaf blade. The leaf hydraulic conductance was then corrected for eventual temperature changes to account for changes in water viscosity.

After each experiment, projected leaf areas (LA*;* m^2) were measured with a leaf area meter (AM-100 Area Meter, Analytical Development Co. Hoddesdon, UK), and leaf and lamina maximum hydraulic conductivities on a surface area basis were calculated (Kh_{leaf} and Kh_{lamina}, respectively; mmol s^{-1} MPa^{-1} m^{-2}).

Five to eight leaves per treatment were analyzed, from 10 AM to 1 PM when transpiration rates were maximal with leaves maintained under natural sun to minimize the potential impact of diurnal periodicity on leaf hydraulic conductance.

Statistical analysis

Regression coefficients and correlations were calculated with the 8.0 Sigma Plot software package (SPSS; Chicago, IL, USA). Differences between means were assessed by Duncan analyses ($P < 0.05$), performed with the SPSS 17.0 software package.

RESULTS

Experimental conditions

During the experimental period, two sets of experiments were carried on. In the first (July), Chardonnay was used and during this period, the climatic conditions showed some variability among days. Daily irradiance peaked 25.6 MJ $m^{-2}d^{-1}$ (data not shown). Potential evapotranspiration (ET_0) showed average values of 5.7 mm day^{-1} (Table 1) and vapor-pressure deficit values were moderate, most days ranging between 1.0 and 1.6 KPa (Table 1). The mean air temperature was 26.4ºC (Table 1). In August, when Syrah and Grenache were used as experimental plants, the mean air temperature was 26ºC (Table 1) and the mean daily irradiance was 21.7 MJ m^{-2} d^{-1} (Table 1). Vapor-pressure deficit values were similar to those in July, ranging between 1 and 1.5 KPa (Table 1). Potential evapotranspiration (ET_0) showed average values of 4.9 mm day^{-1} (Table 1). In total there were only two cloudy days (D.O.Y 222 and 224), as reflected in lower daily irradiance and ET_0 (data not shown).

Therefore, climate conditions during the experimental period were typical for Mediterranean conditions and climatic differences between different experimental periods were not significant (temperature) or not large, i.e. maximum differences between periods in average values were 3.4 MJ m^{-2} d^{-1} for daily irradiance and 0.8 mm day^{-1} for ET_0.

Table 1. Climatic variables during the experimental period. The displayed values are: daily mean irradiance, total daily evapotranspiration (ET_0), daily mean vapor pressure deficit (VPD) and daily mean air temperature. Data were averaged (Temperature, VPD) or integrated (I, ET_0) over the day. Different letters denote statistically significant differences within each period at $P<0.05$ according to Duncan's test.

	Daily I. ($MJ\ m^{-2}d^{-1}$)	**ET_0 ($mm\ day^{-1}$)**	**VPD (KPa)**	**Temperature (ºC)**
Jul-09	25.1 ± 0.5^{b}	5.7 ± 0.1^{b}	1.27 ± 0.05^{a}	26.4 ± 0.3^{a}
Aug-09	21.7 ± 0.6^{a}	4.9 ± 0.1^{a}	1.21 ± 0.04^{a}	26.0 ± 0.1^{a}

Some significant – although not large – differences in environmental values were observed between varieties for the days corresponding to specific treatments during measurements (Fig. 2). Midday air relative humidity (RH) showed a narrow range (45-55%) in all cases (Fig. 2A), as did midday air temperatures (30-31.5ºC, Fig 2B). However, RH was usually higher and temperature lower during measurements with Chardonnay than with the other two varieties. Moreover, with the exception of the first day of recovery, the difference between leaf and air temperatures (T_{leaf}-T_{air}) were always smaller (less than 0.5ºC) in Chardonnay than in Grenache and Syrah (2-3ºC, Fig. 2C). All together resulted in leaf-to-air vapor pressure deficit values (LAVPD) much lower in Chardonnay than in the other two varieties (Fig. 2D). Since LAVPD largely increased during water stress due to leaf heating (see Fig. 2C), the differences in LAVPD between varieties were maximum under these conditions being 2- 2.4 kPa in Chardonnay, 2.8 kPa in Syrah and up to 3.3 kPa in Grenache (Fig. 2D).

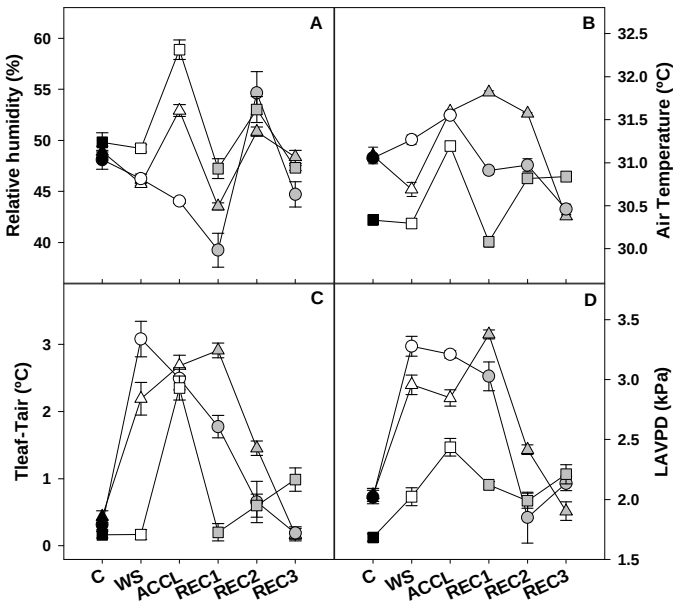

Figure 2. Evolution during the experiment of (A) Relative humidity (RH), (B) Air Temperature, (C) differences between leaf and air temperatures (T_{leaf}-T_{air}) and (B) leaf-to-air vapour pressure deficit (LAVPD). Each symbol represents one specific variety: ■, Chardonnay; ▲, Syrah and ●, Grenache. Black symbols represent irrigated (i.e. control) plants, white symbols represent water stressed and acclimated plants and grey symbols represent plants during recovery. Measuring day abbreviations are: C, average of values of Control plants during the entire experiment; WS, first day under water stress; ACCL, acclimation after 7 days under water stress; REC1, REC2 and REC3 are, respectively, 1, 2 and 7 days after re-watering. Values are means ± SE of 10–12 replicates.

Treatments

In all three genotypes, irrigation was halted until reaching similar decreases in AWA, from about 80% to about 20% (Fig. 3). The rate of AWA decrease differed among varieties, being fastest for Chardonnay (4 days), followed by Syrah (5 days), and slowest for Grenache (7 days). Low AWA was kept constant along the deficit irrigation period (Fig. 3). After re-watering, AWA of stressed pots were fully recovered in about three days.

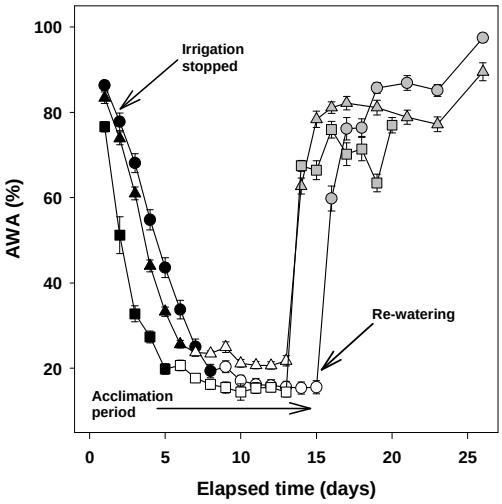

Figure 3. Changes in the amount of water available in the substrate (AWA), determined every day during the experimental period. Symbols and colors as in Figure 2. Values are means ± SE of 15 replicates.

Differences in plant biomass production during the entire experimental period and total leaf area (LA) between treatments were also reflected in significant reductions of both parameters in water stressed plants in respect to control plants (Table 2). Whole plant production revealed reductions of 15, 25 and 36% in water stressed plants in Chardonnay, Syrah and Grenache, respectively. However, the patter was different for leaf area reductions, which were the highest in Chardonnay (Table 2).

Table 2. Plant production and total leaf area per plant (LA) for treated and control plants of Syrah, Grenache and Chardonnay. Data are means ± S.E. of 5 replicates. Different letters within a column indicate a statistically significant difference (P<0.05) according to Duncan's test.

Variety	Treatment	Plant Production (ΔBiomass) (g)	LA (cm^2)
SYRAH	**C**	155.6 ± 19.5^{c}	2228.3 ± 31.0^{d}
	WS	117.2 ± 6.9^{bc}	1846.8 ± 55.5^{c}
GRENACHE	**C**	139.0 ± 15.1^{c}	1447.9 ± 53.6^{b}
	WS	90.2 ± 7.5^{a}	1017.3 ± 37.5^{a}
CHARDONNAY	**C**	98.8 ± 11.7^{b}	1634.5 ± 266.1^{bc}
	WS	83.5 ± 12.4^{a}	920.5 ± 83.4^{a}

Evolution of stomatal conductance and lamina hydraulic conductivity

Under irrigation, stomatal conductance (g_s) presented significant differences between varieties, with Chardonnay showing the largest g_s values and Grenache the lowest, while Syrah displayed intermediate values (Fig. 4A). g_s strongly responded to water withholding, the response being initially higher in Syrah and Grenache than in Chardonnay, although all three varieties attained the same g_s by the end of the acclimation period. After re-watering, recovery took place similarly slowly in all three varieties, so that g_s was kept lower than in control plants for almost 7 days (Fig. 4A).

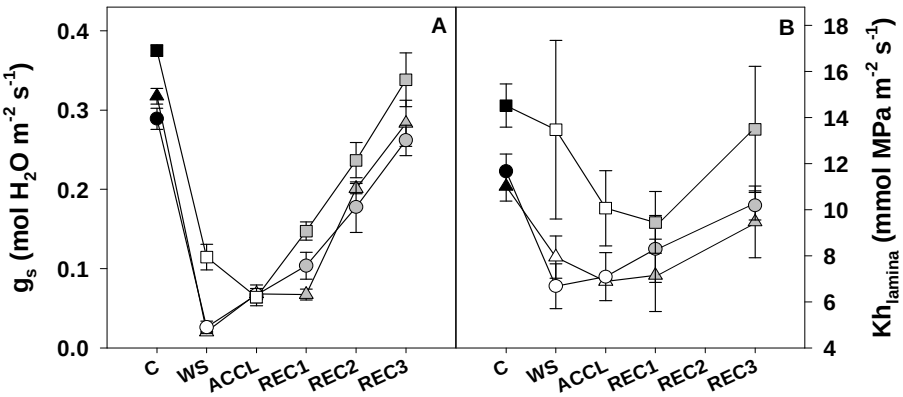

Figure 4. Evolution during the experiment of (A) stomatal conductance (g_s) and (B) lamina hydraulic conductivity (Kh_{lamina}) for each sampling day during the experiment. Symbols and colors as in Figure 2. Values for C are means ± S.E. of 20 replicates, while for other treatments are means ± S.E. of 4-5 replicates.

All varieties also showed significant reductions of Kh_{lamina} as a result of water depletion, with subsequent increases during re-watering (Fig. 4B). In irrigated plants (C), values of Kh_{lamina} were significantly larger in Chardonnay (14.5 mmol MPa^{-1} m^{-2} s^{-1}) than in Grenache and Syrah (11.7 and 11 mmol MPa^{-1} m^{-2} s^{-1}, respectively). When water stressed (WS), Kh_{lamina} of Chardonnay decreased to a lesser extent (dropping to 13.5 mmol MPa^{-1} m^{-2} s^{-1}) than Grenache and Syrah (6.9 and 7.9 mmol MPa^{-1} m^{-2} s^{-1}, respectively, Fig. 4B). After a week of potential acclimation to water stress (ACCL), the differences between varieties were no longer significant. Upon re-watering, and similarly to g_s, Kh_{lamina} showed a slow recovery in all varieties, reaching almost full recovery only 7 days after irrigation. The speed of initial recovery was slightly faster in Grenache, which showed some extent of recovery by

the very first day after re-watering than in Syrah and Chardonnay, which took more days to initiate recovery (Fig. 4B).

Transpiration, photosynthesis and water use efficiency during water stress and recovery

Leaf transpiration displayed a pattern similar to that of g_s with highly significant reductions of E under WS, maintaining lowered values after seven days of water stress period and with a subsequent recovery after re-watering (Fig. 5A). While during C conditions differences in E between varieties were non-significant, under WS Chardonnay kept significantly higher E (2.2 mmol H_2O m^{-2} s^{-1}) than Grenache and Syrah (0.8 and 0.6 mmol H_2O m^{-2} s^{-1}, respectively). During ACCL, and similarly to g_s, values for Chardonnay further decreased while for Grenache and Syrah they increased. During re-watering, all varieties completely recovered control E values 7 days after re-watering.

Water stress imposition also resulted in large reductions in net photosynthesis (A_N) from values around 19 μmol CO_2 m^{-2} s^{-1} to values around 9 μmol CO_2 m^{-2} s^{-1}. During acclimation to water stress, the behaviour of A_N differed among varieties. While in Chardonnay A_N it decreased from ca. 11 μmol CO_2 m^{-2} s^{-1} in the first day of water stress to 9 μmol CO_2 m^{-2} s^{-1} by the last day of acclimation, in Syrah and Grenache A_N initially decreased below 5 μmol CO_2 m^{-2} s^{-1} to then partially recover during the acclimation period to 9 μmol CO_2 m^{-2} s^{-1}. This was related to a small but significant increase in g_s (Fig. 4A) and, especially, an increase in g_m (Fig. 5C) and J_{flu} (Fig. 5D). Upon re-watering, recovery of A_N was almost completed in about 3-4 days in all varieties, but the velocity of recovery was much faster in Chardonnay than in the other two varieties (Fig. 5B).

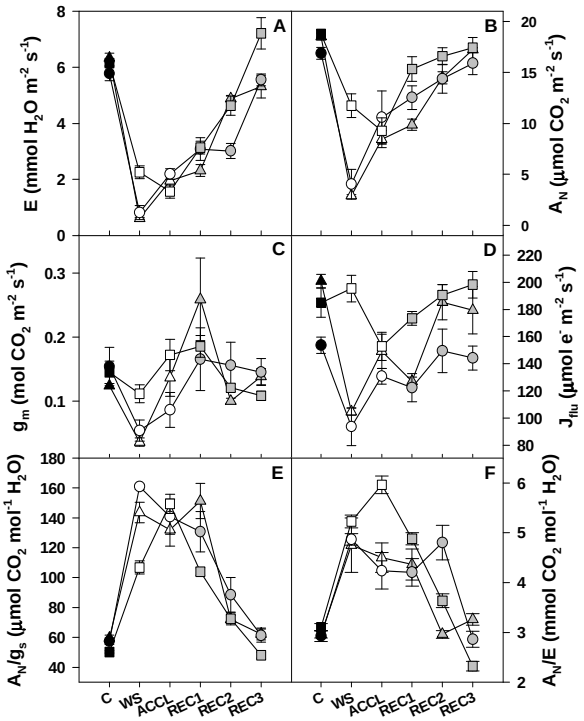

Figure 5. Evolution during the experiment of (A) transpiration (E), (B) net photosynthesis (A_N), (C) mesophyll conductance (g_m), (D) electron transport rate (J_{flu}), (E) intrinsic water use efficiency (A_N/g_s) and (F) instantaneous water use efficiency (A_N/E). Symbols and colors as in Figure 2.Values are means ± S.E. of 10 replicates.

The dynamics of mesophyll conductance to CO_2 (g_m) differed from those of g_s and showed notable differences among varieties. In Chardonnay, it was kept within a narrow range through all the experiment, i.e. water stressed plants showed values similar to control plants (Fig. 5C). In Grenache and Syrah, g_m initially decreased with water stress, for then partially (Grenache) or fully (Syrah) recover during the acclimation period. So, in Chardonnay g_m did not decrease, in Syrah it initially decreased but recovered completely even before re-watering, and in Grenache it decreased to recover only partially during acclimation but fully by the first day of recovery (Fig. 5C). Water stress induced some decrease of J_{flu} as well (Fig. 5D). However, in Chardonnay J_{flu} was depressed only slightly and after 7 days of acclimation, while in Grenache and Syrah J_{flu} was more strongly depressed and from early in the water stress period, but was partially restored before re-watering. In all three varieties, J_{flu} was fully restored two days after re-watering.

Larger reductions of g_s than A_N resulted in an increased ratio of net photosynthesis to stomatal conductance (A_N/g_s), i.e. leaf intrinsic water use efficiency (WUE_i) during water stressed as well as many days after re-watering. As water stress developed, WUE_i increased from around 60 μmol CO_2 mol^{-1} H_2O to around 150 μmol CO_2 mol^{-1} H_2O in all three varieties (Fig. 5E). Increased WUE_i was faster in Grenache and Syrah (maximum values by the first day of water stress) than in Chardonnay (maximum values at the end of the acclimation period). One day after re-watering, WUE_i persisted at the highest values in Grenache and Syrah, while in Chardonnay it started to decline. Recovery was slow in all three varieties, being completed only one week after re-watering (Fig. 5E). The changes in the instantaneous water use efficiency (WUE_{inst}; A_N/E) were similar to that WUE_i, with the highest values during water stress and persisting several days after re-watering (Fig. 5E). However, contrary to WUE_i, the highest WUE_{inst} was achieved by Chardonnay, which showed values ca. 30% higher than Syrah and Grenache (Fig. 5E).

Dependency of gas exchange on vapour pressure deficit

As expected, g_s was strongly and negatively dependent of LAVPD for both irrigated and water stressed plants (Fig. 6A). Interestingly, under water stress the slopes of g_s vs LAVPD were similar in all three varieties, but because of differences among them in LAVPD (see Fig. 2) they appeared as parallel lines. These data suggest that anisohydric behaviour favours a lower LAVPD with beneficial effects on current leaf transpiration. In fact, when comparing WUE_{inst} with LAVPD Chardonnay displays higher WUEinst values for both, water stressed and irrigated plants (Fig. 6B).

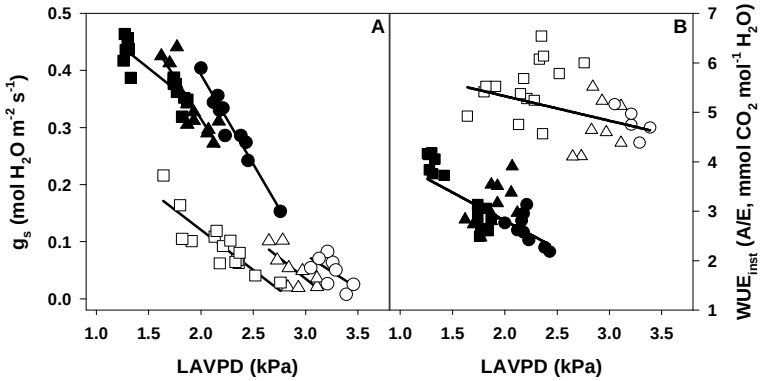

Figure 6. Response of (A) stomatal conductance (g_s) and (B) WUE_{inst} to leaf-to-air vapour pressure deficit (LAVPD) during water stress and acclimation. Symbols and colors as in Figure 2. Each value represents a single data. In the first plot (A) two regressions are assessed for each single variety depending of the treatment. For Control plants: Chardonnay, r^2=0.51, P<0.001; Syrah, r^2=0.74, P<0.001 and Grenache: r^2=0.89, P<0.001. For Water stressed plants: Chardonnay, r^2=0.77, P<0.001; Syrah, r^2=0.51, P<0.001 and Grenache: r^2=0.5, P<0.001. In the second plot (B) regressions are shown for each treatment pooling together data from all varieties (Control: r^2=0.47, P<0.001; Water stress: r^2=0.17, P=0.03).

In this case, when considering all varieties together either under irrigation or water stress, negative relationships were obtained between WUE_{inst} and LAVPD (Fig. 6B) being this relationship stronger and steeper in irrigated than in water stressed plants.

Dependency of gas exchange on hydraulic conductivity

Pooling together all data for irrigation, water stress and recovery, a clear, unique positive linear relationship (r^2= 0.75; *P<0.001*) was observed between g_s and Kh_{lamina}, with no differences between varieties (Fig. 7A). However, no clear relationship was observed between Kh_{lamina} and g_m (data not shown).

As expected, a curvilinear relationship was observed between A_N and g_s when combining all data from this experiment (data not shown). Consequently, WUE_i increased from 50 to 150 μmol CO_2 mol^{-1} H_2O when g_s decreased in water stressed plants. Since g_s and Kh_{lamina} were strongly interdependent, an inverse, highly significant (r^2 = 0.86; P<0.001) exponential relationship was observed between WUE_i and Kh_{lamina} (Fig. 7B).

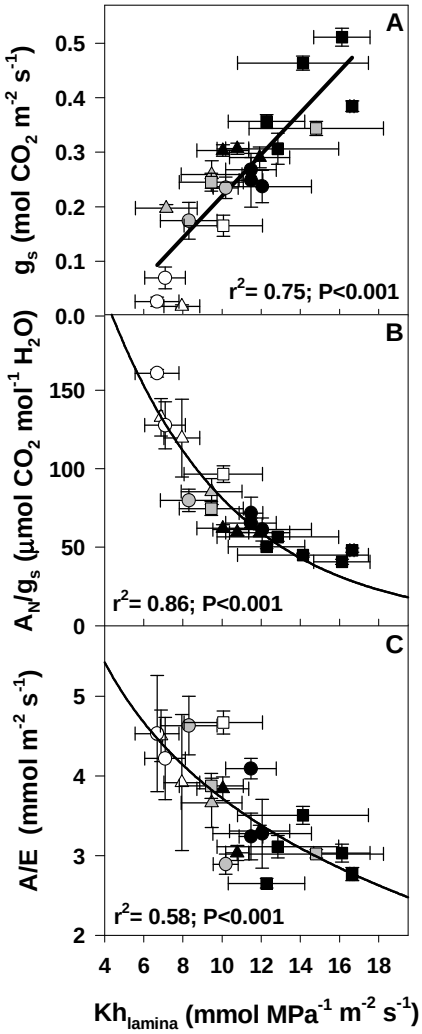

Figure 7. The relationships between lamina hydraulic conductivity (Kh_{lamina}) and (A) stomatal conductance (g_s), (B) intrinsic water use efficiency (WUE_i) and (C) instantaneous water use efficiency (WUE_{inst}). Symbols and colors as in Figure 2. Kh_{lamina} values are means ± S.E. of 4-5 replicates while values of g_s and A_N are means ± S.E. of 10 replicates. Logarithmical curves were fitted to the data for A_N/g_s and A_N/E as a function of Kh_{lamina}.

A similar curvilinear relationship was observed between WUE_{inst} and Kh_{lamina} when combining all data in the same plot (Fig. 7C) but, as expected, WUE_{inst} values became less determined by Kh_{lamina} with r^2 of 0.58 ($P<0.001$) as a consequence of differences in E between varieties (Fig. 6A) induced by their different LAVPD (Fig. 2D).

DISCUSSION

Grenache confirmed its reputation as isohydric (Schultz 2003) and Chardonnay its reputation as anisohydric (Vandeleur et al. 2009). Syrah, in contrast, did not show its expected anisohydric behaviour (Schultz 2003; Soar et al. 2006). While its physiological behaviour in response to water stress and recovery was generally intermediate between the other two varieties, it was closer to Grenache than to Chardonnay, i.e., it displayed near-isohydric behaviour. Discrepancies in iso- and anisohydric behavior with respect to literature reports are not surprising, since it has been shown that the same variety could behave as iso- or anisohydric depending on experimental conditions (Medrano *et al.* 2003; Chaves *et al.* 2010; Lovisolo *et al.* 2010). In the present study, although environmental conditions were generally similar, a somewhat higher daily irradiance and ET_0 in July – when the experiments with Chardonnay were done – and August – when Grenache and Syrah were evaluated – may have induced differences in the hydric behaviour between varieties. Other factors that could have influenced these behaviours are the fact that different rootstocks were used in the different varieties, as well as initial differences in total leaf area among them (Table 2). In summary, although we cannot say that we examined iso-hydric versus aniso-hydric *varieties*, certainly we have analyzed the responses to water stress and recovery in plants displaying iso-hydric versus aniso-hydric *behaviours* under similar climatic conditions.

So, irrespective of whether their iso- or anisohydric behaviours observed here are genetically-, structurally- and/or environmentally-driven, Grenache presented lower stomatal conductance and transpiration even under irrigation, for which it took more time to deplete water from the substrate (Fig. 3). These plants had been previously shown to keep midday leaf water potential higher than -0.9 MPa through the entire experiment (Pou *et al.* Unpublished). Chardonnay instead showed the largest g_s and E under irrigation, it rapidly depleted water and reached the desired low AWA, and suffered the largest drops of Ψ_{MD}, to -1.3 MPa, despite presenting a lower total leaf area, i.e. lower evaporative surface (Table 2). In the present study Syrah presented a near-isohydric behaviour, contrary to some reports (Schultz 2003; Soar *et al.* 2006) but in agreement with others (Collins *et al.* 2010).

Although iso- and anisohydric behaviours have been reported to depend on ABA physiology (Tardieu and Simoneau, 1998; Soar *et al.*, 2006), in grapevines and other woody species it was previously suggested to also strongly depend on plant and leaf hydraulics (Schultz 2003; Bucci *et al.* 2005; Vandeleur *et al.* 2009; Lovisolo *et al.* 2010). We previously showed that the relationship between the whole plant hydraulic conductivity (Kh_{plant}) – as estimated from Ohm's law – and Ψ_{MD} was steeper in Grenache and Syrah than in Chardonnay, i.e. the varieties showing isohydric behaviour in this study (Pou *et al.* Unpublished). Therefore, Grenache and Syrah showed a stronger response of Kh_{plant} to water stress. Furthermore, in the present study, larger maximum Kh_{lamina} and higher g_s were observed in Chardonnay than in the two isohydric-behaving varieties (Fig. 4), thus expanding the previous observations. Moreover, a stronger linear dependency was observed between Kh_{lamina} and g_s (Fig. 7A), i.e. the isohydric-behaving plants showing stronger decreases of Kh_{lamina} in response to stress also close stomata to a larger extent, avoiding decreases in Ψ_{MD}. A number of previous studies have demonstrated a correlation between the liquid phase conductance from soil-to-leaf and g_s or E (Meinzer and Grantz 1990; Meinzer *et al.* 1995; Saliendra *et al.* 1995). The correlation results from an active response of stomata to leaf hydraulics because when the latter is experimentally changed, there is an almost immediate change in g_s (Salleo *et al.* 2000; Hubbard *et al.* 2001). However, and contrary to what was expected, both Kh_{lamina} and g_s recovered faster after re-watering in Chardonnay than in the other two varieties despite larger Kh_{lamina} decreases. This suggests that cavitation, if any, was not large under the present moderate water stress conditions. Cavitation thresholds of -1.5 MPa are often described in grapevines (Lovisolo *et al.* 2008a, 2010), although lower thresholds have been reported in other studies (Schultz 2003). It is likely that regulation of Kh_{lamina} in the plants studied may have been driven by different mechanisms, which perhaps could involve aquaporins. Indeed, leaf aquaporins decrease their expression during acclimation of grapevine plants to water stress and specially during re-watering (Galmés *et al.* 2007), and blocking aquaporin activity with mercurial largely reduces hydraulic conductance (Lovisolo and Schubert 2006; Lovisolo *et al.* 2008b). Moreover, it has been suggested that aquaporins are involved in the recovery after

water stress of shoots (Lovisolo and Schubert 2006) and roots (Lovisolo et al. 2008b) of grapevines. Finally, aquaporins have been described to be on the basis of iso- versus anisohydric behaviour in grapevines (Vandeleur *et al*. 2009).

It was also confirmed that responses of g_s to water stress are faster and stronger in the plants showing isohydric behaviour than in those showing anisohydric response, while its recovery upon re-watering is faster in the anisohydric (Fig. 4A). This causes the maintenance of higher Ψ_{MD} in isohydric plants during water stress, but at the expense of reduced net CO_2 assimilation (Pou et al. unpublished). The maintenance of higher g_s in Chardonnay during water stress and recovery, while water availability was identical to the other varieties, may be due to either different ABA synthesis and/or stomatal response to ABA (Davies *et al.* 2002; Soar *et al.* 2006), to larger water extraction due to increased root aquaporins (Vandeleur *et al.* 2009) or to osmotic adjustments contributing to the maintenance of open stomata at lower water potentials (Patakas and Noitsakis, 1997). However, contrary to early reports (Soar *et al.* 2006), the responses of g_s to increased LAVPD are not stronger in isohydric plants. Although the ranges of LAVPD attained in this experiment by each variety differed, being the lowest in Chardonnay, extrapolation of the slopes of g_s response to LAVPD may allow comparisons among varieties. In irrigated plants, the slope of this relationship (Fig. 6A) was somewhat steeper in Grenache than in Syrah, confirming the results of Soar et al. (2006). However, under water stress the relationships for the three varieties appeared as parallel, i.e. with the same slope. The extrapolation of these linear relationships shows that, at common LAVPD, Grenache would show the largest g_s rather than the lowest, which would correspond to Chardonnay. In another variety showing anisohydric behaviour, such as Semillon (Rogiers *et al.* 2009), higher maximum g_s resulted in a higher sensitivity of g_s to changes in LAVPD than either Syrah or Grenache. This study and the present confirm previous reports that species with high g_s at low VPD had greater sensitivity to VPD (Oren et al. 1999). Stomatal responses of isohydric and anisohydric-behaving plants to air humidity deserve better attention in future studies.

As for water use efficiency, Poni *et al.* (2007) showed that in isohydric plants the increase of intrinsic water use efficiency (WUE_i), i.e. A_N/ g_s in response to water

stress was more pronouncedly than in anisohydric plants. Instead, Lovisolo *et al.* (2010) did not find any difference in WUE_i in a broader comparison of many iso- and anisohydric varieties. In the present study, A_N/g_s of all three varieties reached similar values after one week of water stress acclimation (Fig. 5E). However, the two isohydric-behaving varieties increased A_N/g_s earlier in the imposition of water stress and kept it larger during the first day of recovery. Therefore, averaging the entire experimental period A_N/g_s was higher in isohydric than in the anisohydric plants. A sustained down-regulation of stomatal conductance after re-watering at time with substantial photosynthesis recovery kept WUE_i of previously stressed plants higher than in controls for a week after recovery. This has been already observed in several species, including grapevines (Bogeat-Triboulot *et al.* 2007; Gallé *et al.* 2007; Flexas *et al.* 2009). While the reasons are not fully clear, Pou *et al.* (2008) showed it to be related with incomplete recovery of hydraulic conductivity. This is confirmed by the present results, since Kh_{lamina} also shows slow recovery after water stress (Fig. 4B) and the linear correlation between g_s and Kh_{lamina} is maintained during recovery (Fig. 7A).

WUE_i is a parameter often used when comparing WUE of different species and varieties because it allows comparisons at different LAVPD, and hence between different experimental conditions (Osmond et al. 1980), and because A_N/g_s and not A_N/E is directly related to other more stable indicators of WUE, such as $\delta^{13}C$ which is often used for broad comparisons among many varieties and breeding programs (Gibberd *et al.* 2001; Souza *et al.* 2005 Schultz and Stoll 2010). However, it should be kept in mind that what matters in terms of carbon and water balances is A_N/E (WUE_{inst}), i.e. the carbon gain per unit real water loses. Schultz and Stoll (2010) already alerted that frequently A_N/g_s and A_N/E can go in opposite directions. Since $E = g_s \times LAVPD$, and reduced g_s results in increased LAVPD due to leaf heating, A_N/E could be maintained or even reduced while A_N/g_s is increased. Schultz and Stoll (2010) showed a good example of this in an experiment with Syrah in which, under moderate water stress WUE_i increased while WUE_{inst} was kept constant, and under severe stress WUE_i was further increased while WUE_{inst} decreased below irrigated values. A similar pattern occurs here when comparing isohydric and anisohydric

plants. Because of its ability to keep leaf temperature closer to air temperature, Chardonnay endured lower LAVPD during the experiment. This is partly due to its higher transpiration rates under irrigation and during the imposition of water stress, but also because of slight climatic differences between the days the different varieties were measured (for instance, in the 'acclimation' period g_s and T_{leaf}-T_{air} were identical for the three varieties, yet Chardonnay still presented lower LAVPD due to higher relative humidity on that particular day). Consequently, during acclimation to water stress, while Chardonnay showed higher g_s, E was lower (Fig. 5A) and hence, while A_N/g_s was lower (in WS and REC1) or similar (in ACCL) in Chardonnay as compared with the the other two varieties, A_N/E was significantly higher (Fig. 5F). Therefore, while WUE_i average over the entire experimental period was higher in Grenache and Syrah than in Chardonnay, the opposite was true for $WUE_{inst.}$ That is, on average, the real WUE at the leaf level was higher for the anisohydric-behaving Chardonnay.

The maintenance of higher average WUE_{inst} during the experiment in Chardonnay is also partly because this variety maintains better photosynthesis stability. In addition to somewhat lower stomatal closure, the other two main determinants of photosynthesis, i.e. mesophyll conductance to CO_2 (g_m) and photosynthetic photo- and biochemistry (reflected here by J_{flu}), remained higher under water stress in Chardonnay than in the other two varieties. g_m was unaffected during the entire experiment in Chardonnay, while in Grenache and Syrah it strongly decreased early under water stress to partially recover during acclimation (Fig. 5C). J_{flu} was initially unaffected in Chardonnay and slightly decreased by the end of acclimation, rapidly recovering one day after irrigation. In Grenache and Syrah, instead, J_{flu} was largely affected at the beginning of water stress, it partially recovered during acclimation and it was fully recovered only two days after re-watering. In consequence, A_N was on average much larger in Chardonnay than in Syrah and Grenache. In grapevines, as in other species, photosynthetic metabolism has been shown to directly depend on g_s, not Ψ_{MD} (Flexas et al. 2002, 2004; 2006a). Based on the status of the photosynthetic apparatus, Medrano et al. (2002) established three ranges of g_s corresponding to mild (photosynthesis limited only by diffusional

limitations), moderate (g_m limitations and some biochemical limitations appear) and severe water stress (photosynthesis is mostly limited by biochemical limitations). The threshold value between mild and moderate water stress was at g_s around 0.15 mol H_2O m^{-2} s^{-1}, and that between moderate and severe stress 0.05-0.10 H_2O m^{-2} s^{-1}. By closing stomata earlier in response to water stress, isohydric plants reach the moderate water stress threshold faster than anisohydric ones, and hence suffer g_m and metabolic limitations at the same AWA at which anisohydric plants' photosynthesis is still limited by stomatal closure only. This may allow maintaining higher photosynthesis under water stress as well as faster recovery after re-watering (Flexas et al. 2006b), as indeed occurred in the present study.

In summary, the results of the present study reinforce the view that a given variety can behave as iso- or anisohydric depending on the study conditions. While Grenache confirmed its reputation as isohydric and Chardonnay as anisohydric, Syrah, a variety often considered as an anisohydric model plant showed here a near-isohydric behaviour. Moreover, the results do not support the idea that isohydric behaviour results in a better performance under water stress conditions. Indeed, under the moderate water stress established in this study and frequently occurring by vineyards under Mediterranean conditions, Chardonnay showed some advantages over the two varieties displaying near-isohydric behaviour. By delaying its adjustment of Kh_{lamina} in response to water stress, Chardonnay delays stomatal closure, which in turn results in the maintenance of higher photosynthesis and photosynthetic capacity, favoring faster recovery upon re-watering. Furthermore, a recent study on *Lolium perenne* (Marika *et al.* 2010) has shown that a rapid hydraulic recovery and an hydraulic "buffering" effect on assimilation rate under water stress, enable this species for a substantial increase of dry matter yield per unit of water added (i.e. water use efficiency) by displaying anisohydric behaviour. Consistent with this, Chardonnay showed the smallest reduction of whole plant biomass production under watered stress (15% reduction, as compared to 27% and 36% reductions in Syrah and Grenache, respectively). In contrast total leaf area suffered the greatest reductions in this cultivar (Table 2), possibly favoring faster recovery of the remaining leaves after re-watering. Contrarily, at similar water availability, the two

varieties showing isohydric behaviour showed strong adjustments of Kh_{lamina} and g_s, lowering photosynthesis and photosynthetic capacity and delaying recovery. Therefore, spanned over a period of about 20 days consisting of water stress imposition, acclimation and recovery Chardonnay displayed higher CO_2 assimilation than Grenache and Syrah, which implies a higher yield potential under these conditions, as well as, clearly higher WUE_{inst} under water stress and early recovery. In contrast, the conservative behaviour of Grenache and Syrah allowed them to maintain high minimum leaf water potentials, while in Chardonnay the values dropped close to those commonly inducing cavitation in grapevines. These results suggest that under more severe stress conditions isohydric-behaving plants may have some advantage over anisohydric ones, but to confirm this further experiments should be performed.

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4.4. PHOTOSYNTHESIS LIMITATIONS DURING WATER STRESS ACCLIMATION AND RECOVERY IN THE DROUGHT-ADAPTED VITIS HYBRID RICHTER-110 (*V. berlandieri* × *V. rupestris*)

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Running title: Photosynthesis during acclimation to and recovery after water stress.

ABSTRACT

The hybrid Richter-110 (*Vitis berlandieri* × *Vitis rupestris*) has the reputation of being a genotype strongly adapted to drought. A study was performed with plants of R-110 subjected to sustained water withholding to induce acclimation to two different levels of water stress, followed by re-watering to induce recovery. The goal was to analyze how photosynthesis is regulated during acclimation to water stress and recovery. In particular, the regulation of stomatal conductance (g_s), mesophyll

conductance to CO_2 (g_m), leaf photochemistry (chlorophyll fluorescence and thermoluminescence) and biochemistry ($V_{c,max}$) were assessed.

During water stress, g_s declined to 0.1 and 0.05 mol CO_2 m^{-2} s^{-1} in moderately and severely water stressed plants, respectively, and was kept quite constant during an acclimation period of one-week. Leaf photochemistry proved to be very resistant to the applied water stress conditions. In contrast, g_m and $V_{c,max}$ were affected by water stress, but they were not kept constant during the acclimation period. g_m was initially unaffected by water stress, and $V_{c,max}$ even increased above control values. However, after several days of acclimation to water stress both parameters declined below (g_m) or at ($V_{c,max}$) control values. For the latter two parameters there seemed to be an interaction between water stress and cumulative irradiance, since both recovered to control values after several cloudy days despite water stress. A photosynthesis limitation analysis revealed that diffusional limitations and not biochemical limitations accounted for the observed decline in photosynthesis during water stress and slow recovery after re-watering, both in moderately and severely stressed plants. However, the relative contribution of stomatal (SL) and mesophyll conductance (MCL) limitations changes during acclimation to water stress, from predominant SL early during water stress to similar SL and MCL after acclimation. Finally, photosynthesis recovery after re-watering was mostly limited by SL, since stomatal closure recovered much more slowly than g_m.

Keywords

Stomatal conductance, mesophyll conductance, water stress, drought, photosynthetic limitations, photochemistry, Rubisco, thermoluminescence.

Abbreviations

ABA, abscisic acid; A_N, light-saturated net photosynthesis; C_a, atmospheric CO_2 concentration; C_c, chloroplast CO_2 concentration; C_i, sub-stomatal CO_2 concentration; C_i*, C_i at the CO_2 compensation point in the absence of mitochondrial respiration; g_m, mesophyll conductance to CO_2; g_s, stomatal conductance; HTL, high temperature thermoluminescence; J_{flu}, electron transport rate determined by chlorophyll fluorescence; J_{max}, maximum rate of electron transport; R-110, Richter-110 (an hybrid of *Vitis berlandieri* × *Vitis rupestris*); R_D, leaf respiration in the dark; R_L, leaf respiration in the light; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; RuBP, ribulose-1,5-bisphosphate; TL, thermoluminescence; τ, Rubisco specificity factor; Γ*, C_c at the CO_2 compensation point in the absence of mitochondrial respiration; $V_{c,max}$, maximum rate of carboxylation; VPD, vapor pressure deficit.

INTRODUCTION

Low water availability is the main environmental factor limiting plant growth and yield worldwide, and global change will likely make water scarcity an even greater limitation to plant productivity across an increasing amount of land (Chaves *et al*., 2008). The limitation of plant growth imposed by low water availability is mainly due to reductions of plant carbon balance, which is largely dependent on photosynthesis. For this reason, photosynthesis responses to water stress have been subject of study and debate for decades, in particular concerning which are the most limiting factors for photosynthesis under water stress (Flexas and Medrano, 2002; Lawlor and Cornic, 2002).

Reduced CO_2 diffusion from the atmosphere to the site of carboxylation is the main cause for decreased photosynthesis under most water stress conditions (Centritto *et al*., 2003; Flexas *et al*., 2004; Grassi and Magnani, 2005; Chaves e*t al*., 2008; Erismann *et al*., 2008; Peeva and Cornic, 2009). Although in many water stress situations photosynthetic reductions cannot be fully explained by stomatal closure alone, early experiments by Kaiser and others (reviewed in Kaiser, 1987 and Cornic *et al*., 1992) showed total restoration of photosynthesis when very high CO_2 concentrations was applied. Moreover, Cornic *et al*. (1989), using chlorophyll fluorescence emission, suggested the importance of changes under drought of the CO_2 resistance path from ambient air to carboxylating sites. Therefore, reduced leaf diffusive capacity is not only due to stomatal closure, but also to reduced mesophyll conductance to CO_2 (g_m). Both the physiological bases and the role of g_m remain elusive although there is now evidence that g_m can vary at least as fast as stomatal conductance (Flexas *et al*., 2007*a*, 2008), and it has been suggested that some aquaporins are involved in its regulation (Hanba *et al*., 2004; Flexas *et al*., 2006*a*), particularly under water stress (Miyazawa *et al*., 2008). Regardless of the mechanisms for regulation of g_m, the response of photosynthesis to soil water shortage can be divided into two distinct phases: during the first stage, characterized by a daily maximum stomatal conductance (g_s) above 0.05-0.10 mol H_2O m^{-2} s^{-1}, photosynthesis is mostly limited by restricted CO_2 diffusion (decreased g_s plus decreased g_m); during the second stage, characterized by stomatal conductance below

that threshold, a general metabolic impairment can eventually occur, particularly under conditions of high light intensity that favor oxidative stress (Flexas *et al*., 2004, 2006*b*; Zhou *et al*., 2007).

The photosynthetic responses described above proceed, in general, from studies in which water stress was applied to plants over relatively short periods, and in which measurements are taken on specific, often few days during the experimental period. However, under natural conditions water stress normally develops much more gradually, over periods comprising weeks or months, and hence it is possible that some acclimation occurs, in addition to day-to-day variations in response to variable environmental conditions (Flexas *et al*., 2006*b*). Acclimation to water stress may comprise responses involving gene expression and modification of plant physiology and morphology, taking place in days to weeks, which lead to a homeostatic compensation for the initial negative effects of water stress on photosynthesis. In leaves acclimated to water stress during their development, a higher photosynthesis rate than in non-acclimated leaves if often associated with morphological adaptations and higher electron transport rates (Maury *et al*., 1996, Kitao *et al*., 2003; Galmés *et al*., 2006). However, less is known about short-term acclimation to water stress in already developed leaves. Although the general idea is that morphological changes and osmotic adjustments may be a long-term acclimation, recent studies on transcriptomics and proteomics in plants subjected to water stress showed that the genes or proteins associated with metabolism display acclimation responses in less than a week after drought stress imposition (Watkinson *et al*., 2003; Bougeat-Triboulot *et al*., 2007). In such studies, photosynthetic pathways are in general not among the most altered by the stress (reviewed in Chaves *et al*., 2008). Even in those photosynthetic genes responding to stress, the most common trend is a down-regulation, i.e., they would not contribute to acclimation of photosynthesis, but rather to its further decline. In addition, the alterations found at transcriptomic level are larger (5-10%) than at protein level (usually less than 1%). In summary, there is little evidence for acclimation of photosynthesis to water stress conditions in the short term, but studies that specifically address this issue are needed. Moreover, day-to-day

variations of photosynthesis during an acclimation period may lead to erroneous conclusions when measurements are taken on a single day during the period.

Besides acclimation, the carbon balance of a plant during a period of water stress and recovery may depend as much on the velocity and degree of photosynthetic recovery as it depends on the degree and velocity of photosynthesis decline during water depletion (Flexas *et al*., 2006*b*). In general, plants subjected to severe water stress recover only 40-60% of the maximum photosynthesis rate during the day after re-watering, and recovery continues during the next days, but maximum photosynthesis rates are not always recovered (Kirschbaum, 1987, 1988; Sofo *et al*., 2004). The strong influence of previous water stress severity in the velocity and extent of photosynthesis recovery has been illustrated in kidney bean by Miyashita *et al*. (2005) and has been suggested for *Vitis vinifera* as well (Gómez-del-Campo *et al*., 2007). Over the last three years, many studies have addressed the response of photosynthesis to re-watering after water stress, which highlights the importance of the issue, but none of these studies have analyzed all the potential physiological limitations to recovery (dos Santos *et al*., 2006; Grzesiak *et al*., 2006; Hura *et al*., 2006; Bogeat-Triboulot *et al*., 2007; Gallé and Feller, 2007; Gallé *et al*., 2007; Gómez-del-Campo *et al*., 2007; Montanaro et al., 2007; Pérez-Pérez *et al*., 2007; Gomes *et al*., 2008; Luo *et al*., 2008). Early works by Kirschbaum (1987, 1988) suggested that recovery after a severe drought was a two-stage process, the first involving leaf re-watering and stomata re-opening, and the second, long lasting period requiring de novo synthesis of photosynthetic proteins to over-come biochemical limitations. However, it should be noticed that at that time g_m was not considered a possible limitation to photosynthesis. Hence any mechanism not involving stomata was ascribed to a biochemical limitation, i.e., to impairment of the primary photosynthetic machinery of the plant. More recently, Gomes et al. (2008) applied a photosynthesis limitation analysis to show that, in general, mesophyll limitations were more important than stomatal limitations during recovery, but also in this study the role of biochemistry and mesophyll diffusion conductance on mesophyll limitations were not separated. Galmés *et al*. (2007*a*) were the first to apply the photosynthesis limitation analysis proposed by Grassi and Magnani (2005)

to ten different Mediterranean species, and showed that limited recovery of g_m was the main limiting factor for photosynthesis recovery the day after re-watering in many of these plants. However, it would be necessary to apply this analysis in plants subjected to different water stress intensities, and to span it to longer time periods. On the other hand, in some species including the *Vitis* hybrid R-110 (*Vitis berlandieri* x *rupestris*), a sustained down-regulation of stomatal conductance after re-watering imposes a substantial limitation to photosynthesis recovery, at the time that it increases the intrinsic water-use efficiency (Bogeat-Triboulot *et al*., 2007; Gallé and Feller, 2007; Gallé *et al*., 2007; Pou *et al*., 2008).

Therefore, current knowledge about physiological limitations to photosynthesis during short-term acclimation to different water stress intensities and recovery after re-watering is scarce, but crucial to improve the understanding of plant responses to drought and for the development of water saving irrigation schedules in agriculture (Flexas *et al*., 2006*b*). Of particular interest would be to analyze such responses in species adapted to water stress conditions, such as those found in Mediterranean regions. Among well-adapted crops, grapevine (*Vitis vinifera* L.) is especially interesting since it performs most of its phenological cycle during summer under non-irrigation conditions. The hybrid *Vitis* Richter-110 (*Vitis berlandieri* × *Vitis rupestris*) is often used as a rootstock in semi-arid viticultural areas, and it is especially well adapted to water stress conditions. In previous works, we have shown that R-110 presents a completely isohydric behavior under water stress, during acclimation and during recovery after re-watering, i.e. it is able to maintain homeostasis in its leaf water relations regardless of decreased soil water availability and replenishment (Pou *et al*., 2008). This is achieved by keeping high root and stem hydraulic conductivities, which involves complex changes in the expression of all major putative aquaporins (Galmés *et al*., 2007*b*), as well as a strong fine stomatal regulation in response to water stress and leaf-to-air vapor pressure deficit, which is achieved by a mechanism combining variations in leaf abscisic acid content and hydraulic conductivity, leading to high water-use-efficiency (Pou *et al*., 2008). In summary, R-110 seems especially well adapted to drought with respect to other *Vitis* genotypes and, under the experimental stress conditions applied in the present study,

it shows higher photosynthesis, growth and water use efficiency than cultivars of *Vitis vinifera* such as Grenache and Syrah (Pou *et al*., unpublished results).

The aims of the present work were to analyze in the drought-adapted R-110 how photosynthesis is regulated by different physiological limitations under water stress imposition, acclimation and recovery. Our hypothesis were: (1) that down-regulation of mesophyll conductance to CO_2 may impose a limitation to photosynthesis of similar magnitude to that imposed by stomatal closure during acclimation to water stress in such drought-adapted species, while impairment of leaf photochemistry and biochemistry may be low; (2) that during acclimation, day-to-day variations in leaf photosynthetic properties may play a role in setting the overall photosynthesis limitations during the period; and (3) that photosynthesis recovery after re-watering may be mostly limited by diffusional limitations, rather than by biochemical limitations.

MATERIAL AND METHODS

Plant material and water stress treatments

Plants of Richter-110 (*Vitis berlandieri* × *Vitis rupestris*) were subjected to water withholding followed by re-watering during summer 2005 at the Universitat de les Illes Balears (Mallorca, Spain), as described with detail by Pou *et al*. (2008). Briefly, one-year old plants were used, growing outdoors in 30 L pots filled with a mixture of clay soil and organic substrate. Control plants were daily irrigated at field capacity, while plants in which irrigation was stopped were divided in two groups, corresponding to two different levels of water stress defined by the leaf maximum daily stomatal conductance (g_s), as suggested by Flexas *et al*. (2002): moderate water stress (g_s near 0.15 mol H_2O m^{-2} s^{-1}) and severe water stress (g_s near 0.05 mol H_2O m^{-2} s^{-1}). The first level of g_s was reached four days after stopping irrigation, while the second was achieved eight days after stopping irrigation. Once the desired water stress was obtained, plants were maintained at constant water stress for a week to assess possible acclimation. This was achieved by daily replacing the amount of water consumed, as determined by weighting of pots every evening. After one week

at the established soil water deficit, all plants were re-watered to field capacity and recovery was followed for several days.

Instantaneous gas exchange and chlorophyll fluorescence measurements, and corrections for C_i

Instantaneous gas-exchange and chlorophyll fluorescence measurements were taken daily, between noon and 1 P.M. local time, on 10-12 leaves from different plants per treatment, using an open gas-exchange system (Li-6400; Li-Cor, Inc., Lincoln, NE, USA) with an integrated fluorescence chamber head (Li-6400-40; Li-Cor Inc., Nebraska, USA). No measurements were taken by days 4 and 5 due to rainfall, and by day 9 due to technical problems with the Li-6400. All measurements were made on young, fully expanded leaves, at 1500 μmol m^{-2} s^{-1}, with a CO_2 concentration in the leaf cuvette of 400 μmol CO_2 mol^{-1} air. Block temperature was kept at 30ºC during all measurements, and the registered leaf temperatures ranged between 30 and 34ºC (Fig. 1). Respiration in the light or 'day' respiration (R_d), the apparent CO_2 photocompensation point (C_i*), and photosynthesis responses to CO_2 (A_N-C_i curves) were determined only on five specific sampling days per each treatment: the day the desired stomatal conductance was first achieved, seven days after sustaining the plants at constant soil moisture, just before re-watering ('acclimation'), and then 1, 3 and 7 days after re-watering.

From instantaneous measurements, net CO_2 assimilation (A_N), stomatal conductance (g_s) and the sub-stomatal CO_2 concentration (C_i) were recorded. However, C_i values can be overestimated due to two main problems that have been described particularly under water stress: an increasing importance of the cuticular conductance to vapour pressure as stomata get closed (Boyer *et al*., 1997) and heterogeneous ('patchy') stomata closure (Laisk, 1983; Buckley *et al*., 1997). We calculated leaf cuticular conductance and tested for patchy stomatal closure as detailed below.

Vitis are hypostomatous species, and hence cuticular conductance was estimated in three different ways: (1) measuring it with the IRGA on leaves with the abaxial surface covered with silicone grease and a polyethylene filter to prevent stomatal gas exchange (Boyer *et al*., 1997); (2) measuring gas exchange of leaves at

night (Kerstiens, 1996), although there is now evidence that this could overestimate cuticular conductance due to incomplete stomatal closure at night (Kerstiens, 2006); and (3) by determining cuticular transpiration after turgor loss during the measurements of pressure-volume curves (Burghardt and Riederer, 2003). The three methods yielded similar values, of 0.007 ± 0.001, 0.006 ± 0.001 and 0.008 ± 0.001 mol H_2O m^{-2} s^{-1}, respectively, which were similar to those found by Boyer *et al.* (1997) for another *Vitis* species but, contrary to Boyer et al. (1997) without any significant difference between treatments. Therefore, a value of 0.007 mol H_2O m^{-2} s^{-1} was used to re-calculate g_s and C_i as described previously (Boyer *et al.*, 1997; Flexas *et al.*, 2002).

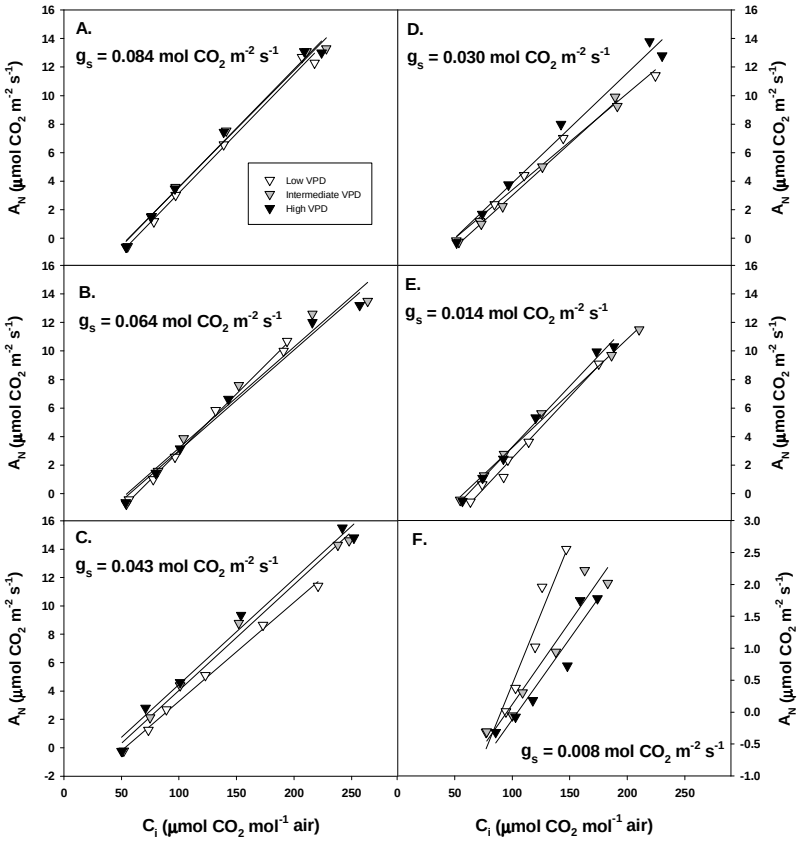

Figure 1. The relationship between net photosynthesis (A_N) and substomatal CO_2 concentration (C_i) on leaves subject to increasing VPD. Representative examples of leaves differing in stomatal conductance are shown. VPD conditions: white triangles represent low VPD (typically 1.7-2.1 KPa), grey triangles represent intermediate VPD (typically 2.2-2.5 KPa), and black triangles represent high VPD (typically 2.6-3.6 KPa).

In order to detect symptoms of stomatal patchiness, two different checks were performed. In the first, chlorophyll fluorescence was measured in different areas of the leaf blade (Flexas *et al.*, 2002). Five to six patches were measured over each leaf, and the differences in fluorescence parameters were usually lower than 10%, except over leaf veins (data not shown). In the second, the initial slope of several phototosynthetic response curves to intercellular CO_2 concentration (A_N-C_i curves) on the same leaf was determined under conditions of increasing vapor pressure deficit (VPD) and decreasing stomatal conductance, following Grassi and Magnani (2005). As it can be observed (Fig. 1), when g_s was above 0.06 mol CO_2 m^{-2} s^{-1} all three curves looked identical, which was interpreted as an evidence of the absence of patchiness (Fig. 1A, B). For g_s values ranging between 0.01 and 0.06 mol CO_2 m^{-2} s^{-1} some deviation was observed (Fig. 1C-E), but still very minor to consider it causing a significant bias in the value of C_i. Only when g_s dropped below 0.01 mol CO_2 m^{-2} s^{-1} (Fig. 1F) there was clear evidence of impairment of the calculation of C_i. Since these low g_s values were never averaged by any treatment during the experiment, no correction to account for patchiness was done in the calculation of C_i.

Concerning chlorophyll fluorescence, the actual photochemical efficiency of photosystem II (ϕ_{PSII}) was determined by measuring steady-state fluorescence (Fs) and maximum fluorescence during a light-saturating pulse of ca. 8000 μmol m^{-2} s^{-1} (Fm') following the procedures of Genty *et al*. (1989):

$$\phi_{PSII} = (Fm'-Fs)/Fm' \qquad [1]$$

The electron transport rate (J_{flu}) was then calculated as:

$$J_{flu} = \phi_{PSII} \cdot PPFD \cdot \alpha \cdot \beta \qquad [2]$$

where PPFD is the photosynthetically active photon flux density, α is leaf absorptance and β reflects the partitioning of absorbed quanta between photosystems II and I. The product $\alpha \cdot \beta$ was determined, following Valentini *et al*. (1995), from the relationship between ϕ_{PSII} and ϕ_{CO2} obtained by varying either light intensity under non-photorespiratory conditions in an atmosphere containing less than 1% O_2. The obtained relationship showed a slope of 10.6 with almost zero-intercept, and no significant differences were observed between treatments (Fig. 2A).

The maximum quantum efficiency of PSII (Fv/Fm = (Fm-Fo)/Fm) was determined for two different days at pre-dawn (6:00AM local time). NPQ at mid-morning was calculated each day using the closest measured pre-dawn Fm (NPQ = (Fm-Fm')/Fm').

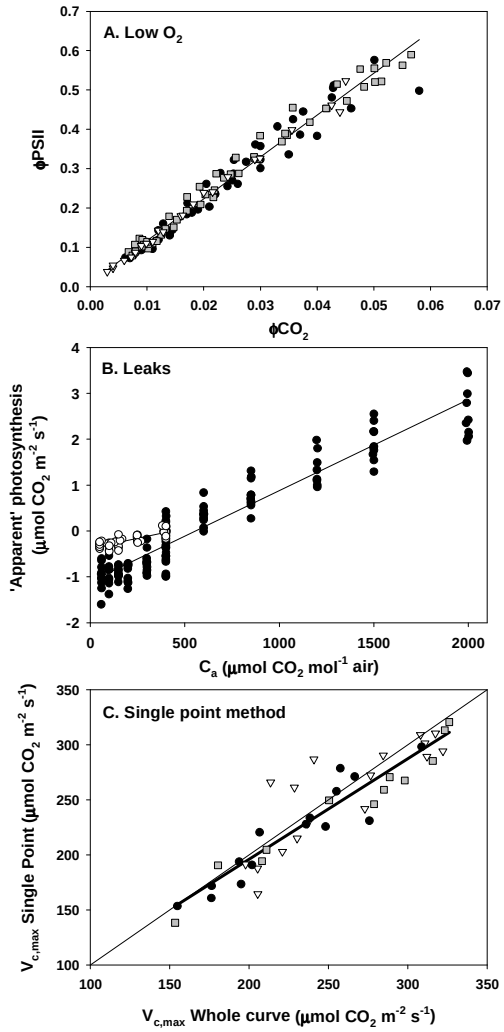

Figure 2. (A) The relationship between photochemical efficiency of photosystem II (Φ_{PSII}) and Φ_{CO2} [(A_N + R_d)/PPFD] under non-photorespiratory conditions (less than 1% O_2) in Richter-110 leaves under irrigation (black circles), moderate drought (gray triangles) and severe drought (white triangles). (B) The responses of leakage CO_2 flow from the gas exchange cuvette ('apparent net photosynthesis') to CO_2 concentration (C_a) in the 2 cm^2 chamber (filled circles) and the 6 cm^2 chamber (empty circles) filled with a dead leaf. Ranges of C_a are different in each case because a 2 cm^2 chamber was used for entire A_N-C_i curves, while a 6 cm^2 chamber was used for the 'Laisk method', with a more limited C_a range (see Material and Methods). (C) The relationship between the maximum capacity for carboxylation ($V_{c,max}$) determined using the single point method and using the whole A_N-C_i curve in single replicates for plants under irrigation (black circles), moderate water stress (gray squares) and severe water stress (white triangles). The thick line is the best-fit while the thin line represents the 1:1 relationship.

Respiration in the light, apparent CO_2 photocompensation point and A_N-C_i curves

Respiration in the night (R_n) was determined on several days at pre-dawn using the Li-6400. Respiration in the light or 'day' respiration (R_d) and the apparent CO_2 photocompensation point (C_i*) were determined according to the method of Laisk (1977) as described in von Caemmerer (2000). Briefly, A_N-C_i curves were measured at three different PPFDs (50, 200, and 500 μmol m^{-2} s^{-1}) at six different CO_2 levels ranging from 300 to 50 μmol CO_2 mol^{-1} air, using the 6-cm^2 leaf chamber. The intersection point of the three A_N-C_i curves was used to determine C_i* (x-axis) and R_d (y-axis). C_i* was used as a proxy for the chloroplastic CO_2 photocompensation point (Γ*), according to Warren and Dreyer (2006). According to Galmés *et al*. (2006), only C_i* values for irrigated plants were considered, which averaged 42.0 ± 0.9 μmol CO_2 mol^{-1} air at a leaf temperature of 30ºC, i.e. a Γ* of 43.1 μmol CO_2 mol^{-1} ($\Gamma^* = C_i^* + R_d/g_m$), corresponding to a Rubisco specificity factor of 90. Considering published Γ* response functions to temperature for several species (reviewed by Warren and Dreyer, 2006), this would correspond to a Rubisco specificity factor of about 100 at 25ºC, i.e., totally agreeing with the actually determined value for *Vitis* (Bota *et al*., 2002).

CO_2-response curves were performed in light adapted leaves of different plants for each day and treatment, using two Li-6400 units simultaneously. Photosynthesis was induced with a PPFD of 1500 μmol m^{-2} s^{-1} (previously performed light response curves had shown this to be sufficient light to saturate photosynthesis) and 400 μmol mol^{-1} CO_2 surrounding the leaf (C_a). The amount of blue light was set to 10% PPFD to maximize stomatal aperture. Air temperature was kept at 30ºC, and leaf-to-air vapor pressure deficit was different depending on the day and treatment, but kept within a variation of 0.5 KPa during the performance of a single curve. Once steady state was reached (usually 30 minutes after clamping the leaf), a CO_2-response curve was performed. Gas exchange and chlorophyll fluorescence were first measured at 400 μmol mol^{-1}, then C_a was decreased stepwise until 50 μmol mol^{-1}. Upon completion of measurements at low C_a, this was returned to 400 μmol mol^{-1} to restore the original A_N. Then C_a was increased stepwise until 2000 μmol mol^{-1} to

complete the curve. The number of different C_a values used for the curves was 12 and the time lag between two consecutive measurements at different C_a was restricted to 2-4 minutes, so that each curve was completed in 30-40 minutes.

Leakage of CO_2 in and out the leaf cuvette was determined as described for the range of CO_2 concentrations used in this study with photosynthetically inactive leaves (obtained by heating the leaves until no variable chlorophyll fluorescence was observed) enclosed in the leaf chamber (Flexas *et al*., 2007*b*). The leakage values obtained did not differ significantly among days, so they were pooled together (Fig. 2B) and the average relationship was used to correct the measured leaf fluxes for the entire experiment. The higher number of points at the lower end of the relationship (Fig. 2B) was because test for leakage was made also for the 6-cm^2 leaf chamber used for the 'Laisk' method.

Estimation of photorespiration and g_m by gas exchange and chlorophyll fluorescence

From combined gas-exchange and chlorophyll fluorescence measurements, the photorespiration rate (P_r) was calculated according to Valentini *et al*. (1995). In their model, they assumed that all the reducing power generated by the electron transport chain is used for photosynthesis and photorespiration, and that chlorophyll fluorescence gives a reliable estimate of the quantum yield of electron transport.

Thus, P_r can be solved from data of A_N, R_d and J_{flu}, and from the known stochiometries of electron use in photorespiration, as follows (Valentini *et al*., 1995):

$$P_r = 1/12\ [J_{flu} - 4\ (A_N + R_d)].$$

The method by Harley *et al*. (1992) was used to make estimations of g_m as:

$$g_m = A_N / (C_i - (\Gamma^* \cdot (J_{flu} + 8 \cdot (A_N + R_d)) / (J_{flu} - 4 \cdot (A_N + R_d)))) \qquad [3]$$

were A_N and C_i are taken from gas exchange measurements at saturating light and Γ^* and R_d were estimated using the Laisk (1977) method (see previous section).

The calculated values of g_m were used to convert A_N-C_i curves into A_N-C_c curves using the following equation:

$$C_c = C_i - (A_N / g_m) \qquad [4]$$

From A_N-C_c curves, the maximum carboxylation capacity ($V_{c,max}$) and the maximum capacity for electron transport rate (J_{max}) were calculated using the temperature dependence of kinetic parameters of Rubisco described on a C_c basis by Bernacchi et al. (2002), whereby net assimilation rate is given as:

$$A_N = \min \{A_c, A_q\} - R_d \quad [5]$$

With:

$$A_c = V_{cmax} \frac{C_c - \Gamma *}{C_c + K_c[1 + (o_i / K_o)]} \quad [6]$$

$$A_q = \frac{J(C_c - \Gamma *)}{4(C_c + 2\Gamma *)} \quad [7]$$

where A_c and A_q represent photosynthesis limited by carboxylation and RuBP regeneration, respectively, K_c and K_o are the Rubisco Michaelis-Menten constants for carboxylation and oxygenation, respectively, and o_i is the leaf internal oxygen concentration (assumed equal to the external).

Complete A_N-C_i curves (and hence A_N-C_c curves) were performed in several days. From them, it was confirmed that at ambient CO_2 concentration, net photosynthesis was always in the $V_{c,max}$ region and not in the J_{max} region, regardless of the day and treatment. In order to estimate $V_{c,max}$ for each day of the experiment, even for days in which only instantaneous measurements at ambient CO_2 were available, the 'single point' method described by Wilson *et al.*(2000), modified by Grassi and Magnani (2005) to account for g_m, was used. This method consisted in an estimation of $V_{c,max}$ using a single value of A_N and C_c (at ambient CO_2) plus R_d. For the cases were the whole CO_2 response curve was available together with instantaneous measurements, it was verified that the single point method yielded results similar to those adjusting the entire curve (Fig. 2C).

Quantitative limitation analysis

To partition photosynthesis limitations into components related to stomatal conductance (S_L), mesophyll conductance (MC_L) and leaf biochemical characteristics

(B_L), a modification of the approach proposed by Grassi & Magnani (2005) was considered. At ambient CO_2 concentration, light-saturated photosynthesis is generally limited by substrate availability, which was verified by A_N-C_i curves in the present data for each species and treatment (previous section), i.e. photosynthesis can be expressed using equation [6] (Farquhar *et al.*, 1980). To compare their relative limitations to assimilation due to water stress, acclimation and recovery, photosynthetic limitations were partitioned into their functional components following the approach proposed by Grassi & Magnani (2005), with some modifications. This approach which requires the measurement of A_N, g_s, g_m and $V_{c,max}$, makes it possible to partition photosynthesis limitations into components related to stomatal conductance (S_L), mesophyll conductance (MC_L) and leaf biochemical characteristics (B_L), assuming that a reference maximum assimilation rate can be defined as a standard. In the current study, the maximum assimilation rate, concomitantly with g_s, g_m and $V_{c,max}$, was generally reached under well-watered conditions, therefore the control treatment was used as a reference. However, since A_N of irrigated plants declined during the experiment, presumably due to leaf ageing, the values for irrigated plants *for each day* were considered as the reference for the moderate and stressed plants determined during the same day. In doing so, photosynthesis limitations due to leaf ageing in irrigated plants were assessed by comparing the values along the experiment with the maximum values observed (obtained by the third day of experiment). On the other hand, 'pure' water stress limitations (i.e., without interaction with leaf ageing) were obtained for moderate and severely stressed plants. Whenever one of the involved parameters (g_s, g_m and $V_{c,max}$) was higher in stressed than in irrigated plants, its corresponding limitation was set to zero, and the other limitations re-calculated accordingly.

Finally, non-stomatal limitations were defined as the sum of the contributions of mesophyll conductance and leaf biochemistry ($NS_L = MC_L + B_L$), while diffusive limitations were the sum of stomatal and mesophyll conductance components ($D_L = S_L + MC_L$).

Thermoluminescence measurements

Thermoluminescence (TL) glow curves of *Vitis* R-110 leaf discs were measured using home-built apparatus (SBE-INRA/CEA-Saclay, France), as described with detail by Ducruet (2003), with modifications as in Sajnani *et al*. (2007). Data acquisition and signal analysis were performed using dedicated software developed in Saclay (see Ducruet, 2003, and references therein). The sample cuvette consists in a horizontal cylindrical chamber (2.5 cm diameter) with a copper film on the bottom, stuck on a thermoelectric Peltier plate (model DT 1089-14; Marlow Industries, USA) below the chamber for temperature regulation. A thin thermocouple is placed under the copper film in the centre of the plate. The bottom face of the Peltier element is maintained at a constant temperature by a water flow. A drop of water (100 µl) is placed on the centre and the leaf disc is pressed on the bottom by a washer. A circular Pyrex window between the leaf disc and the washer reduces water loss from the sample during warming (except for high temperature thermoluminescence, HTL, measurements). The common side of a 5-arms light guide (Walz, Effeltrich, Germany) was placed 5 mm above the sample, one arm being used for conveying the luminescence emission to a red-sensitive Hamamatsu H5701-50 analogue photomultiplier through a red filter (>670 nm) and the other arms for different types of illumination. In standard experiments, leaf discs, from plants dark adapted during short (120 min: Day) or long periods (8 h: Night), were punched out under a dim green light then incubated also in darkness for 2 min at 20ºC and cooled to 1ºC during 1 min. At the end of this period leaf discs were subsequently illuminated with one or three saturating single-turnover flashes, of white xenon light XST-103 (Walz, Germany) separated by 1 s or alternatively with a Far Red light (FR) LED 102-FR (Walz) operated at setting 10 during 30 s. Luminescence emission was immediately recorded while warming the sample from 0ºC to 80ºC at a heating rate of 0.5ºC s^{-1}.

HTL measurements were carried out with the same set-up using a similar protocol, but without a covering window to allow drying of the sample during warming, in order to prevent hydrolysis of peroxides at high temperature (Ducruet and Vavilin, 1999). However, the heating rate of the sample was of 0.1ºC s^{-1} and the

measuring range from 10ºC to 160ºC. HTL bands reflect lipid peroxidation in stressed samples.

Determination of ascorbate

Ascorbate (Asc) and dehydroascorbate (DHAsc) were determined using modified bipyridyl methods of Okamura (1980) and Knörzer *et al*. (1996). 0.3-0.4 g of fresh leaf tissue was grind to fine powder in liquid nitrogen, and then homogenized by adding 2 mL of cold metafosphoric acid 5% (w/v). The homogenate was clarified by centrifuging at 13,200 rpm for 15 min at 4ºC. 125 μL supernatant aliquots were used for subsequent determinations. Sample A, for determination of reduced ascorbate (Asc), contained 270 μL of the extract, neutralized with 27 μL 1 N NaOH, 300 μL 150mM sodium phosphate buffer (pH 7.4) and 200 μL H_2O. Sample B, for determination of total ascorbate (Asc+DHAsc), consisted of 270 μL of the extract neutralized as above, mixed with 300 μL 150mM sodium phosphate buffer (pH 7.4) and 100 μL 0.1M dithiothreitol (DTT) to reduce the DHAsc present in the extract. After 15 min incubation at room temperature, the excess of DTT was removed by addition of 75 μL of 0.5% (w/v) N-ethylmaleimide, and the sample was incubated further for 30 s. Subsequently, samples A and B were treated identically. The samples were mixed with 300 μL of 10% (w/v) trichloroacetic acid, 300 μL of 44% (v/v) phosphoric acid, 200 μL of 4% 2,2-bipyridyl (w/v in 70% ethanol) and 150 μL of 3% (w/v) $FeCl_3$, incubated for 1 h at 37ºC, and the A_{525} was recorded. In parallel, standard samples with known amounts of ascorbate, treated identically as the extract probes, were measured. Ascorbate concentration was calculated on the basis of the standard curve. The DHAsc concentration was calculated by subtracting the Asc concentration measured in sample A from the total Asc determined in sample B.

RESULTS

Experimental conditions and plant water status

Climate conditions during the experiment (July-August 2005) were those typical for Mediterranean regions, with midday air temperatures usually above 30ºC and relative humidity below 60% (Pou *et al*., 2008), leading to leaf-to-air vapor

pressure deficits (VPD) typically above 2 KPa and as high as 3.5 KPa when plants were water stressed (Fig. 3A), and to leaf temperatures between 31ºC and 34ºC, the highest corresponding to the most stressed plants (Fig. 3B). There were two days (4 and 5 after the onset of the experiment) complete cloudy and rainy, with decreased day temperatures, during which no measurements could be taken, and a second period of only partially cloudy days (8 to 12 after the onset of the experiment), during which measurements were preformed normally. During this period, leaf-to-air VPDs were the lowest and all leaf temperatures remained close to 30ºC, regardless of the treatments.

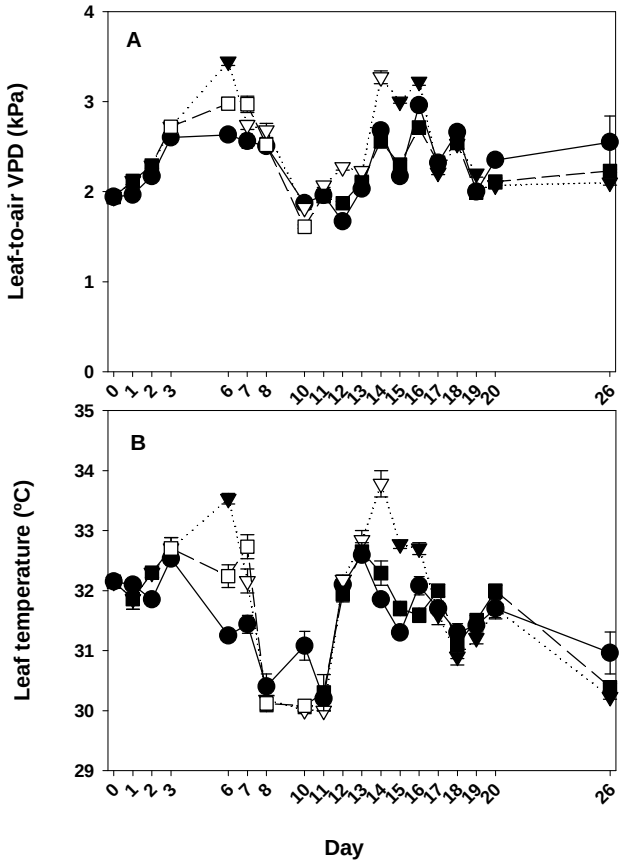

Figure 3. Evolution during the experiment of (A) leaf-to-air vapour pressure deficit (VPD) and (B) leaf temperature. Values represent means ± SE of 10-12 replicates per treatment. The treatments were irrigation (circles), moderate water stress (squares) and severe water stress (triangles). Day 0 corresponds to the first day of water stress application. Empty symbols correspond to the days were plants were at the desired water stress level (acclimation period), filled symbols represent the days previous to reach this level as well as the days of recovery.

There was a gradient of substrate water availability from the highest values in irrigated plants to the lowest in severely stressed. During the acclimation period, substrate water availability was maintained constant. The two-day rainy period did not affect substrate water content because all plants were preventively placed inside a greenhouse. Despite differences in substrate water availability, pre-dawn leaf water potential was kept always above -0.2 MPa, and midday water potential between -1.0 and -1.4 MPa, the lowest values not corresponding to the most severely water stressed plants (see Pou *et al*., 2008).

Evolution of photosynthetic parameters

Net photosynthesis (A_N) of irrigated plants progressively declined during the experiment, from about 18 μmol CO_2 m^{-2} s^{-1} by day 0 to values slightly above 15 μmol CO_2 m^{-2} s^{-1} by day 26 (Fig. 4A). However, water stress imposition resulted in larger reductions, to 12-13 μmol CO_2 m^{-2} s^{-1} under moderate and to less than 5 μmol CO_2 m^{-2} s^{-1} under severe stress (Fig. 4A). During acclimation to water stress, A_N oscillated within a range, particularly for severely stressed plants. Upon re-watering, recovery of A_N was almost complete in about 3 days both in moderately and severely stressed plants (Fig. 4A).

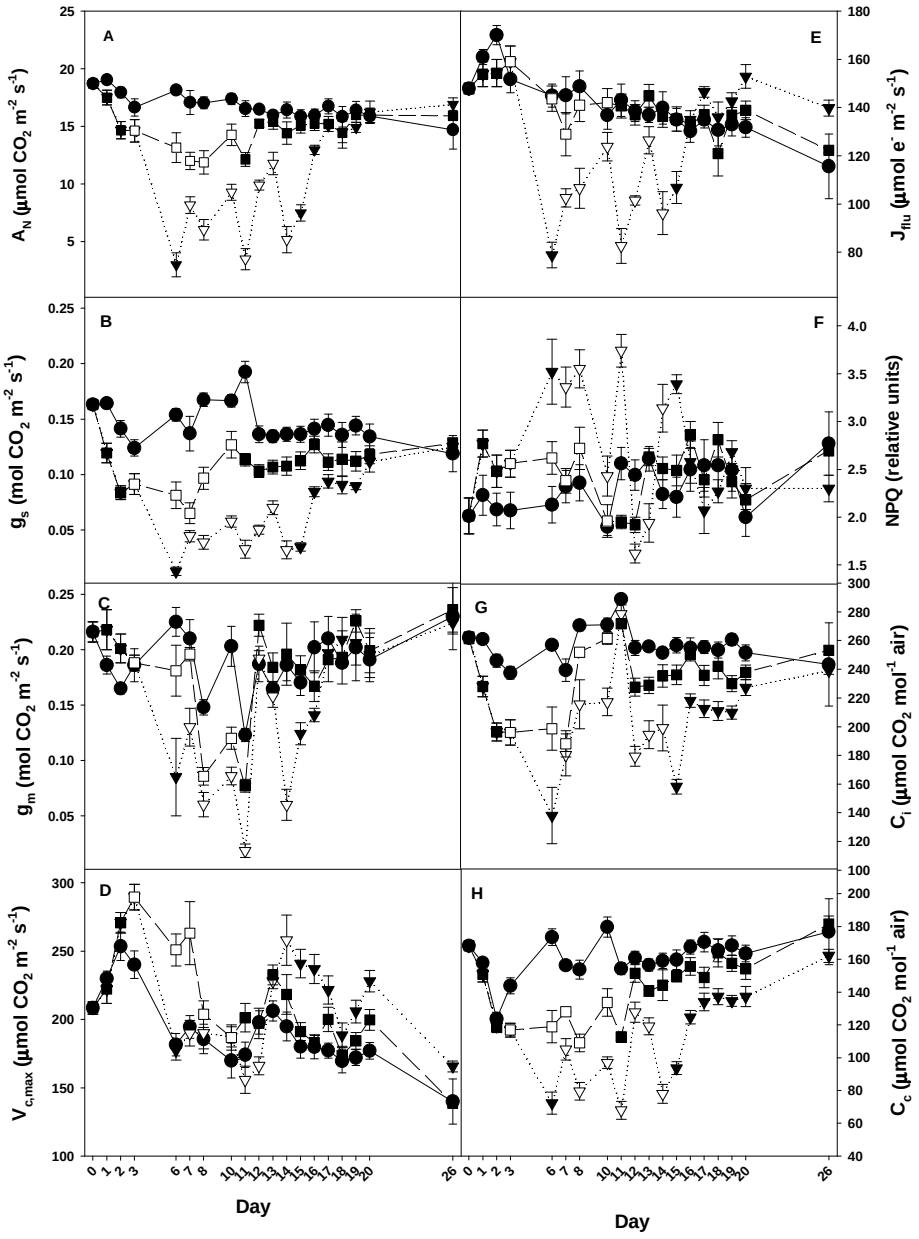

Figure 4. Evolution during the experiment of (A) net photosynthesis (A_N), (B) stomatal conductance (g_s), (C) mesophyll conductance (g_m), (D) maximum capacity for carboxilation ($V_{c,max}$), (E) electron transport rate (J_{flu}), (F) non-photochemical quenching of chlorophyll fluorescence (NPQ), (G) sub-stomatal CO_2 concentration (C_i) and (H) chloroplast CO_2 concentration (C_c). Values represent means ± SE of 10-12 replicates per treatment. Symbols as in Fig. 3.

These variations in photosynthesis were accompanied by similar variations in stomatal conductance (g_s), which in irrigated plants was kept around 0.15 mol CO_2 m^{-2} s^{-1} (i.e., 0.24 mol H_2O m^{-2} s^{-1}), while in moderately and severely stressed plants it declined to the levels established in Material and Methods, i.e. to 0.1 and 0.05 mol

CO_2 m^{-2} s^{-1} in moderately and severely water stressed plants, respectively (Fig. 4B). Oscillations of g_s among days during the acclimation period were also present, although less marked than for A_N. Unlike A_N, recovery of g_s after re-watering was slow, taking about 2 weeks to restore the values found in irrigated plants (Fig. 4B). Mesophyll conductance to CO_2 (g_m) followed a somewhat different pattern (Fig. 4C). In moderately stressed plants g_m did not decline after five days of acclimation, but it suddenly halved during the last two days of acclimation. During re-watering g_m stayed low the first day and fully recovered to control values by day two. On the other hand, g_m was strongly reduced during the first days of acclimation to severe water stress (Fig. 4C). However, during acclimation to severe water stress, g_m was totally recovered during the two semi-cloudy and more humid days (Fig. 3, see also Pou *et al*., 2008), in coincidence with the highest A_N determined in severely stressed plants during the acclimation period (Fig. 4A). One day later (i.e., they day after re-watering) low values were again observed. Upon re-watering, g_m of severely stressed plants recovered to control values within three days.

During the first three days of experiment, $V_{c,max}$ of irrigated plants slightly increased, and then progressively declined during the rest of the experiment (Fig. 4D), similar to A_N (Fig. 4A) and J_{flu} (Fig. 4E). For water stressed plants, the initial period of increase was extended, so that the maximum values achieved were higher than in irrigated plants. However, prolonged water stress induced some decrease of $V_{c,max}$ (Fig. 4D). For moderately stressed plants, $V_{c,max}$ was depressed only during the last two days of acclimation (i.e., the same days in which g_m was depressed), being restored to values *above* irrigated plants three days after re-watering. For severely stressed plants, $V_{c,max}$ was depressed since early in the acclimation period but was restored *above* irrigated plants before re-watering (Fig. 4D), also at the end of the semi-cloudy period but one day later than g_m restoration (Fig. 4C). J_{max} could be determined only in those days in which complete A_N-C_i curves were performed. Similarly to $V_{c,max}$, J_{max} decreased from values of ca. 200 μmol m^{-2} s^{-1} by day 3 in both irrigated and moderately stressed plants, to rates ranging from 130 to 170 μmol m^{-2} s^{-1} after day 7, with no significant differences between treatments (data not shown). The rate of chloroplast electron transport (J_{flu}) in moderately stressed plants

did not differ from that of irrigated plants during the experiment. In severely stressed plants J_{flu} was generally lower during water stress, totally recovering after two days of re-watering (Fig. 4E). During acclimation to water stress, non-photochemical quenching of chlorophyll fluorescence (NPQ) was slightly higher and substantially higher than in irrigated plants in moderately and severely stressed plants, respectively, but recovery was fast after re-watering (Fig. 4F).

As a consequence of decreased g_s during water stress, the sub-stomatal CO_2 concentration (C_i) was also depressed (Fig. 4G). The depression was more marked for the chloroplast CO_2 concentration (C_c), which was more differentiated between treatments (Fig. 4H). It is worth noting that some changes in C_i did not simply follow those changes in g_s. For instance, in moderately stressed plants, during the last two days of acclimation, C_i increased to control values (Fig. 4G) due to simultaneous slight increase of g_s (Fig. 4B) and decrease of g_m (Fig. 4C) and $V_{c,max}$ (Fig. 4D). Similarly, in severely stressed plants, by day 14 (i.e., after the semi-cloudy period) C_i was increased to control values, because low g_m was restored after temporary increase (Fig. 4C) but $V_{c,max}$ was kept still high (Fig. 4D). These particular changes of C_i were not observed for C_c. After re-watering, due to slow recovery of g_s, C_i and C_c were maintained lower in previously stressed than in irrigated plants (Fig. 4G, H).

As a consequence of reduced C_c, A_N was lower under water stress. However, $V_{c,max}$ was kept at or above control values, and J_{flu} was not decreased in moderately stressed plants and less decreased than A_N in severely stressed plants. This was due to increased (moderately stressed plants) or sustained (severely stressed plants) photorespiration (P_r) during the acclimation period, which was not fully reversed after re-watering (Fig. 5A). Indeed, P_r was strongly correlated with $V_{c,max}$ during the entire experiment (Fig. 5B).

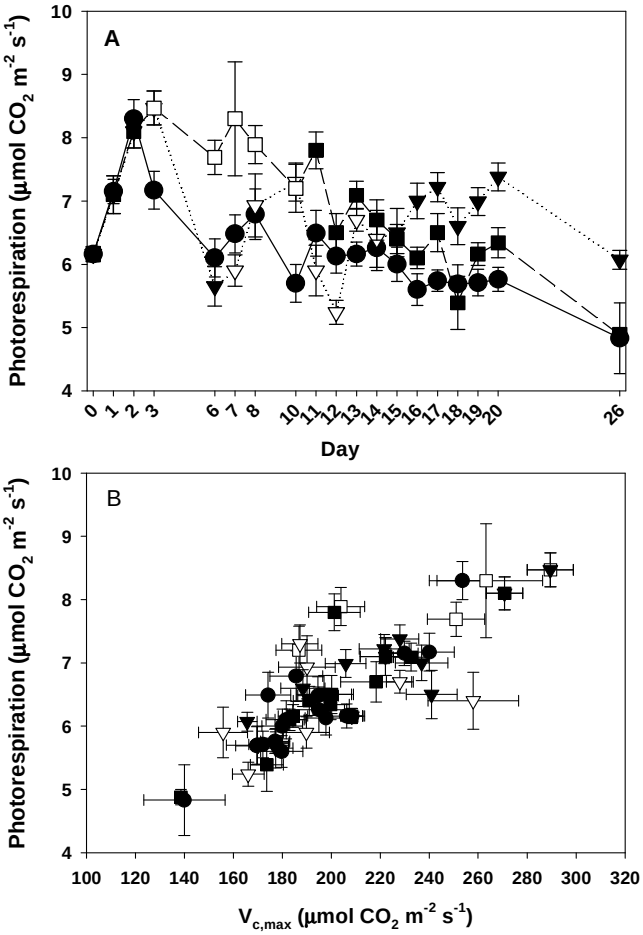

Figure 5. Evolution of photorespiration during the experiment (A) and the relationship between photorespiration and the maximum capacity for carboxylation (B). Values represent means ± SE of 10-12 replicates per treatment. Symbols as in Fig. 3.

In addition to J_{flu} and NPQ, leaf primary photochemistry was assessed independently by measuring (1) pre-dawn maximal photochemical efficiency of PSII (Fv/Fm), (2) thermoluminescence, and (3) determining ascorbate pools as indicative of possible oxidative stress. Fv/Fm was kept above 0.8 during the entire experiment (Table 1), and it by the end of acclimation period it was indeed significantly higher in moderately (0.82) and severely stressed plants (0.83) than in irrigated plants (0.80). Similarly, the total pool of ascorbate did not change significantly during the experiment (data not shown), and the ratio of reduced to total ascorbate was kept between 0.95 and 0.97 regardless of the treatment (Table 2).

Table 1. Pre-dawn Fv/Fm in leaves by the day each treatment was achieved (Day 1) and seven days after acclimation to each treatment (Day 7). Values are means ± S.E. of 10 to 15 replicates per treatment.

	Well irrigated	Moderate stress	Severe stress
Day 1	0.819 ± 0.002	0.820 ± 0.002	0.826 ± 0.003
Day 7	0.800 ± 0.003	0.817 ± 0.003	0.828 ± 0.001

Table 2. Reduced / Total ascorbate in leaves by the day each treatment was achieved (Day 1), seven days after acclimation to each treatment (Day 7) and the first day upon re-watering (Day 8). Values are means ± S.E. of 6 replicates.

	Well irrigated	Moderate stress	Severe stress
Day 1	0.96 ± 0.02	0.95 ± 0.03	0.96 ± 0.02
Day 7	0.97 ± 0.01	0.96 ± 0.01	0.95 ± 0.02
Day 8 (1^{st} recovery)	0.97 ± 0.01	0.95 ± 0.01	0.97 ± 0.01

TL curves were measured in leaves with a dark adaptation period of 8h, under three flashes (3F), one flash (1F) and far red light (FR). 3F measurements induced a B band corresponding to "pure" S3 states, which are more sensitive than S2 (usually induced with 1F) to lumen acidity. I.e., the peak temperature of the B band Tm(B) under 3F was lower than Tm(B) under 1F. Tm(B) under 3F was about 25°C (solid line Fig. 6A) in irrigated plants, whereas under 1F the Tm was around 30ºC (dashed line Fig. 6A). The differences observed in intensity between young and mature leave were simply due to the total chlorophyll content of the leaves. FR light produced a curve with a B band with a Tm near 25ºC and an AG band near 45°C (Fig. 6B). 3F did not induce an AG band in *Vitis* (Fig. 6A), even under water stress (Fig. 6C, D). However, water stress induced differences in TL curves obtained under 3F, consisting in a downshift of the B band from around 28ºC to 17ºC, although these differences were similar regardless of stress intensity (Fig. 6C, D). During the recovery phase the down-shift of the B band under 3F persisted, at least seven days after re-watering, which was the last day we measured TL. HTL measurements were also obtained during the experiment, and no differences were observed between control and treated plants (data not shown).

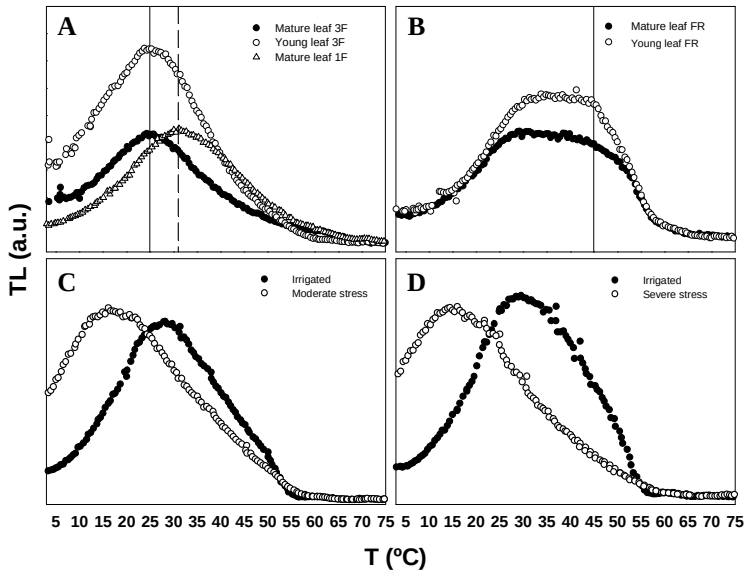

Fig. 6. TL curves obtained in 8h dark-adapted mature leave from hybrid Richter-110 under 1F and in mature and young leaves under 3F (A) and under FR light (B). TL curves obtained after one week from irrigated plants, plants with moderate (C) and severe water stress (D) under 3F after an 8h dark adaptation period.

Photosynthesis limitations

In irrigated plants, the total photosynthesis limitation increased from 0% at the beginning of the experiment to about 20% at the end, ca. one month later (Fig. 7A). This limitation was mostly due to a biochemical limitation (BL, i.e., decreased $V_{c,max}$), while stomatal and mesophyll conductance limitations were of minor importance. In contrast, although moderate water stress led to a similar total limitation (25-30%) during the acclimation period (Fig. 7B), this was mostly due to diffusional limitations (stomatal, SL plus mesophyll conductance, MCL). The same occurred in severely stressed plants, in which a total limitation as high as >80% was fully accounted by the sum of SL and MCL, with no incidence of BL (Fig. 7C).

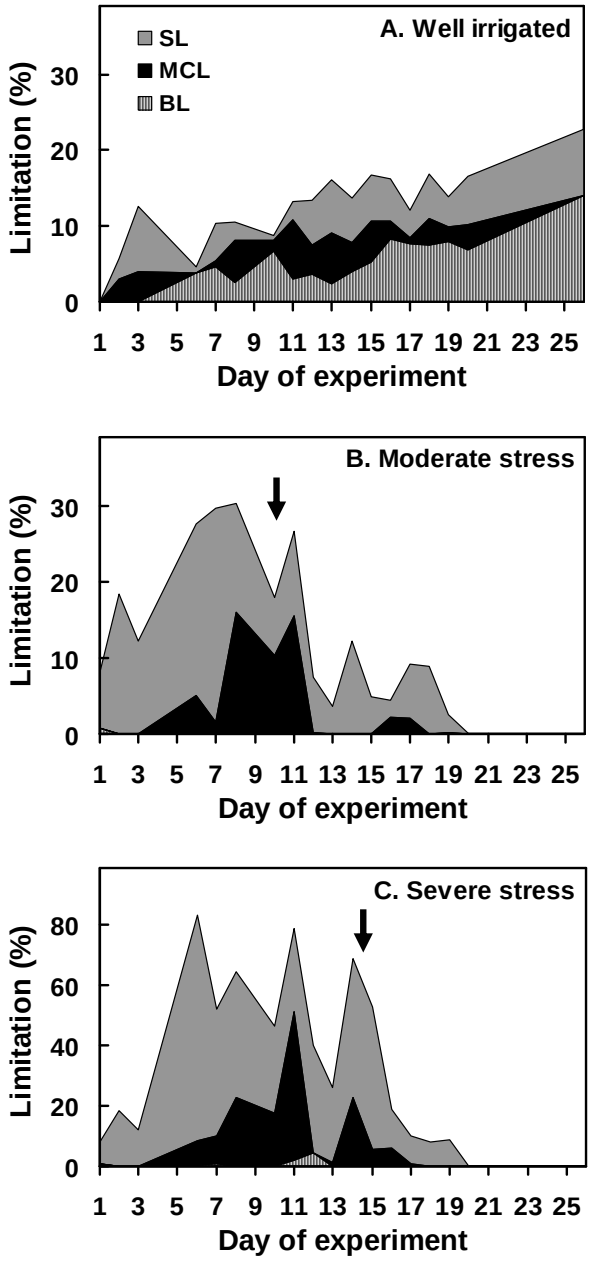

Fig. 7. Quantitative limitation of photosynthesis in well irrigated (A), moderately (B) and severely (C) water stressed grapevines. SL, MCL and BL denote for stomatal, mesophyll and biochemical limitations, respectively.

Among the two diffusional limitations, SL appeared early during the imposition of water stress, and was of higher importance than MCL during the first days of acclimation to both moderate and severe water stress (Fig. 7B, C). However, MCL increased during the acclimation period, being of similar magnitude under

moderate stress or even higher under severe stress than SL before re-watering. However, MCL was rapidly reversed after re-watering, totally disappearing in two (moderate stress) or three (severe stress) days, while SL lasted for at least one week after re-watering (Fig. 7B, C).

DISCUSSION

Regulation of photosynthesis under water stress imposition, short-term acclimation and recovery, was analyzed in a 28-day lasting experiment using the plants of the drought-adapted *Vitis* hybrid R-110, growing outdoors under typical Mediterranean conditions (i.e., high temperature and irradiance). During this period, photosynthesis progressively declined in continuously irrigated plants, which was not due to diffusional limitations, but rather to decreased photosynthetic capacity, as reflected by both decreased $V_{c,max}$ and J_{flu}. These symptoms are typical of leaf ageing, particularly in deciduous species like *Vitis* (Grassi and Magnani, 2005) but also in evergreens (Niinemets *et al*., 2005). Once this ageing effect was considered or removed (limitation analysis), the effects of both moderate and severe water stress on photosynthesis were consistent with previous reports, i.e., the decrease in A_N was mostly due to diffusional limitations, consisting in decreased stomatal and mesophyll conductance to CO_2 (Flexas *et al*., 2004, 2006*b*). Also in *Vitis* sp. it has been verified many times that moderate water stress decreased photosynthesis by diffusional limitations only, and irrigated and water stressed plants often show similar $V_{c,max}$, J_{flu} and Fv/Fm (Flexas *et al*., 1998, 1999*a*, 2002; De Souza *et al*., 2003, 2005). However, under more severe water stress and in some cultivars, decreased $V_{c,max}$ (Maroco *et al*., 2002), J_{max} (De Souza *et al*., 2003, 2005), J_{flu} (Flexas *et al*., 1998, 1999*a*,*b*, 2002) and even Fv/Fm (Flexas *et al*., 1998, 2002) have been observed.

In vitro studies have shown that this is mostly due to reduced activity of fructose-1,6-biphosphate phosphatase and eventually some other enzymes involved in the Calvin cycle (Maroco *et al*., 2002; De Souza *et al*., 2005), as well as to decreased activity of Rubisco (Maroco *et al*., 2002) due to both decreased concentration and activation state (Bota *et al*., 2004). However, transcriptomic analysis also in *Vitis* have shown that some photosynthetic genes, like that of Rubisco activase, some

Calvin cycle enzymes and some PSI and PSII-related genes are instead up-regulated during acclimation to water stress (Cramer *et al*., 2007). Although proteomic analysis showed that some photosynthetic proteins were down-regulated during water stress, it also confirmed that some – notably Rubisco and sedoheptulose-1,5-bisphosphatase – were indeed up-regulated (Vincent *et al*., 2007). In the present study, by determining day-to-day $V_{c,max}$, both effects were observed: on one hand, early acclimation to water stress increased $V_{c,max}$ as compared to irrigated plants, on the other hand, under prolonged water stress $V_{c,max}$ declined (Fig. 4D). Cramer *et al*. (2007) proposed the following explanation: as stomatal conductance decreases with water deficit, internal CO_2 concentrations in the leaf are predicted to be reduced, thus causing a slower rate of photosynthesis. Under these conditions, increases in Rubisco activase could improve photosynthetic efficiency by increasing the amount of Rubisco that is activated for CO_2 fixation, thus compensating for the reduced stomatal conductance. The present results are in accordance with this hypothesis: increased $V_{c,max}$ in response to decreased C_c indeed improved photosynthetic efficiency during the early days of water stress and, most notably, during recovery. We suggest that the decline of $V_{c,max}$ during water stress was due to oxidative stress affecting Rubisco, as demonstrated by Zhou *et al*. (2007) and suggested by the presence of degradation products of Rubisco during water stress (Vincent *et al*., 2007). Indirect evidence for this comes from the fact that, in moderately stressed plants, decreased $V_{c,max}$ did not occur during the first days but only three days after the first cloudy period, i.e., after several days of substantial irradiance in addition to water scarcity. Moreover, in severely stressed plants $V_{c,max}$ was totally restored prior to re-watering, in coincidence with the end of the second cloudy period. Although in this study, Rubisco activity was not measured with biochemical methods, and $V_{c,max}$ determinations could be questioned, previous work in several species including *Vitis* showed good agreement between the initial Rubisco activity determined *in vitro* and $V_{c,max}$ derived from A_N-C_c curves, but not $V_{c,max}$ derived from A_N-C_i curves (Bota *et al*., 2004). Therefore, we feel confident that the estimations of $V_{c,max}$ (and J_{max}) presented in this research are reliable and reflect the discussed mechanisms.

Contrary to carboxylation, leaf photochemistry was more stable during the experiment and in response to water stress, in contrast to previous reports (Maroco *et al*., 2002; Xu and Baldocchi, 2003; Misson *et al*., 2006). While J_{max} did not show any difference between treatments, decreased J_{flu} occurred only in severely stressed plants and can be interpreted in terms of dynamic down-regulation due to increased NPQ (Flexas *et al*., 2002). Indeed, Fv/Fm was even higher in the most stressed plants than in irrigated plants, with moderately stressed plants showing intermediate values. This has already been shown in some species, particularly in those showing increased paraheliotropism in response to water stress (Kao and Tsai, 1998; Pastenes *et al*., 2005), including *Vitis californica* (Gamon and Pearcy, 1990). That leaf photochemistry was resistant to water stress was confirmed by thermoluminescence analysis. The downshift of the B band during water stress was expected (Miranda and Ducruet, 1995). The lower Tm(B) could be due a residual pH gradient (ΔpH) following proton pumping under light, which has been suggested to be enhanced by a water stress-induced cyclic electron transport (Golding and Johnson, 2003) or to a dark stable pH gradient maintained by chlororespiration (Rumeau *et al*., 2007). However, 3F did not induce an AG band in *Vitis* (Fig. 5A), even under water stress (Fig. 5C, D), unlike pea, tobacco or barley but similar to maize, this flash-induced AG band being dependent on species (Ducruet, unpublished). Increased pH gradient could be due to increased photorespiration and/or electron transport to alternative sinks under water stress (Flexas *et al*., 1999*b*), which were apparent during water stress (Fig. 5), and/or to impaired proton pumping during photophosphorylation (Tezara *et al*., 2008). Regardless of its nature, an increased pH gradient producing a downshift of the B band is consistent with the increase of NPQ (Fig. 4F), particularly in severely stressed plants, confirming that the electron transport is resistant to water stress and that the electron-proton system is not uncoupled, as previously suggested by Kaiser *et al*. (1981a) working in intact chloroplast subjected to osmotic stress. However, the downshift of the B band persisted during recovery (not shown), which could contrast with the very fast recovery of NPQ (Fig. 4F). This difference can be explained by the fact that active NPQ requires a lumen pH of about 6 (Kramer *et al*., 1999) while the downshift of the B band appears even with a lumen pH of 7 to 8

(Miranda and Ducruet, 1995). Finally, the leaves measured did not show HTL bands suggesting that no lipid peroxidation (i.e oxidative stress) was present in stressed *Vitis* plants (Ducruet, 2003), in agreement with constant reduction state of the ascorbate pool during the experiment.

In summary, although some acclimation of $V_{c,max}$ occurred, as well as some inhibition of $V_{c,max}$ itself and, to a lesser extent, of photochemistry (Fig. 7), stomatal closure and down-regulation of mesophyll conductance to CO_2 during water stress were the main photosynthesis limitations in such drought-adapted species. However, the importance of these two limitations varied along the period of acclimation to water stress, as well as during recovery after re-watering. When measured only on specific days during a water stress experiment, typically all sunny days, g_s and g_m use to change almost in parallel in response to water stress, i.e., the correlation between both is very high (Flexas *et al*., 2002; Warren, 2008). The present daily results show that the two parameters did not correlate so well. Hence, during the early days of water stress only g_s declined while g_m remained constant. Both under moderate and severe water stress, MCL increased lately during the acclimation period while SL decreased, so that both achieved a similar magnitude at the end of the period. Therefore, it will appear that acclimation to water stress involves balancing stomatal and non-stomatal limitations. Although the function of such balancing is unknown, it has been suggested that it may help to keep C_i sufficiently high as to not to induced a feedback stomatal re-opening (Flexas *et al*., 2008; Peeva and Cornic, 2009). C_i indeed increased during the acclimation period, from the lowest value the day water stress was achieved (both moderate and severe) to values closer to irrigated plants by the end of acclimation (Fig. 4G). Despite of the present results, the opposite seems to happen in other species (i.e., g_m tends to recover during the acclimation period, lowering MCL) such as tobacco or holm oak (Gallé, unpublished results). Hence more studies are needed to achieve a general view of how diffusion limitations interact during acclimation to water stress.

On the other hand, once water stress was established and maintained, g_s was much more stable than g_m. An intriguing behavior of the latter was a total recovery of g_m during the severe water stress period, in coincidence with a two-day cloudy

period. This recovery preceded by one day that of $V_{c,max}$, and was fully reversible within one day. Gallé *et al*. (submitted to this issue) have shown in tobacco that, while the response of g_s to water stress, acclimation and recovery is similar under several light conditions, g_m declines the most and recovers the less under high light conditions, while under low light conditions it does not decrease under water stress. Considered together with the present results, it appears that the response g_m to water stress may be dependent of the prevailing light conditions, similarly to what was demonstrated for Rubisco activity and photochemistry (Zhou *et al.*, 2007). The mechanistic basis for this differential response remains unknown. Clearly, further studies are needed to understand the interactions between water stress and other environmental variables on g_m.

Finally, stomatal limitations appeared to be the most important in delaying photosynthesis recovery after re-watering. This is in agreement with reports by Gallé and Feller (2007) and Gallé *et al*. (2007), who showed a sustained reduction of g_s lasting for weeks after re-watering in some tree species. Sustained stomatal limitations in this genotype do not appear to be related to abscisic acid (ABA) metabolism, since leaf xylem ABA levels were fully restored to control values immediately after re-watering (Pou *et al*., 2008). On the other hand, ABA has been shown to have a similar effect on g_s and g_m (Flexas *et al*., 2006c), while only g_s showed delayed recovery in the present study. Most likely, hydraulic limitations are responsible for sustained low g_s (Pou *et al*., 2008) although the development of organic structures occluding stomata pores such as those described by Gallé and Feller (2007) cannot be ruled out.

The present results are not in complete disagreement with those of Galmés *et al*. (2007a), who showed that g_m was the most limiting factor for photosynthesis recovery in most species analyzed. This is because, in their report, only recovery after 24h of re-watering was analyzed. In the present results, mesophyll conductance limitations the day after re-watering were still substantial (in moderately stressed plants even higher than stomatal limitations), but they vanished in 2-3 days while stomatal limitations lasted for at least one week after re-watering. Early suggestions that photosynthesis recovery after water stress was mostly limited by sustained

impairment of several different components of leaf biochemistry (Kaiser *et al*., 1981a, b; Kirschbaunn, 1987; Ehnnali and Earl, 2005) and disruption of chloroplast membrane integrity (Kaiser *et al*., 1981a, b) seem not to apply in drought-adapted species like *Vitis* sp.

In conclusion, the present results reinforce the idea that, at least in drought-adapted species, diffusional limitations account for most of the observed water stress-induced depression of photosynthesis. However, the relative contribution of stomatal and non-stomatal limitations changes during acclimation to water stress, which also involves up-regulation of photosynthetic capacity. Moreover, the intense campaign of measurements revealed that g_s, g_m and $V_{c,max}$ are not as closely regulated under water stress as often reported. The former appears to be more independent of environmental conditions except VPD (Pou *et al*., 2008), while for the latter two there seems to be an interaction between water stress and cumulative irradiance, although this remains to be confirmed. Finally, it is shown that photosynthesis recovery after re-watering is mostly limited by diffusional limitations rather than by biochemical limitations, and particularly by sustained stomatal closure, which recovers much slowly than g_m.

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4.5. OXYGEN ISOTOPE ENRICHMENT IN LEAF WATER REFLECTS CHANGES WITH DROUGHT PROGRESSION IN HYDRAULIC CONDUCTIVITY AND MESOPHYLL CONDUCTANCE FOR CO_2 IN GRAPEVINE

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Running Head: Isotopes, leaf hydraulics and mesophyll

ABSTRACT

Isotopic composition of leaf water reflects evaporative enrichment during transpiration. Diffusion of enriched water from the sites of evaporation to the rest of the leaf is counteracted by the input of unenriched water through the transpiration flow, modulated by the effective path length (*L*), a fitting parameter of enrichment models. Up to now, the mechanisms behind observed *L* differences are still unclear. The aim of this work was to establish a link between *L* and measurable physiological variables that are likely to respond to similar processes in the mesophyll. For this

purpose, we studied the response to drought and vein severing of leaf lamina hydraulic conductivity (*Kh*), mesophyll conductance for CO_2 (g_m) and leaf water isotope enrichment in *Vitis vinifera* L. We hypothesised that restrictions in water pathways would cause an increase in both *Kh* and *L*. As a secondary hypothesis, we proposed that, since these changes involve common pathways for water and CO_2, a similar response should be found in g_m. Our results showed that *L* was strongly related to mesophyll variables, such as *Kh* or g_m, showing stronger relationships than with variables included as input parameters for the models, such as transpiration. The strong correlation found between *L* and g_m supports the idea that water and CO_2 share an important part of their diffusion pathways through the mesophyll.

INTRODUCTION

Evaporative enrichment of leaf water and its effect on newly produced assimilates are considerably well characterised and can be described with mechanistic models (Barbour & Farquhar 2003; Farquhar & Cernusak 2005; Cuntz et al. 2007). Briefly, the isotopic composition of mean lamina leaf water reflects variations in 1) source water isotope signature (i.e. xylem water) and 2) the evaporative enrichment during transpiration (Yakir 1992a; Farquhar & Lloyd 1993). The isotopic enrichment at the site of evaporation can be described using the Craig & Gordon (1965) model for evaporation in water surfaces, adapted for plants by Dongmann et al. (1974). However, this model overestimates mean lamina leaf water enrichment during the day, as the diffusion of enriched water from the sites of evaporation to the rest of the leaf is counteracted by the input of unenriched water through the transpiration flow, what is known as the *Péclet* effect (Farquhar & Lloyd 1993). The Péclet effect is mainly determined by the magnitude of the transpiration flow, modulated by the "scaled effective path length" (*L*), a fitting parameter that must be determined empirically (Farquhar & Lloyd 1993). Up to now, *L* has been generally assumed to be a species-specific constant, associated to the anatomical properties of the leaf (Wang et al. 1998; Barbour et al. 2004; Kahmen et al. 2008; Ripullone et al. 2008). However, short-term variations in leaf parameters in response to environmental conditions might cause changes in *L*, as has been already suggested elsewhere

(Barbour & Farquhar 2003; Keitel et al. 2006), and recently confirmed by experimental studies (Ripullone et al. 2008; Ferrio et al. 2009). Moreover, although some attempts have been made to relate *L* with measurable leaf parameters (Barbour & Farquhar 2003; Kahmen et al. 2008; Kahmen et al. 2009), the mechanistic reasons underlying observed *L* differences are still unclear. Thus, there is a need to characterise the variability of this parameter, and in particular to further assess whether or not it can change with environmental conditions. In this context, establishing a link between *L* and measurable physiological variables that are likely to respond to similar processes in the mesophyll would help to define a mechanistic explanation for *L* variability.

The scaled effective pathlength *L* is defined as the product of the real distance of the water pathway by a scaling factor that accounts for leaf tortuosity, which can range from 10^2 to 10^3 (Farquhar & Lloyd 1993; Barbour et al. 2000b). Leaf tortuosity stands for the complexity of the water pathway, which in turn affects water flow resistance (Steudle & Frensch 1996; Sack & Holbrook 2006). After leaving the xylem, water can move through three main water pathways (Steudle & Frensch 1996): the apoplastic pathway, i.e. around the protoplasts; the symplastic pathway, travelling along the cytoplasmic continuum through plasmodesmata; and the transcellular or vacuolar pathway, which takes place across membranes and is dominated by transmembrane water channels (aquaporins). Although early studies concluded that water exits the xylem through cell walls, the presence of aquaporins and the high surface area for water transport across the membranes of bundle sheath cells suggests that transcellular water movement may play a significant role in leaf hydraulics (Kaldenhoff & Eckert 1999; Martre et al. 2002; Sack & Holbrook 2006; Katsuhara et al. 2008). Evidence so far has shown that due to changes in aquaporin expression and activity, leaf hydraulic conductivity (*Kh*) can be highly dynamic and respond rapidly and reversibly to changes in temperature, irradiance, and water supply (Kaldenhoff & Eckert 1999; Martre et al. 2002; Sack & Holbrook 2006; Cochard et al. 2007; Katsuhara et al. 2008; Mahdieh et al. 2008). Similarly, changes in mesophyll hydraulic properties, and in particular the regulation of the transcellular pathway by aquaporins would modify the proportion between the different water

pathways, and thus leaf tortuosity and *L* (Yakir 1992b; Farquhar & Lloyd 1993; Barbour et al. 2000b; Ferrio et al. 2009). Moreover, water and CO_2 diffusion share at least in part common diffusion pathways in the mesophyll, including the transcellular pathway (Evans et al. 2009; Terashima et al. 2011), in which the transport of both water and CO_2 is facilitated by aquaporins (Uehlein et al. 2003; Flexas et al. 2006; Kaldenhoff et al. 2008; Heinen et al. 2009; Otto et al. 2010). For this reason, assessing to what extent the regulation of water transport pathways in the mesophyll also affects mesophyll diffusion conductance to CO_2 (g_m) has been recently highlighted as a research priority (Flexas et al. 2008).

In the only work comparing *L* with leaf hydraulic conductivity, Kahmen et al. (2009) did not find a clear relationship between them. Nevertheless, in this study both variables showed little variation in response to the applied treatments. We are not aware of any attempt to relate mesophyll conductance for CO_2 (also mediated by aquaporins) with leaf hydraulic conductivity and leaf water enrichment. Therefore, research on potential changes in the scaled effective pathlenght *L* under conditions providing a clear response in leaf hydraulic conductivity and mesophyll conductance for CO_2 is still needed. For this purpose, we studied simultaneously the response of leaf lamina hydraulic conductivity, mesophyll conductance for CO_2 and leaf water isotope enrichment to drought and vein severing in grapevine. We hypothesise that restrictions in water pathways caused by drought and/or vein severing would result in a concomitant increase in leaf lamina hydraulic resistance and thus in *L*. As a secondary hypothesis, we propose that, provided that these changes partly involve variations in common pathways for water and CO_2, the transport of the latter should also be affected, resulting in a decrease in mesophyll conductance for CO_2. Additional comparisons between values of *L* and hydraulic conductivity were performed using data from a literature survey with further enhancement of our central hypothesis.

MATERIALS AND METHODS

Plant material and treatments

The experiment was carried out in July 2008 on two-year-old grapevine plants (*Vitis vinifera* L cv. Grenache) grafted on Richter-110. Plants were grown outdoors at the experimental field of University of the Balearic Islands (Mallorca, Spain) in 15 L pots filled with a mixture of soil and organic substrate and were irrigated daily from April to mid July. Supplemental Hoagland's solution at 50% was given once per week. Thereafter, half of plants were kept as controls, daily irrigated to field capacity, while irrigation was limited to one half of daily water loss (determined gravimetrically) in drought-exposed plants. Before water withholding was applied, two leaves per plant in both control and drought treatments were subjected to *in vivo* severing treatments. The treatment consisted in cutting the lamina across the midrib, 1 cm from the petiole-lamina junction, and severing secondary and tertiary veins by using a scalpel while supporting the leaf on a cardboard, i.e. fully eliminating water transport through the xylem (Sack et al. 2003; 2004). Abaxial and adaxial cuts of the leaf were taped over with a 1.9mm transparent waterproof tape. Every treated leaf was paired with a nearby control leaf (uncut leaf) on the same branch matched approximately in same size and light exposure. Plants were protected from direct sunlight until wounds healed (approximately after 3-4 days), then returned to their position and left to re-acclimate for 1-2 days. Approximately one fourth of the severed leaves died before being included in the experiment.

Experimental schedule

Sampling and gas-exchange measurements in intact leaves were performed daily, starting the day after the beginning of drought treatment. Two sampling/measurement rounds were performed each day, one in the morning (*ca.* 10-12h) and one in the afternoon (*ca.* 13-15h). Leaves from three plants per treatment were sampled for stable isotope determinations at each round. Just before harvesting, leaf temperature, gas exchange and chlorophyll fluorescence measurements were performed as detailed in the next section. Main veins were removed, and the petiol and two thirds of the leaf lamina were stored separately in glass tubes and inmediatly frozen for subsequent water distillation. The remaining third of the leaf was kept to

determine water concentration (% in weight of water divided by fresh weight) per leaf area (m^{-2}). Simultaneously, one severed leaf per treatment and day was measured and harvested as described above. Only healthy leaves were selected, discarding those with clear symptoms of desiccation or providing negative assimilation rates.

Additionally, 3-5 leaves per treatment (including both intact and severed leaves) were excised under water for hydraulic conductivity determinations (see below). Due to time-consuming measurements, it was not possible to determine hydraulic conductivity for all treatments in the same day, and thus leaves from different treatments were sampled consecutively throughout the experiment, between 10 AM to 14 PM, when transpiration rates were maximal, to minimize the potential impact of diurnal periodicity on leaf hydraulic conductivity.

Instantaneous gas exchange and chlorophyll fluorescence measurements, and estimation of g_m

Instantaneous gas-exchange and chlorophyll fluorescence measurements were taken using an open gas-exchange system (Li-6400; Li-Cor, Inc., Lincoln, NE, USA) with an integrated fluorescence chamber head (Li-6400-40; Li-Cor Inc., Nebraska, USA). All measurements were made on young, fully expanded leaves, at 1500 μmol m^{-2} s^{-1} to ensure light saturation, with a CO_2 concentration in the cuvette of 400 μmol CO_2 mol^{-1} air, and using the 2-cm^2 leaf chamber. From instantaneous measurements, A_N, g_s and the sub-stomatal CO_2 concentration (C_i) were recorded. Leaf temperature under ambient conditions was determined just before gas-exchange measurements attaching a leaf thermocouple (Li-6400-04; Li-Cor Inc., Nebraska, USA) coupled to a digital thermometer (model 51 II; Fluke Corp., USA) to the abaxial surface of the leaf.

Photochemical efficiency of photosystem II (ϕ_{PSII}) was determined by measuring steady-state fluorescence (Fs) and maximum fluorescence during a light-saturating pulse of ca. 8000 μmol m^{-2} s^{-1} (Fm') following the procedures of Genty et al. (1989):

$$\phi_{PSII} = (Fm'-Fs)/Fm' \quad [1]$$

The electron transport rate (J_{flu}) was then calculated as:

$$J_{flu} = \phi_{PSII} \cdot PPFD \cdot \alpha \cdot \beta \quad [2]$$

where PPFD is the photosynthetically active photon flux density, α is leaf absorptance and β reflects the partitioning of absorbed quanta between photosystems II and I. The product $\alpha \cdot \beta$ was determined, following (Valentini et al. 1995), from the relationship between ϕ_{PSII} and ϕ_{CO2} obtained by varying light intensity under non-photorespiratory conditions in an atmosphere containing less than 1% O_2. Leakage of CO_2 into and out the leaf cuvette was determined with photosynthetically inactive leaves (obtained by heating the leaves until no variable chlorophyll fluorescence was observed) enclosed in the leaf chamber (Flexas et al. 2007). The method by Harley et al. (1992) was used to make estimations of g_m as:

$$g_m = A_N / (C_i - (\Gamma^* \cdot (J_{flu} + 8 \cdot (A_N + R_d)) / (J_{flu} - 4 \cdot (A_N + R_d)))) \quad [3]$$

where A_N and C_i are taken from gas exchange measurements at saturating light. C_i^* was used as a proxy for Γ* following Warren & Dreyer (2006). C_i^* and R_d were determined according to the 'Laisk-method' (Laisk 1977). Briefly, A_N-C_i curves were measured at three different PPFDs (50, 150, and 750 μmol m^{-2} s^{-1}) at six different CO_2 levels ranging from 400 to 50 μmol CO_2 mol^{-1} air. The intersection point of the three A_N-C_i curves was used to determine C_i^* (x-axis) and R_d (y-axis).

Hydraulic conductivity (*Kh*)

Maximum leaf *Kh* was determined using a high pressure flow meter (HPFM; Dynamax Inc.Houston TX, USA), as described in Tyree et al. (1995). Detached leaves were excised under water and allowed to reach a transpirational steady-state while attached to a flow meter through the petiole using compression fittings. 15mM KCl solution filtered at 0.1μm was forced into the leaves at a pressure (*P*; MPa) up to 0.4 MPa, while measuring the instantaneous flow (*F*; mmol s^{-1}) every 8 s. Corresponding hydraulic conductances (*K*; mmol s^{-1} MPa) were computed as $K = F/P$. During measurements, leaf temperature was monitored by a thermocouple, and maintained between 20ºC and 25ºC by adding water uniformly over the leaf blade. The leaf hydraulic conductance was then corrected for eventual temperature changes to account for changes in water viscosity. *K* decreased during the early phases of

measurements as the likely effect of progressive infiltration of leaf air spaces, and reached stable values after 25-30 minutes. After *K* was recorded, leaf blades were removed using a fresh razor blade. The hydraulic conductance of the petiole ($K_{petiole}$) was measured and the lamina hydraulic conductance (K_{lamina}) (K_{lamina}=1/R) was calculated as:

$$1/K_{lamina} = (1/K_{leaf}) - (1/K_{petiole})$$

After each experiment, projected leaf areas (*LA*; m^2) were measured with a leaf area meter (AM-100 Area Meter, Analytical Development Co. Hoddesdon, UK), and leaf and lamina maximum *Kh* on a surface area basis were calculated (mmol s^{-1} MPa^{-1} m^{-2}).

Water vapour collection, water distillation and mass spectrometry measurements

During each sampling/measuring round, atmospheric water vapour was collected by cryogenic condensation (Roden & Ehleringer 1999). Briefly, air was pumped at 1 l min^{-1} for about 2 h through a trap filled with ethanol and dry ice (ca. -70 °C). Petiole and leaf lamina water were extracted by cryogenic vacuum distillation (Ehleringer & Dawson 1992). For both, water vapour and distilled water, cryogenically trapped water was transferred immediatly into sealed 2 ml crimp cap vials (Infochroma, Zug, Switzerland) and kept cooled until isotope analysis. An aliquot of 0.6 µl of each water sample was then injected in a High Temperature Combustion Elemental Analyzer (TC/EA, Thermo Finnigan, Bremen, Germany), pyrolized at 1450 °C on glassy carbon to CO, the oxygen isotope ratio of which was determined by isotope ratio mass spectrometry (Delta plus XP, Thermo Finnigan, Bremen) and the values expressed as deviations in per mil (‰) from the international standard VSMOW ($\delta^{18}O$). Overall precision was better than 0.2‰.

Leaf water models and determination of effective pathlength L

Isotopic enrichment of mean lamina leaf water above source water (Δ_L, in ‰) was calculated as $\Delta_L=(\delta_L-\delta_S)/(1+\delta_S)$, where δ_L and δ_S stand for the isotopic composition of leaf lamina (after removing main veins) and source water, respectively. Petiol

water ($\delta^{18}O_P$) was considered here to be representative for source water. Steady-state isotopic enrichment at the site of evaporation (Δ_e) was modelled according to Craig & Gordon (1965) and Dongmann et al. (1974):

$$\Delta_e = \varepsilon^+ + \varepsilon_k + \left(\Delta_v - \varepsilon_k\right)\frac{e_a}{e_i} \qquad (1)$$

where ε^+ is the equilibrium fractionation between liquid water and vapour (Majoube 1971); ε_k is the kinetic fractionation of vapour diffussion from the leaf to the atmosphere (Farquhar et al. 1989), Δ_v is the isotopic enrichment of atmospheric water vapour, and e_a/e_i is the ratio of ambient to intercellular vapour pressures.

The steady-state isotopic enrichment of mean lamina mesophyll water (Δ_{Lss}) was calculated by correcting for the gradient from xylem source water to enriched water at the evaporating sites, the so-called *Péclet* effect (Farquhar & Lloyd 1993):

$$\Delta_{Lss} = \Delta_e \frac{1 - e^{-\wp}}{\wp} \qquad \text{with } \wp = \frac{E \cdot L}{C \cdot D} \qquad (2)$$

where $\wp$ is the *Péclet* number, E the leaf transpiration rate (mol m^{-2} s^{-1}), L the scaled effective path length (m) for water movement from the veins to the site of evaporation, C the molar concentration of water (55.56 10^3 mol m^{-3}), and D the tracer-diffusivity (m^2 s^{-1}) of the heavy water isotopologue ($H_2{}^{18}O$) in 'normal' water. The effective pathlength L under the steady state assumption (L_{ss}) was determined by fitting Eq. 2 to measured Δ_L. L_{ss} values were fitted independently for each leaf.

Non-steady-state effects in lamina mesophyll water enrichment (Δ_{Lnss}) were tested using the simplified non-steady-state *Péclet* description (Farquhar & Cernusak 2005):

$$\Delta_{Lnss} = \Delta_{Lss} - \frac{\alpha^+\alpha_k}{g_t w_i}\frac{1 - e^{-\wp}}{\wp}\frac{d\left(V_m \Delta_{Lnss}\right)}{dt} \qquad (3)$$

where $\alpha = 1+\varepsilon$, (α^+ and α_k are corresponding to ε^+ and ε_k, respectively), V_m is lamina leaf water molar concentration (mol m^{-2}), t is time (s), g_t is the total conductance for water vapour of stomata and boundary layer (mol m^{-2} s^{-1}), and w_i is the mole fraction of water vapour in the leaf intercellular air spaces (mol mol^{-1}). The

term $d(V_m\Delta_{Lnss})/dt$ stands for the rate of change in "isostorage" ($V_m\Delta_{Lnss}$) between a given measuring time-point (t_0) and a previous measurement, used as reference (t_{-1}), and is applied to estimate the "net isoflux" during transpiration (Farquhar & Cernusak 2005). According to previous studies on Grenache grapevines (Schultz 2003; Soar et al. 2006; Vandeleur et al. 2009; Lovisolo et al. 2010), leaves were considered to behave isohydrically, and no changes in V_m were included in the non-steady state model. Indeed, we did not find any significant effect of the treatments for this parameter, so we took it as a constant throughout the experiment (V_m=10.9 ±0.02 mol H_2O m^{-2}). To prevent artefacts due to differences between the individual leaves sampled at each time-point, we calculated Δ_{Lnss} using average values for each time-point and treatment (N=3). *Sensu stricto*, the model could only be applied to the values from intact leaves in the afternoon, using morning data as reference values (t_{-1}). Nevertheless, we also applied the non-steady state model to severed leaves, and to morning values in intact leaves. In these cases, in which measured values for t_{-1} were not available, the rate of changes in Δ_L was estimated from the average and the 95% confidence interval for the differences in Δ_L between morning and afternoon values (2.60 ‰ and 1.66-3.53 ‰, respectively), and the average dt (8324 s). Although the non-steady state model under these assumptions may have lost its predictive value, the comparison between L_{ss} and L_{nss}, as well as the sensitivity of L_{nss} to Δ_L changes, provided a qualitative test of the potential risks associated to assuming steady-state conditions.

Equilibrium fractionation ε^+ was calculated after Majoube (1971), and kinetic fractionation ε_k was calculated after Farquhar et al. (1989) with the diffusional fractionation factors of (Cappa et al. 2003). Tracer-diffusivity D as depending on temperature was estimated after Cuntz et al. (2007):

$$D = a_D a_1 \exp\left(-\frac{a_2}{T - a_3}\right) \qquad (4)$$

with $a_1 = 100 \cdot 10^{-9}$, $a_2 = 577$, $a_3 = 145$ and $a_D = 1/1.026$ for $H_2{}^{18}O$.

Effective pathlength L under non-steady state conditions (L_{nss}) was calculated by fitting iteratively Eq. 3 until Δ_{LnP} values at both sides of equation differed by less than 0.01 ‰.

Literature survey

To further assess the link between leaf *Kh* and *L*, we compiled published data from both variables for different species and growing conditions (see details in supplementary material, Table S1). We are only aware of one work reporting both variables determined in the same experiment (Kahmen et al. 2009). Thus, in most cases data for *L* and *Kh* were derived from independent sources, which may differ in environmental conditions, or represent data from different species within the same genus. Compiled *Kh* values were determined by either the evaporative flux (Brodribb & Holbrook 2003) or the Ohm's Law (Van den Honert 1948) methods. An attempt was made to select the most comparable species and/or growing conditions, taking g_s, when available, as the main reference criterion for the water stress severity endured by the plants under study (Medrano et al. 2002). In other cases, other indicators of plant water status were used (e.g. transpiration rate, water potential, reported environmental conditions).

Statistical analysis

The effect of treatment on physiological variables and isotopes was first assessed by means of analyses of variance (ANOVA) including water status (control, drought) and leaf treatment (non-severed, severed) as fixed factors. Additionally, the effect of day and measuring time were considered by adding them as fixed factors to the model, but due to the lack of replicates this was only possible for non-severed plants. All the ANOVAs were performed using standard SAS–STAT procedures (SAS 1988). The relationship between pairs of variables was assessed by means of linear regressions and simple correlation analyses, indicating determination coefficients and *P*-values as main statistics. Unless otherwise stated, averages are reported together with the standard errors of the mean.

RESULTS

Experimental conditions

Conditions close to steady-state were kept during the measurement time (*ca.* 10h-15h) in all measurement days. Nevertheless, environmental conditions varied from one day to another (Supplementary material, Fig. S1). From July 18th to 20th relative air humidity decreased, recovering again from July 21st until the end of the experiment. July 22nd was a cloudy day and thus gas-exchange and sampling for isotopes were not performed. Water availability (expressed as % of field capacity) decreased steadily throughout the experiment. $\delta^{18}O$ of water vapour ($\delta^{18}O_v$) also varied from day to day, following fluctuations in relative humidity.

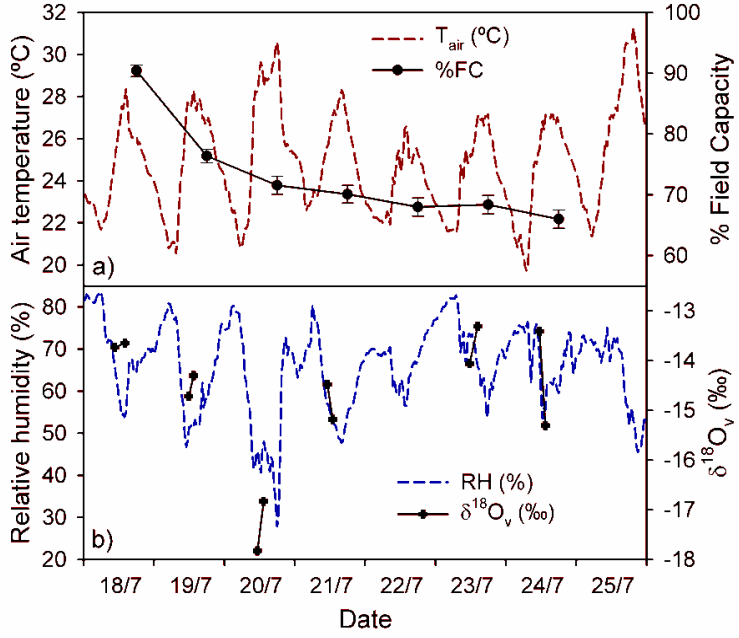

Figure S1. Main environmental variables during the experiment. Time axis ticks are located at midnight. Percentage of field capacity (%) was determined in drought-exposed plants in the evening of each experiment day. $\delta^{18}O_V$, stable isotope composition of atmospheric water vapour during each measuring time.

Leaf physiological response to drought and vein severing

Values of physiological variables and isotope measurements and treatment are shown in Tables 1, 2 and 3. According to the general ANOVA (Table 4), both drought and leaf severing treatments affected significantly (P<0.001) the main gas exchange

variables (net photosynthesis -A_N-, stomatal conductance -g_s- and transpiration -*E*-), with a strongly significant interaction term (P<0.001). g_m showed an effect of leaf severing (P=0.014) and, to lesser extent, drought (P=0.053). Leaf temperature was significantly affected by drought (P=0.033), showing only a weak effect of leaf severing (P=0.096). Lamina *Kh* was significantly affected only by leaf severing (P=0.021), while leaf *Kh* showed a weak effect of leaf severing (P=0.099). More detailed ANOVA, including day and time effects in the model, confirmed these trends for non-severed leaves. A_N, g_s and E showed again a strongly significant (P<0.001) response to drought, responding to day and measuring time at different levels (Table 4). The direct effect of drought on g_m was weak (P=0.054), but showed a significant interaction with day (P=0.010).

Table 1. Main physiological variables and isotope values in intact leaves (average and standard error of three replicates). A_N, net photosynthesis; g_s, stomatal conductance; E, transpiration rate; g_m, mesophyll conductance for CO_2; T_{leaf}, leaf temperature; $\delta^{18}O_P$, petiol water isotope composition; Δ_L, measured isotopic enrichment in leaf lamina water; Δ_e, modelled isotopic enrichment at the site of evaporation assuming steady-state conditions; L_{ss}, scaled effective pathlenght assuming steady-state conditions. (average and standard error of three replicates).

Treatment	Date	A_N (µmol CO_2 m^{-2} s^{-1})	g_s (mmol H_2O m^{-2} s^{-1})	E (mmol H_2O m^{-2} s^{-1})	g_m (mmol CO_2 m^{-2} s^{-1})	T_{leaf} (ºC)	$\delta^{18}O_P$ (‰)	Δ_L (‰)	Δ_e (‰)	L_{ss} (mm)
Control	18/07	16.80 ±0.71	205 ±14.0	4.00 ±0.13	229 ±14.1	30.2 ±0.54	-7.5 ±1.66	15.0 ±2.09	20.7 ±0.12	26 ±8.7
		15.48 ±0.86	216 ±13.6	4.70 ±0.31	176 ±12.7	30.4 ± 1.07	-7.9 ±0.14	19.1 ±0.85	21.5 ±0.13	7 ±0.3
	19/07	13.33 ±0.16	253 ±20.1	5.69 ±0.56	190 ±9.6	32.5 ±0.20	-8.2 ±0.59	19.6 ±1.25	23.1 ±0.11	10 ±3.7
		11.58 ±0.31	206 ±18.0	5.36 ±0.47	173 ±11.5	34.4 ± 1.06	-6.1 ±1.04	21.8 ±1.73	24.5 ±0.12	7 ±2.5
	20/07	13.43 ±1.20	227 ±41.3	5.39 ±0.79	164 ±24.6	32.2 ±0.65	-5.4 ±0.29	17.6 ±0.81	22.2 ±0.12	15 ±6.0
		12.57 ±1.45	196 ±35.3	5.54 ±0.79	178 ±12.7	34.8 ± 0.32	-7.4 ±1.45	20.7 ±0.68	25.4 ±0.16	13 ±3.6
	21/07	14.45 ±1.00	204 ±20.6	4.30 ±0.42	166 ±3.7	28.3 ±0.72	-8.6 ±0.43	17.9 ±0.21	21.0 ±0.14	10 ±1.7
		14.67 ±0.83	215 ±1.9	5.02 ±0.04	154 ±21.9	29.7 ±0.60	-8.3 ±0.55	18.3 ±0.40	22.1 ±0.16	11 ±1.6
	23/07	16.34 ±0.22	274 ±14.4	6.31 ±0.25	207 ±33.2	29.6 ±0.28	-5.9 ±0.46	14.4 ±1.58	17.1 ±0.15	9 ±5.0
		15.48 ±0.97	239 ±19.6	5.68 ±0.63	162 ±28.1	31.5 ± 0.49	-7.0 ±0.80	15.3 ±0.67	19.8 ±0.03	15 ±3.0
	24/07	15.42 ±0.92	274 ±12.0	5.31 ±0.38	142 ±15.1	28.6 ±0.12	-9.5 ±0.02	13.4 ±0.78	18.2 ±0.00	17 ±2.0
		16.25 ±0.25	260 ±11.0	5.74 ±0.22	158 ±7.2	30.8 ±0.20	-8.7 ±0.44	17.0 ±1.60	20.9 ±0.06	12 ±5.5
Mean ±SE		**14.58 ±0.34**	**229 ±7.0**	**5.24 ±0.16**	**175 ±6.2**	**31.2 ±0.38**	**-7.5 ±0.29**	**17.7 ±0.51**	**21.5 ±0.44**	**13 ±1.4**
Drought	18/07	6.96 ±0.88	54 ±15.1	1.30 ±0.35	197 ±58.4	32.2 ±0.20	-6.1 ±0.70	19.8 ±0.64	22.8 ±0.02	41 ±16.2
		4.51 ±2.49	35 ±19.0	1.02 ±0.55	114 ±50.3	36.5 ±0.00	-7.9 ±0.70	25.2 ±0.72	27.3 ±0.04	33 ±9.8
	19/07	12.42 ±0.52	117 ±3.5	3.09 ±0.02	270 ±3.4	35.8 ±2.20	-6.4 ±0.36	22.6 ±0.18	25.5 ±0.03	13 ±5.7
		5.21 ±3.71	41 ±26.9	1.31 ±0.84	167 ±104.0	37.9 ±0.29	-7.5 ±1.88	25.3 ±1.45	28.2 ±0.07	70 ±50.7
	20/07	6.64 ±2.77	70 ±30.2	2.03 ±0.74	92 ±32.2	35.7 ±1.18	-6.8 ±0.35	20.2 ±0.76	26.6 ±0.03	62 ±21.5
		4.48 ±1.43	45 ±15.2	1.60 ±0.49	69 ±9.9	37.3 ±0.30	-5.9 ±0.65	24.1 ±0.84	27.6 ±0.07	35 ±11.5
	21/07	10.63 ±0.37	118 ±8.1	2.75 ±0.20	191 ±38.3	30.0 ±0.29	-7.0 ±0.31	20.0 ±1.53	22.2 ±0.04	12 ±10.8
		6.45 ±0.45	51 ±5.6	1.50 ±0.19	239 ±12.7	33.4 ±0.49	-8.3 ±0.47	23.2 ±0.95	25.9 ±0.02	22 ±6.9
	23/07	9.09 ±1.46	114 ±41.8	2.81 ±0.93	168 ±33.0	34.2 ±1.33	-4.5 ±0.73	17.3 ±1.38	21.7 ±0.02	22 ±2.6
		6.72 ±1.72	70 ±21.1	1.99 ±0.55	121 ±22.0	33.5 ±1.39	-6.9 ±0.59	19.0 ±0.78	22.3 ±0.01	33 ±14.7
	24/07	3.09 ±1.18	31 ±12.3	0.84 ±0.28	56 ±21.3	34.2 ±0.28	-8.0 ±1.53	20.6 ±1.24	24.2 ±0.01	75 ±25.8
		3.46 ±0.40	27 ±4.9	0.88 ±0.16	101 ±18.2	33.7 ±2.61	-7.2 ±0.54	20.7 ±1.71	23.9 ±0.02	53 ±0.5
Mean ±SE		**6.67 ±0.60**	**66 ±7.6**	**1.78 ±0.18**	**149 ±14.5**	**34.3 ±0.47**	**-6.8 ±0.27**	**21.3 ±0.49**	**24.7 ±0.44**	**39 ±5.6**

Table 2. Main physiological variables and isotope values in vein-severed leaves (individual leaf values). A_N, net photosynthesis; g_s, stomatal conductance; E, transpiration rate; g_m, mesophyll conductance for CO_2; T_{leaf}, leaf temperature; $\delta^{18}O_P$, petiol water isotope composition; Δ_L, measured isotopic enrichment in leaf lamina water; Δ_e, modelled isotopic enrichment at the site of evaporation assuming steady-state conditions; L_{ss}, scaled effective pathlenght assuming steady-state conditions.

Treatment	Date	A_N (µmol CO_2 m^{-2} s^{-1})	g_s (mmol H_2O m^{-2} s^{-1})	E (mmol H_2O m^{-2} s^{-1})	g_m (mmol CO_2 m^{-2} s^{-1})	T_{leaf} (ºC)	$\delta^{18}O_P$ (‰)	Δ_L (‰)	Δ_e (‰)	L_{ss} (mm)
Control	18/07	6.67	50	1.08	246	34.5	-7.9	21.4	25.4	53
	19/07[a]	*0.10*[a]	*8*[a]	*0.28*[a]	*4*[a]	38.3	-7.6	22.7	28.9	300
	20/07	9.35	97	2.43	200	33.4	-7.3	21.8	25.2	19
	21/07	1.44	20	0.70	17	33.2	-6.7	19.4	25.4	123
	23/07	2.64	30	0.97	86	31.4	-7.5	18.9	24.3	80
Mean ±SE		**5.0 ±1.82**	**49 ±17.1**	**1.30 ±0.39**	**137 ±52.3**	**33.1 ±0.63**	**-7.4 ±0.24**	**20.4 ±0.72**	**25.1 ±0.26**	**69 ±22.0**
Drought	18/07[a]	*0.09*[a]	*13*[a]	*0.32*[a]	*3*[a]	36.8	-7.5	17.0	27.2	538
	19/07	2.90	24	0.73	14	33.0	-7.2	20.8	25.2	83
	20/07	3.60	29	1.13	113	38.0	-7.0	22.9	28.5	68
	21/07[a]	*0.02*[a]	*8*[a]	*0.27*[a]	*3*[a]	35.0	-6.2	17.9	26.6	503
	23/07[b]	1.39	*13*[b]	*0.48*[b]	45	35.6	-9.6	22.5	25.7	93
	24/07	3.20	28	0.78	69	34.9	-8.8	21.6	25.0	62
Mean ±SE		**2.8 ±0.48**	**23 ±3.6**	**0.78 ±0.13**	**60 ±20.9**	**35.4 ±1.03**	**-8 .2 ±0.64**	**22.0 ±0.49**	**26.1 ±0.81**	**76 ±7.1**

[a] discarded as outliers in all calculations; [b] discarded as outlier only for L_{nss} calculations (see Fig. 3). In both cases, the particular variables in which the samples were recognised as outliers are highlighted.

Table 3. Individual leaf values of leaf hydraulic conductivity. C-severed, D-severed: vein-severed leaves in control and drought-exposed plants, respectively.

		Max. conductivity (*Kh*) (mmol H_2O s^{-1} MPa^{-1} m^{-2})	
Treatment	**Date**	**Leaf**	**Lamina**
Control	19/07	3.6	4.1
	19/07	10.5	10.9
	22/07	9.6	10.6
	24/07	4.3	5.3
Mean ±SE		**7.0 ±1.77**	**7.7 ±1.77**
C-severed	19/07	4.2	4.6
	22/07	2.7	2.9
	22/07	5.0	5.3
	22/07	4.5	5.0
	24/07	4.3	4.4
Mean ±SE		**4.1 ±0.39**	**4.4 ±0.42**
Drought	18/07	1.8	1.9
	23/07	3.9	5.4
	23/07	9.1	11.2
	24/07	6.1	6.5
	24/07	3.2	7.2
Mean ±SE		**4.8 ±1.27**	**6.4 ±1.50**
D-severed	18/07	6.2	6.3
	23/07	1.9	2.5
	24/07	2.3	2.5
Mean ±SE		**3.5 ±1.36**	**3.8 ±1.28**

Regarding isotope values, treatments did not affect significantly the isotope composition of petiol water ($\delta^{18}O_P$), whereas measured leaf water enrichment (Δ_L) showed a significant effect of drought (P=0.016), but no significant effects of leaf severing (Table 4). Modelled enrichment at the site of evaporation (Δ_e) was significantly affected by both treatments (P=0.026 and P=0.009 for drought and leaf severing, respectively). Calculated steady-state effective pathlength (L_{ss}) showed a strongly significant response to leaf severing (P<0.001), and a weak effect of drought (P=0.059). Looking in depth at non-severed leaves, the ANOVAs including day and time in the model showed a strongly significant effect of drought (P<0.001), day and time on $\delta^{18}O_L$, Δ_L and Δ_e, whereas L_{ss} was only significantly affected by the drought treatment (P<0.001). In all cases interaction terms were weak or non significant.

Table 4. Summary of the results of ANOVAs for the most relevant variables studied. A_N, net photosynthesis; g_s, stomatal conductance; E, transpiration rate; g_m, mesophyll conductance for CO_2; T_{leaf}, leaf temperature; $\delta^{18}O_P$ and $\delta^{18}O_L$, petiol and leaf lamina water isotope composition; Δ_L, measured isotopic enrichment in leaf lamina water; Δ_e, modelled isotopic enrichment at the site of evaporation assuming steady-state conditions; L_{ss}, scaled effective pathlenght assuming steady-state conditions; Kh_{leaf} and Kh_{lam}, leaf and lamina maximum specific hydraulic conductivity. *** P<0.001; ** P<0.01; * P<0.05; † P<0.1; n.s., P≥0.1.

Factor	A_N	g_s	E	g_m	T_{leaf}	$\delta^{18}O_P$	$\delta^{18}O_L$	Δ_L	Δ_e	L_{ss}	Kh_{leaf}	Kh_{lam}
General ANOVA												
Drought	***	***	***	†	*	n.s.	*	*	*	†	n.s.	n.s.
Leaf Severing (LS)	***	***	***	*	†	n.s.	n.s.	n.s.	n.s.	***	†	*
Drought x LS	***	***	***	n.s.	n.s.	n.s.	†	n.s.	*	n.s.	n.s.	n.s.
ANOVA non-severed												
Drought	***	***	***	†	***	n.s.	***	***	***	***	-	-
Day	n.s.	†	†	**	***	**	***	***	***	n.s.	-	-
Drought x day	*	*	n.s.	*	n.s.	n.s.	†	n.s.	n.s.	†	-	-
Time	*	*	n.s.	*	***	n.s.	***	***	***	n.s.	-	-
Drought x time	†	n.s.	†	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	-	-
day x time	n.s.	n.s.	n.s.	*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	-	-

Relationship between *Kh*, gas exchange variables and effective pathlength *L*

Whereas treatment averages of leaf *Kh* showed the strongest correlation with *E*, lamina *Kh* was not significantly correlated with this variable (Fig. 1a,c). On the other hand, L_{ss} was negatively correlated with leaf and lamina *Kh* (Fig. 1b,d). In particular, among all the physiological variables studied, the strongest relationship across treatment averages was found between L_{ss} and lamina *Kh* (r^2=0.991; see Fig. 1d). L_{ss} showed also a weak negative trend with *E* (r^2=0.855, *P*=0.075). Correlations between g_s and both *Kh* and L_{ss} showed the same pattern found for *E*, but generally weaker (r^2=0.748-0.962, *P*=0.019-0.135). Treatment averages of g_m showed a weak correlation with lamina *Kh* and L_{ss} (r^2=0.870, *P*=0.067 and r^2=0.822, *P*=0.093, respectively) but were not significantly correlated with leaf *Kh*, *E* and g_s (*P*=0.133-0.247).

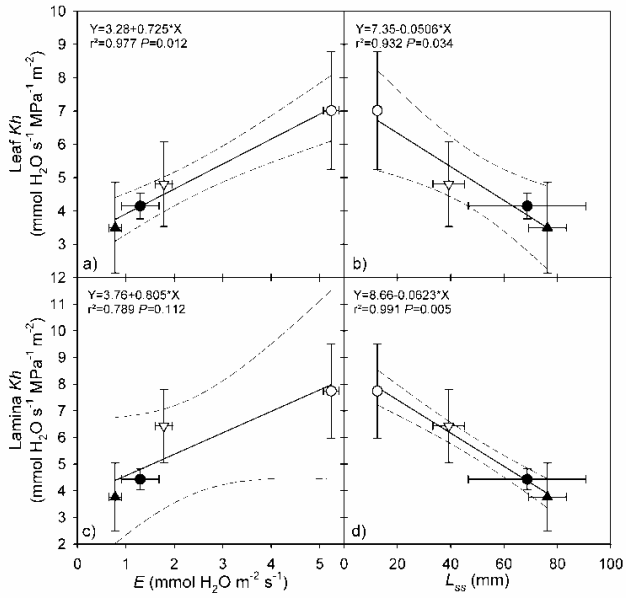

Figure 1. Relationship between treatment averages for transpiration rates (*E*), scaled effective pathlength (L_{ss}, determined with the steady state model), and both leaf and lamina maximum specific hydraulic conductivity (*Kh*). Circles: control plants, triangles: drought plants. Open symbols: intact leaves. Closed symbols: vein-severed leaves.

When individual replicates were plotted, L_{ss} was only correlated with *E* and g_s in their lowest range of values (*E*< 4 mmol H_2O m^{-2} s^{-1} and g_s< 150 mmol H_2O m^{-2} s^{-1}), which excluded nearly all samples from control plants (r^2=0.540 for *E*; r^2=0.513

for g_s; see Fig. 2a). Similarly, g_m was linearly correlated with L_{ss} in its lowest range of values (g_m<200 mmol H_2O m^{-2} s^{-1}; r^2=0.625; Fig. 2b), although in this case this condition was fulfilled by most of the samples (about 79% and 71% of control and drought plants, respectively). Looking at treatment averages after excluding samples with high apparent g_m, we found significant correlations for this variable and the other two mesophyll parameters, lamina *Kh* (r^2=0.952; Fig. 2b, inset) and L_{ss} (r^2=0.979, P=0.011). After applying this low-pass filter to g_m, other correlations emerged across treatment averages: with leaf *Kh* (r^2=0.906; P=0.048), E (r^2=0.852, P=0.077) and g_s (r^2=0.816, P=0.097).

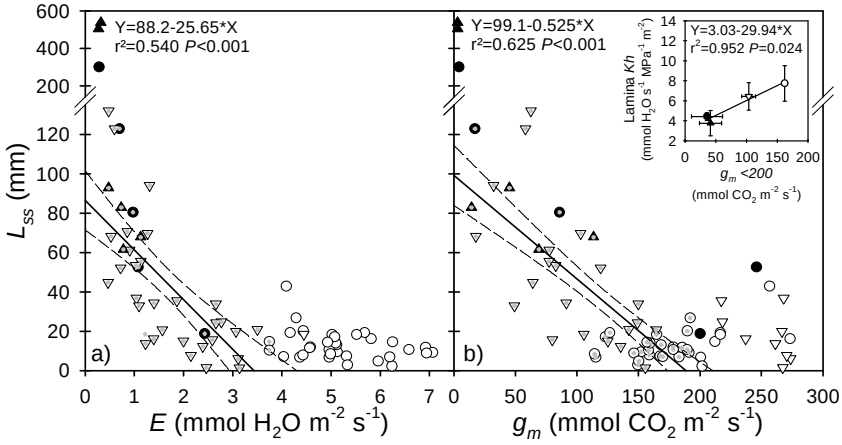

Figure 2. Relationship of individual leaf values for transpiration rates (E) and mesophyll conductance for CO_2 (g_m) with scaled effective pathlength (L_{ss}, determined with the steady state model). Inset: relationship between treatment averages of g_m (excluding samples with g_m>200 mmol CO_2 m^{-2} s^{-1}), and lamina maximum specific hydraulic conductivity (*Kh*). Circles: control plants, triangles: drought plants. Open symbols: intact leaves. Closed symbols: vein-severed leaves. Gray dots: samples included in regression models.

Divergence between steady state and non-steady state models

The application of non-steady state models to calculate the effective pathlenght (L_{nss}) of the afternoon data confirmed the observations derived from the steady-state models (L_{ss}), showing a strong correlation between L_{nss} and L_{ss}, as well as a similar positive correlation between L_{nss} and g_m (Fig. 3a, b). Extending non-steady state models to those samples in which the values at t_{-1} were not available gave comparable results, showing a good correlation between the outputs of steady state and non-steady state models, and most samples showed small estimation errors (Fig.

3c, d). The resulting correlation between treatment averages of L_{nss} and lamina *Kh* was comparable to that obtained with the steady state models (r^2=0.960; see Fig. 3d, inset).

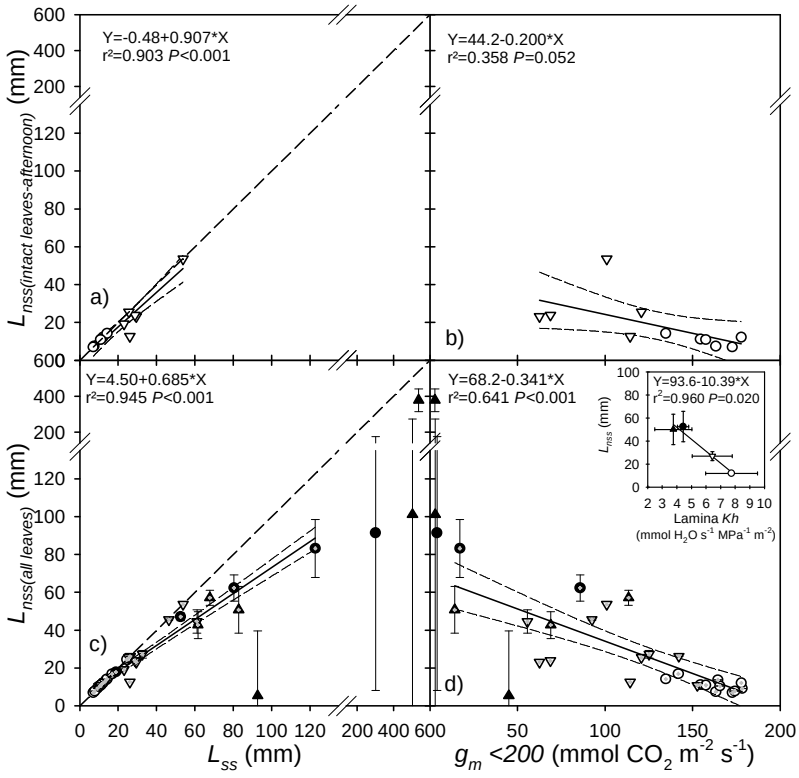

Figure 3. Relationship between averages per measuring time and treatment for scaled effective pathlength (L_{ss} and L_{nss}, determined with the steady state and non-steady state models, respectively), and mesophyll conductance for CO_2 (g_m). In a) and b) L_{nss} calculated for intact leaves in the afternoon, using morning data as reference values (see text for details); In c) and d) L_{nss} calculated for all the samples using the average (symbol) and the 95% confidence interval (error bars) for the differences between morning and afternoon values in intact leaves (see text for details). Inset: relationship between treatment averages of leaf lamina maximum specific hydraulic conductivity (*Kh*) and L_{nss}. Circles: control plants, triangles: drought plants. Open symbols: intact leaves. Closed symbols: vein-severed leaves. Gray dots: samples included in regression models.

Results from the literature survey

Comparing literature data for effective pathlenght *L* and leaf *Kh* showed a relationship between both variables that was consistent with that found in the present experiment (Fig. 4). The relationship was steeper in the lowest range of *L*, and thus the best fit was obtained with a logarithmic model (r^2=0.596). Monocot species (wheat and maize) clearly appeared as outliers for the general relationship, built

mainly from broad-leaved dicots. Pines also appeared as potential outliers, probably due to their particular leaf anatomy. Nevertheless, excluding them from the model gave almost identical results (*Kh*=21.76-3.957**Ln*(*L*); r^2=0.536, *P*<0.001).

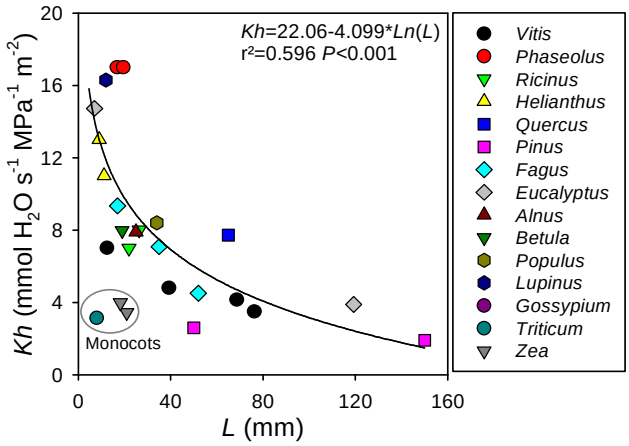

Figure 4. Relationship between effective pathlength (*L*) and leaf-specific hydraulic conductance (*Kh*) across different genus and growing conditions. *Vitis* data correspond to the present study, the rest was derived from literature data. Source: Gregory & Eastham (1996); Gallardo et al. (1996); Bond & Kavanagh (1999); Barbour et al. (2000a); Gan et al. (2003); Barbour et al. (2004); Costa e Silva et al. (2004); Farquhar & Cernusak (2005); Aranda et al. (2005); Li et al. (2005); Liu et al. (2005); Brandes et al. (2006); Barnard et al. (2007); Kahmen et al. (2008); Ripullone et al. (2008); Sellin et al. (2008); Ferrio et al. (2009); Domec et al. (2009). See Table S1 in supplementary material for details.

DISCUSSION

Sources of variability for leaf water enrichment

As expected, the combination of changing environmental conditions from day to day, together with drought and leaf severing treatments resulted in a wide range of leaf water status, and this was reflected in Δ_L (Tables 1-2). However, although leaf temperature was affected by both treatments, and a subsequent effect on Δ_e was predicted, Δ_L was only significantly affected by drought (Table 4). According to theory (Equation 2), average leaf water is expected to be isotopically depleted compared to the site of evaporation due to the *Péclet* effect. Such a depletion would be related to the mass flow of water from the xylem to the stomata, which is in turn assumed to mainly depend on *E* (Farquhar & Lloyd 1993; see Eq. 2). Consequently, the decrease in *E* in response to treatments (Tables 1 and 2) should have reduced the difference between Δ_L and Δ_e, thus contributing to an even higher ^{18}O enrichment of

the leaf lamina water in response to drought and leaf severing. Indeed, assuming the rest of parameters (including *L*) defining the *Péclet* number as constant, variations in modelled isotopic enrichment of leaf water under steady state (Δ_{Lss}) across treatments would be almost two-fold higher than modelled Δ_e (Δ_{Lss}=1.73*Δ_e-19.7; r^2=0.999; P<0.001, for L=13 mm and D=2.79*10^9 m^2 s^{-1}). In contrast, observed lamina leaf water Δ_L showed even less variation than modelled Δ_e (Δ_L=0.91*Δ_e-1.83; r^2=0.924; P=0.039). Among the parameters constituting the *Péclet* number, *C* is a physical constant and variations in *D* are small and well characterised (Cuntz et al. 2007). Thus, changes in *L* appear as the most likely explanation for the observed discrepancies between modelled and measured values, as discussed in detail in the next section. Nevertheless, and despite *L* variations, Δ_L changes were mainly controlled by the driving forces of evaporative enrichment, thus showing a good correlation with Δ_e both at the treatment level (see above) and at the leaf level (Δ_L=0.95*Δ_e-2.57; r^2=0.731; P<0.001). Similarly, assuming a constant L=13 mm, predicted values for lamina leaf water enrichment using the steady-state *Péclet* model (eqn. 2) correlated reasonably well with measured values at the leaf level (Δ_L=0.68*Δ_{Lss}+5.65; r^2=0.653; P<0.001). Thus, we can confirm the main conclusions of previous works (e.g. Ripullone et al. 2008; Ferrio et al. 2009; Kahmen et al. 2009), stating that in most cases *L* changes do not preclude the applications of evaporative enrichment models as leaf water isotopic enrichment is strongly determined by leaf temperature and air humidity. However, variable *L* in response to restricted water availability may be an important source for discrepancies between modelled and measured data, which is not considered by current models and might need to be included to quantitatively predict Δ_L.

Pathlength *L* response to environmental variables: a matter of scale?

Initially, *L* was assumed to be a species-specific constant, potentially associated to the anatomical properties of the leaf (Farquhar & Lloyd 1993; Barbour et al. 2004). Accordingly, most efforts to characterise this parameter have been focused on interspecific variability (e.g. Wang et al. 1998; Barbour et al. 2004; Kahmen et al. 2008). In this regard, *L* values of grapevine under well-watered

conditions (L_{ss}=13 ±1.4 mm**)** agree with the typical range of values observed in other species with high g_s and E (6.25-13.5 mm) (cf. Flanagan et al. 1994; Barbour & Farquhar 2000; Barbour et al. 2000b). Nevertheless, this parameter also showed significant changes with drought, becoming nearly 3-fold greater in water-stressed plants (see table 2). Similarly, Ferrio et al. (2009) found a comparable increase in L from control to drought plants in beech seedlings. In addition, vein excision tended to increase L, although the differences were not significant due to the strong variability in the physiological response to this treatment. Other attempts to assess the response of this parameter to environmental conditions have given contrasting results: whereas Ripullone et al. (2008) showed only a weak but significant relationship between L and atmospheric vapour pressure deficit (VPD) in cotton plants, Kahmen et al. (2009) did not find any significant variation in L with changing relative humidity for common bean, castor bean and sunflower. Thus, the magnitude of L response to environmental changes appears to be species-specific.

Alternatively, it may be argued that non-steady-state effects are behind observed deviations under drought stress, due to changes over time in both water concentration and isotopic composition of leaf water (Farquhar & Cernusak 2005). However, in our case changes in volumetric water content (V_m) were negligible, and the rate of variation in Δ_L was generally small, resulting in little deviations between steady state and non-steady state estimates for effective pathlength (see Fig. 3). In addition, we observed in most cases a weak sensitivity to varying the rate of change in "isostorage" ($V_m \Delta_{Lnss}$), as shown by the small variations in L_{nss} estimates along the 95% confidence interval for this value (Fig. 3c). This finding indicates that leaf water enrichment was relatively insensitive to non-steady state effects in most of the samples. Indeed, the only exceptions were the four samples with extremely low E and g_s that were originally flagged as outliers (see Table 2), suggesting that they were far from steady-state conditions. Altogether, our data exemplifies how the risk of potential deviations due to non-steady state conditions can be detected *a priori* based on gas exchange variables, and can be further confirmed by applying a rather simple sensitive analysis to changes in isostorage: the greater the deviations obtained, the higher the risk associated with the steady state assumption. This procedure might not

be extensible to all species and growing conditions, but is likely to be suitable for isohydric species, such as grapevine.

Mechanisms explaining observed variability in pathlength *L*

Some attempts have been made to relate *L* with measurable leaf parameters (Barbour & Farquhar 2003; Kahmen et al. 2008; Kahmen et al. 2009), and the most consistent pattern found until now is an inverse relationship between *L* and *E*. This trend has been observed across species (Kahmen et al. 2008; Kahmen et al. 2009), leaf ontogeny (Barnard et al. 2007), or in response to water status (Ripullone et al. 2008; Ferrio et al. 2009); this work, see Fig. 2). The mechanisms underlying such relationship are still unclear, but it has been speculated that, according to theory, *L* may reflect changes in the hydraulic properties of the leaf mesophyll (Keitel et al. 2006; Ripullone et al. 2008; Ferrio et al. 2009), which in turn are related with transpiration rates (Sack & Holbrook 2006). Theory predicts an inverse relationship between mesophyll hydraulic conductance and *L*, since, as formulated by Farquhar & Lloyd (1993), the latter can be interpreted as an expression of hydraulic resistance. In practice, however, the main limitations arise from the assumptions required for the determination of *L* (Barbour et al. 2004). The most critical assumption is that of steady state conditions (Farquhar & Cernusak 2005; Cuntz et al. 2007). In this work it has been shown that at least for certain species, non-steady state effects are generally small and can be quantified in a relatively simple way (Fig. 3). Another limitation is that leaf morphology and the presence of casparian strips appear to modify the relationship between *L* and *Kh*, and thus a species-specific calibration may be required. Still another concern is that determining water isotopes in plant tissues is destructive, and requires a costly and time-consuming procedure for water distillation and subsequent analysis. Nevertheless, recent advances in laser spectroscopy offer the possibility to easily determine online leaf water enrichment from the analysis of the isotope composition of oxygen in respired CO_2 (Yakir & Sternberg 2000; Barbour et al. 2007).

In the first study trying to test the assumption that variations in *L* were reflecting changes in mesophyll hydraulic resistance, Kahmen et al. (2009) did not find significant differences between humidity treatments for either *L* or *Kh*. In

addition, although they observed significant differences among species in both parameters, they were not correlated, concluding that *L* was a species-specific constant parameter. This contrasts with our results, showing not only a strong variation in *L*, but also a good correlation with leaf and lamina *Kh* (see Fig. 1). On the one hand, such divergent results may indicate that species with little response in terms of *Kh* and strong variations in leaf water content also show little effects on *L*, whereas more isohydric species, such as beech or grapevine, show greater variations in both parameters (Fig. 4). On the other hand, comparing leaf *Kh* among species with contrasting leaf anatomy (including presumable differences in petiol conductance) introduces an additional source of error that makes difficult to establish definitive conclusions when comparing a limited number of species.

To assess the general validity of our findings, we plotted the data from this work together with that of Kahmen et al. (2009) and a survey of independent data on *L* and *Kh*, resulting in a consistent relationship across a broad range of species (Fig. 4). Interestingly, species with particular leaf anatomic features, such as monocots and conifers showed a distinct relationship between leaf *Kh* and *L*. In both cases, the radial effective pathlength, as calculated here, is complicated by longitudinal *Péclet* effects causing an enrichment in xylem water along the leaf xylem (Helliker & Ehleringer 2000; Farquhar & Gan 2003; Barnard et al. 2007). In addition, in pine needles the veins are located in a central cylinder enclosed in a sort of casparian strip, which causes certain compartmentation of water within the needle (Wu et al. 2005), further complicating the application of evaporative models (Barnard et al. 2007). Nevertheless, in spite of the errors associated to different environmental conditions and leaf anatomy, as well as the use of leaf *Kh* instead of lamina *Kh*, the data demonstrates that species with low *Kh* tend to show higher *L*, and that this trend is consistent in most cases when comparing similar growing conditions for the same or closely related species. Thus, the observed trends in our experiment appear to be extensible to most species, confirming the theoretical relationship between *L* and mesophyll hydraulic resistance, which can be considered as an expression of leaf tortuosity (Cuntz et al. 2007). These results suggest that *L* variations can be potentially used as a faster surrogate to determine changes in lamina *Kh*, which is of

practical importance due to the time-consuming measurements of leaf and lamina *Kh*. In addition, the stronger relationship between *L* and g_m than with g_s or *E* further confirms that there is a direct relationship between mesophyll properties and *L*, rather than an indirect relationship associated to the parametrization method, which included *E* (and to a lesser extent, g_s) as input variables (see Fig. 1).

The relationship between pathlength *L* and mesophyll conductance to CO_2

It is known that water, after leaving the leaf xylem is not only moving on apoplastic pathways but also via transcellular and symplastic pathways to the sites of evaporation (Steudle & Frensch 1996; Barbour & Farquhar 2003; Sack & Holbrook 2006). This provides a potential link between *Kh* and g_m, given that fast responses to environmental conditions to occur in both variables, often uncoupled from stomatal responses, and at least partly mediated by aquaporins (Flexas et al. 2002; Sack & Holbrook 2006; Cochard et al. 2007). In the present study, where changes in *Kh* were forced by means of drought imposition and/or leaf vein severing, g_m generally mimicked the variations in both *L* and *Kh*, suggesting a common effect of aquaporin regulation in the three variables. Our results showing that the linear relationship between g_m and both *Kh* and *L* is only valid for values of g_m<200 mmol CO_2 m^{-2} s^{-1},are likely to be at least partly caused by the assumptions required to perform g_m calculations using the Harley et al. method. With this method, calculation uncertainties increase with larger g_m values (Harley et al. 1992; Pons et al. 2009). Despite the negative relationship observed here between *Kh* and g_m and the negative relationship between *L* and g_m, recent studies have shown that even though both leaf hydraulics and CO_2 diffusion respond to changes in aquaporin activity, different combinations of aquaporin subunits in aquaporin tetramers may promote either water or CO_2 transport, or both, depending on the proportion of PIP1 or PIP2 aquaporins (Otto et al. 2010). Thus, although mesophyll conductance for water and CO_2 appeared to respond concomitantly in our study, an uncoupling between water and CO_2 pathways is likely to occur in response to other environmental variables, e.g. light (Kodama et al., unpublished results).

Concluding remarks

As hypothesised, *L* was more consistently related to mesophyll variables, such as *Kh* or g_m, than to other variables that are included as input parameters for the models, such as *E* or g_s. Indeed, the strongest correlation across treatment averages was found between L_{ss} and lamina *Kh*, the latter being the closest estimate for leaf mesophyll conductivity. Thus, *L* variations can be potentially used as a faster surrogate to determine changes in lamina *Kh*. Moreover, the strong correlation found between *L* and g_m largely supports the idea that water and CO_2 share an important part of their respective diffusion pathways through the mesophyll, so that any down-regulation of leaf hydraulics may result not only in reduced g_s but also in reduced g_m, both contributing to reduced photosynthesis. Further research including simultaneous measurements of leaf water enrichment, leaf *Kh* and aquaporin activity in response to environmental conditions may offer not only new avenues to understand leaf water enrichment, but also for the study of short-term changes in leaf hydraulics.

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4.6. AQUAPORIN EXPRESSION IN RESPONSE TO DIFFERENT WATER STRESS INTENSITIES, ACCLIMATION AND RECOVERY IN RICHTER-110 (*Vitis sp.*): RELATIONSHIP WITH ECOPHYSIOLOGICAL STATUS

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ABSTRACT

Aquaporins seem essential for the regulation of plant water status and expenses. Richter-110 is a *Vitis* hybrid (*Vitis berlandieri* x *rupestris*) reputed to be strongly drought-tolerant. Three irrigation treatments were established in Richter-110 plants growing outdoors defined by the resulting maximum stomatal conductance (g_s), and ensuring water stress situations not severe enough as to stop photosynthesis and growth: well-watered plants (g_s about 250 mmol H_2O m^{-2} s^{-1}), moderate water stress (g_s about 150 mmol H_2O m^{-2} s^{-1}) and severe water stress (g_s about 50 mmol H_2O m^{-2} s^{-1}). Plants under water stress were kept at constant water availability for seven days to check for possible acclimation. Finally, plants were re-watered and allowed to recover for three days. Stomatal conductance, leaf water potential, xylem

abscisic acid (ABA) content and root and stem hydraulic conductivity were determined. The relative amounts of expression of mRNA encoding seven putative aquaporins were determined in roots and leaves by RT-PCR.

The decreases in stomatal conductance with the moderate and severe water stress were associated with increasing ABA contents, but not with the leaf water potential and hydraulic conductivities, which remained unchanged during the entire experiment. Aquaporin gene expression varied depending on which aquaporin, water stress level and the plant organ. We suggest that aquaporin expression was responsive to water stress as part of the homeostasis which resulted in constant leaf water potential and hydraulic conductivity.

Keywords

Aquaporins, drought, recovery, Richter-110, *Vitis*, water stress

Abbreviations

ABA, abscisic acid: AQP, aquaporin; MIP, major intrinsic protein; g_s, stomatal conductance; PIP, plasma membrane intrinsic protein; PLC, percentage of loss conductivity; RWC, leaf relative water content; TIP, tonoplast intrinsic protein; Ψ, leaf water potential.

INTRODUCTION

Water shortage is well-known to induce a myriad of changes in physiological processes (Hsiao 1973). Plant water status is determined by the balance between water losses in transpiration to the atmosphere and water uptake from the soil. When transpiration exceeds uptake, cell turgor falls, which negatively affects plant physiology and consequently plant productivity, distribution and competitive relationships (Boyer 1982; Chaves et al. 2003).

Many studies have focused in the analysis of short-term physiological responses, particularly the responses of water relationships (Hsiao 1973; Jones et al. 1981; Lovisolo et al. 2002) and photosynthesis to water stress (Chaves 1991; Lawlor and Cornic 2002; Flexas et al. 2004). Short-tem water stress usually affects these parameters negatively, inducing decreased plant water status, photosynthesis and growth (Chaves et al. 2003). However, under field conditions, the overall time-

integrated response of plants may not be related only to the initial negative response, but also dependent on acclimation as well as on the capacity and rate of recovery after re-watering (Flexas et al. 2006a). Acclimation to water stress may involve changes in gene expression resulting in modification of plant physiology and morphology, which may take place over periods of days or weeks and moderate the initial negative effects of water stress. Acclimated plants would show improved water relations and photosynthesis compared to non-acclimated plants, which may lead to lower growth losses (Flexas et al. 2006a). While advances have been made concerning the physiological mechanisms involved in acclimation, including solute accumulation and up- and down-regulation of specific proteins (Chaves and Oliveira 2004), these responses mostly relate to severe water stress conditions where photosynthesis and growth have ceased. The mechanisms leading to acclimation to more modest water stress are, however, mostly unknown. The increase and/or maintenance of a high hydraulic conductivity, allowing the maintenance of cell turgor, may be crucial for acclimation (Sperry et al. 2002).

Studies on plant AQPs and plant water relations have been carried out for years allowing the discovery of diverse functions played by AQPs. These proteins have been shown to be involved in root conductivity and, therefore, water uptake and transport by roots (Henzler et al. 1999; Tyerman et al. 2002). Furthermore, AQPs involvement in stem conductivity has been documented (Kaldenhoff et al. 1998; Siefritz et al. 2002). In addition, a specific role for aquaporins during recovery in xylem conduit re-filling after drought-induced embolism has been proposed (Holbrok and Zwieniecki 1999; Tyree et al. 1999). Some AQPs have also been shown to perform other functions, such as transport of ammonia and other substances (Tyerman et al. 2002; Luu and Macrel 2005), including CO_2 in the leaf mesophyll during photosynthesis (Uehlein et al. 2003; Hanba et al. 2004; Flexas et al. 2006b). Despite these findings, the potential relationship between the role of AQPs in the regulation of plant water status and the regulation expression of their genes is unclear. Some AQPs are constitutively expressed (Johansson et al. 1996), while the expression of others is regulated by different stimuli, such as developmental stage, hormones, or by adverse environmental conditions such as drought and salinity

(Vera-Estrella et al. 2004). AQP gene expression has been shown to increase, decrease or remain unaffected under short-term water stress conditions (e.g. Alexandersson et al. 2005). In addition, although gene expression responses to environmental changes can occur within hours, the expression pattern of AQPs after long term water shortage, involving acclimation mechanisms, remains to be elucidated. Moreover, 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, photosynthesis, stomatal conductance and hydraulic conductivity.

Despite the extensive research on AQPs, little is known about their physiological roles in plants under favorable conditions or during acclimation to water stress and recovery. This article reports the short and long term expression profiles of seven AQPs, belonging to three different subfamilies, for two different water stress intensities, and during a re-watering treatment. *Vitis* is an excellent plant for studying physiological and molecular responses to water stress under realistic crop conditions because most of its vegetative development takes place during Mediterranean summer. The selection of R-110 rootstock, a hybrid of *Vitis berlandieri* and *Vitis rupestris*, was based on its reported high vigor and resistance to drought stress (Galet 1988). Moreover, R-110 is the unique *Vitis* genotype for which the characterization of a number of putative AQP genes has been published (Baiges et al. 2001). We describe the expression profile of *Vitis* AQP genes relating them to plant water status.

MATERIAL AND METHODS

Plant material and water stress treatments

Sixty plants of Richter-110 (*Vitis berlandieri* × *Vitis rupestris*) were grown from cuttings outdoors at the University of the Balearic Islands in 30 L pots filled with a mixture of soil and organic substrate. The plants were one year old and were irrigated daily with water from April to mid July, with a supplement of 50% Hoagland's solution once per week. At the beginning of the experiment, 20 of plants were kept as controls, irrigated daily to field capacity, while irrigation was stopped in

the other 40 plants. Two levels of water stress were established, defined by the leaf maximum daily stomatal conductance (g_s), as suggested by Flexas et al. (2002): moderate drought (g_s about 150 mmol H_2O m^{-2} s^{-1}; 150T) and severe drought (g_s about 50 mmol H_2O m^{-2} s^{-1}; 50T). Once the first desired level of g_s was achieved (4 days after stopping irrigation), 20 plants were kept for a week at similar g_s of ~150 mmol H_2O m^{-2} s^{-1} daily replacing the amount of water consumed, as determined by weighting pots daily in the evening. The other 20 plants were left non-irrigated until g_s was ~50 mmol H_2O m^{-2} s^{-1} (8 days after stopping irrigation), and then kept for a week at that level of stress as in the previous treatment. After one week maintained at the established soil water deficit, plants of each water stress treatment were sampled again (treatments 150M and 50M). Then all plants were irrigated to field capacity, and irrigated daily to measure recovery after three days (treatments 150R and 50R).

Leaf water potential

Pre-dawn and midday leaf water potentials (Ψ) were determined with a Scholander chamber (Soilmoisture Equipment Corp., Santa Barbara, CA, USA). Five replicates per treatment were obtained in each sampling. Measurements were performed on young, fully expanded, fully exposed apical leaves.

Stomatal conductance

Stomatal conductance (g_s) was measured at mid-morning with an open gas exchange system (Li-6400; Li-Cor, Inc., Lincoln, NE, USA). All measurements were carried out at 1500 µmol photons $m^{-2}s^{-1}$. Cuvette CO_2 concentration was set at 400 µmol CO_2 mol^{-1} air and vapor pressure deficit between 2.0 and 3.4 kPa. Measurements were performed on young, fully expanded, fully exposed apical leaves.

Abscisic acid concentration

Xylem abscisic acid (ABA) concentration was determined at midday in five replicates per treatment at sampling point. Young fully expanded and sun exposed leaves were excised and xylem exudation was collected after applying sufficient

pressure with a leaf pressure chamber (Soilmoisture Equipment). After discarding the first exudation, sap was collected and immediately submerged in liquid nitrogen and kept at -80ºC. The ABA concentrations in xylem exudation were measured with the Phytodetek ABA enzyme immunoassay test kit (Agdia Inc.; Elkhart, IN, USA).

Hydraulic conductivity

Hydraulic conductivity was measured in roots and twigs in three replicates at sampling point. Diameter of samples was less than 0.3 cm. Roots and twigs were detached and the cut surface rapidly sealed with vaseline to prevent embolisms caused by air entering into the cut vessels.

The initial hydraulic conductance (k_i) of each root or twig segment was measured gravimetrically connecting the xylem segment to a low-pressure water source (approximately 0.005 MPa) and registering the weight of the flowing water every second on a balance, until a steady state was reached. Then existing embolisms were flushed out using a 0.15 MPa water flow, coming from a compressed air tank, and the hydraulic conductance (k) was measured again. This process was repeated until k no longer increased after flushing, and this point was taken to be the maximum conductance (k_{max}).

The percent of hydraulic conductance loss (PLC) was calculated as:

$$PLC = 100 \cdot \left(\frac{k_{max} - k_i}{k_{max}} \right)$$

Where k_i and k_{max} represents the initial and maximum xylem hydraulic conductance, respectively.

RNA extraction

Leaves and roots of similar developmental stage and from similar position to those used to determine the plant water status and hydraulic conductivity were harvested on sampling days at 10 a.m. Five replicates of different plants were sampled per treatment and day, immediately frozen in liquid nitrogen and kept at -80 ºC until analysis.

Total nucleic acids were extracted with CTAB buffer (Doyle and Doyle, 1987). Total RNA was then isolated by a conventional chloroform/isoamyl alcohol isolation procedure and precipitated with LiCl. RNA was quantified by spectrophotometry at 260 nm and the integrity of the RNA was checked by electrophoresis on a 1% agarose gel. In order to remove contaminant DNA from the RNA samples, the nucleic acid extract was treated with RNase-free DNaseI (Roche Diagnostic GmbH; Mannheim, Germany), according to the manufacturer's instructions.

Semi-quantitative real-time PCR analysis

For the RNA analysis, cDNA was synthesized using SuperSript^{TM} II RNase H$^-$ Reverse Transcriptase (Invitrogen) according to manufacturer instructions, using oligo d(T)$_{15}$ as a primer. The reactions were incubated at 42ºC for 30 min, 50ºC for 40 min and 95ºC for 5 min.

Semi-quantitative real-time polymerase chain reaction was performed in a LightCycler 2.0 system (Roche) using SYBR Green I sequence non-specific detection (Roche). Transcript abundance of AQPs PIP1.1, PIP1.2, PIP1.3, PIP2.1, PIP2.2, TIP1 and TIP2 were analyzed using specific primers (Table 1) (Baiges et al. 2001). To test the suitability of these primers, the specificity and identity of the reverse transcription (RT)-PCR products was monitored after each PCR by a melting curve analysis of the reaction products, which can distinguish the gene-specific PCR products from the non-specific PCR products. The temperature of PCR products was elevated from 55 to 99ºC at a rate of 1ºC/5 s, and the resulting data were analyzed by using the LightCycler software. Only one single band with a characteristic melting point was observed for each sample, indicating that the RT-PCR reaction produced a product specific to the primers used for the reaction. To further confirm that the primer sets produced only the target genes, the RT-PCR products were separated by electrophoresis and visualized in a 1% agarose gel.

The cDNA was amplified by 30-40 cycles at 94°C for 36 s, 56-60°C (depending on each primer) for 90 s, 72°C for 90 s, followed by a final extension step

of 72°C for 6 min. The reaction mixture contained sufficient cDNA template, 0.5 µM of each primer and an appropriate amount of SYBR Green I master mix (Roche).

Table 1. Gene-specific primers pairs used in the real-time RT-PCR experiments.

Gene	Primer
PIP1;2	Forward; 5′-CCTCCTCCTGAATCTGGATTG-3′
	Reverse; 5′-GCGAGAGAAGCCATTAAAG-3′
PIP1;3	Forward; 5′-GACTTCCATCTCCTCTCTCTT-3′
	Reverse; 5′- ATGGGCAGGGAAGGATAAAAG -3′
PIP2;1	Forward; 5′- TTGCAGAGCCATTTTGATTCC-3′
	Reverse; 5′- GGAAAAGATAAGCATGAAGTGG-3′
PIP2;2	Forward; 5′- CAACTAAAAACCCACAACACCC-3′
	Reverse; 5′- TACACAAAAGCCCAAAGCTAACA-3′
TIP1	Forward; 5′- GTTGTTGTCTCAACCCATTTCC-3′
	Reverse; 5′- ATCACCAACCTCATTCATATGC-3′
TIP2	Forward; 5′- GGAGCTTGCTATGAATTTCAGG-3′
	Reverse; 5′- GTGATTGCAAACAAACCAGACAG-3′
Malic	Forward; 5′-TTCCTGGACAGGCTAACAATG-3′
	Reverse; 5′-TTAGCAGCAATGTGGGCTGAT-3′
Elongation factor 1	Forward; 5′- GAACTGGGTGCTTGATAGGC-3′
	Reverse; 5′- AACCAAAATATCCGGAGTAAAAGA-3′
Ubiquitine	Forward; 5′-AGTAGATGACTGGATTGGAGGT-3′
	Reverse; 5′-GAGTATCAAAACAAAAGCATCG-3′

Values for the threshold (Ct) were determined using the LightCycler software. Relative gene expression numbers were calculated as a percentage of control plants, using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001) with malic enzyme gene as a reference. To further detect changes in transcript abundance, relative gene expression values were also normalized using two other reference genes: ubiquitin and elongation factor 1 (Table 1). No significant changes were observed when normalizing data with any of the three different reference genes.

Statistical analysis

Regressions coefficients and correlations were calculated with the 8.0 Sigma Plot software package (SPSS; Chicago, IL, USA). Differences between means were assessed by Duncan analyses ($P < 0.05$), performed with the SPSS 14.0 software package (SPSS).

RESULTS

Plant water status

Two water stress treatments were set on the basis of the stomatal conductance (g_s): a moderate stress intensity, corresponding to g_s of about 150 mmol H_2O m^{-2} s^{-1}, and a severe water stress intensity, with a g_s of about 50 mmol H_2O m^{-2} s^{-1} (Fig. 1a). Once these g_s values were reached, plants were maintained at constant water availability to check for possible acclimation. However, the treatment did not result in significant changes of g_s after a week ($P < 0.05$) (Fig. 1a). Although the ABA may become more concentrated in the xylem of water-stressed plants due to lower rates of flow, g_s was found to be inversely proportional to the evolution of ABA content throughout the experiment (Fig. 1b), except for data during recovery (see below). In plants subjected to moderate stress the leaf water potential at midday (Ψ) decreased progressively although not to a great extent from short to long term water stress (Fig. 1c). By contrast, severely stressed plants did not show any statistically significant change in Ψ as water stress progressed, but trend was to maintain a higher midday leaf water potentials (Ψ) than plants maintained to moderate stress ($P < 0.05$). Furthermore, maintenance to long term severe water stress did not induce significant changes in Ψ. Pre-dawn Ψ (not shown) showed the same trend described for midday values. The leaf relative water content (RWC) both at pre-dawn and midday were kept constant during the entire experiment, as it was the bulk modulus of elasticity (data not shown). Similarly, stem and root percentage of loss conductivity at mid-morning was maintained constant whatever the treatment and measuring day (Fig. 1d, and not shown).

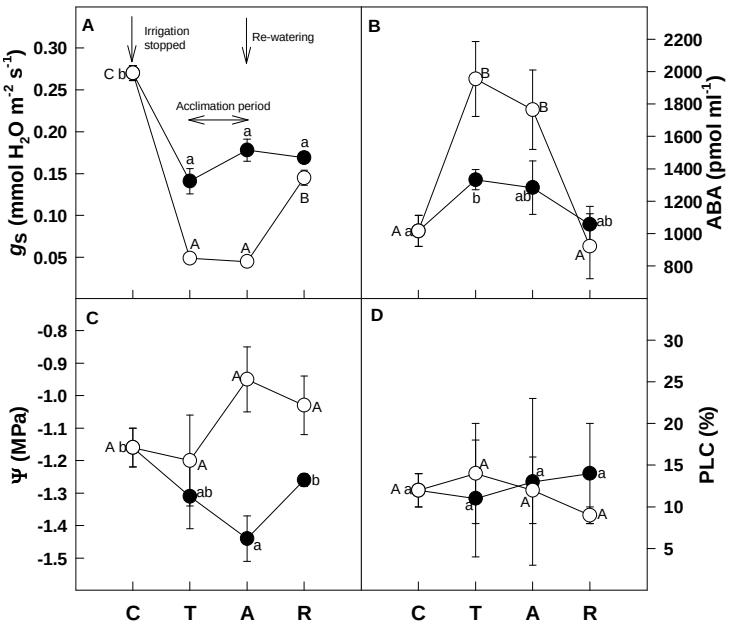

Figure 1. (A) Stomatal conductance (g_s), (B) midday xylem ABA content (ABA), (C) midday leaf water potential (Ψ), and (C) shoot percentage of loss conductivity (PLC). Filled symbols represent g150 treatment and open symbols g50 treatment. Data are means ± SE of 4-6 replicates. Measuring day abbreviations: C, control; T: first day under the correspondent treatment; A: acclimation after 7 days under the correspondent treatment; R: 3 days after re-watering. Different letters denote statistically significant differences by a Duncan's multiple comparison test ($P < 0.05$) within each treatment.

Re-filling pots to field capacity led to different responses depending on the intensity of imposed water deficit. Re-watering moderately water stressed plants resulted in no significant ($P > 0.05$) change in g_s after three days, while g_s of plants subjected to severe stress increased up to ~50% of control values (Fig. 1a). Contrary to g_s, the ABA concentration recovered to control values after re-watering irrespective of imposed stress (Fig. 1b). Midday Ψ was increased three days after re-watering moderatly stressed plants, but no change was observed when re-watering severely stressed plants (Fig. 1c). Finally, values of stem and root hydraulic conductance still remained unaffected after three days re-watering (Fig. 1d and not shown).

Water stress-induced changes in expression profile of AQPs

Using semi-quantitative RT-PCR, the mRNA accumulation of seven AQPs was investigated throughout the experiment. Despite the analysis of two different

stress intensities and their effects at short-term, long-term and recovery, AQP expression changes in leaves ranged only from no change to 10-fold. All seven AQP genes showed a similar pattern of expression in leaves as a result of water stress imposition, stress maintenance and re-watering (Fig. 2). This pattern was firstly characterized by an initial significant decrease of all AQP genes expression in plants subjected to moderate water stress followed by an increase to almost control values of expression after one week at constant soil water deficit.

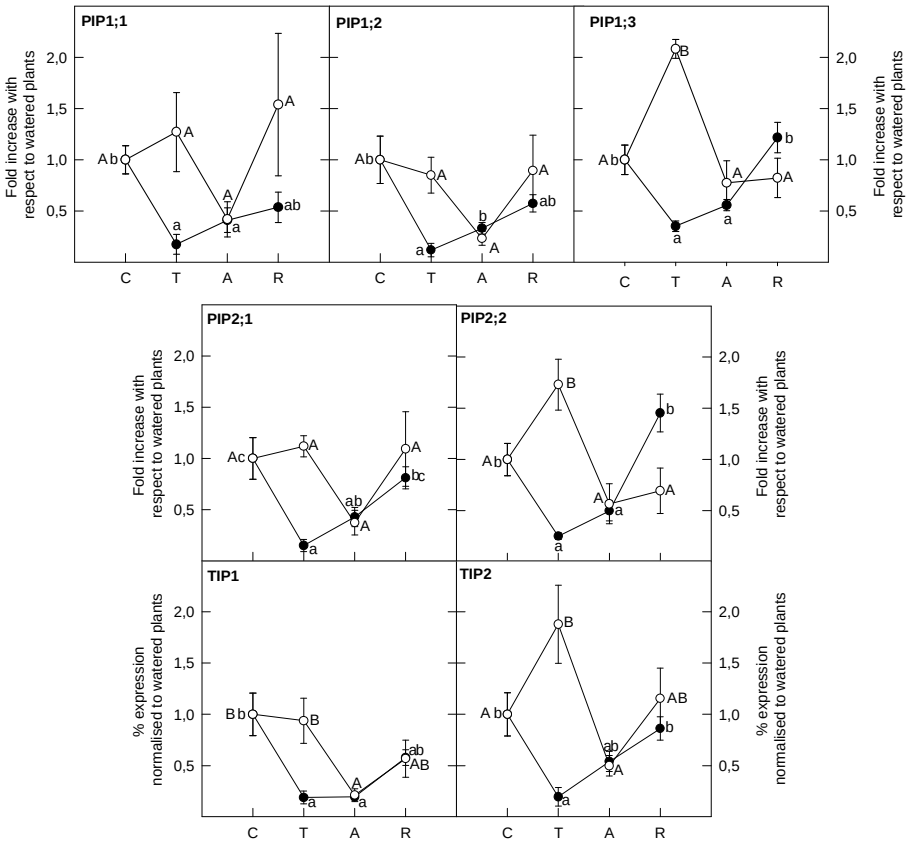

Figure 2. Expression of *Vitis* aquaporins in leaves. Values represent the fold-increase expression for each aquaporin in leaves with respect to control plants. Filled symbols represent g150 treatment and open symbols g50 treatment. Data are average ± SE of 4-6 replicates. Measuring day abbreviations: C, control; T: first day under the correspondent treatment; A: acclimation after 7 days under the correspondent treatment; R: 3 days after re-watering. Different letters denote statistically significant differences by a Duncan's multiple comparison test ($P < 0.05$) within each treatment.

In severely stressed plants, by contrast, the expression of all AQP genes was similar to or significantly higher than control values, depending on the particular gene, but they were also restored to control values after one week at constant soil

water deficit. Because at day 'T' for g150 plants both g150 and g50 plants were enduring exactly the same water stress, the values shown as g150T are also representative of interim values between g50C and g50T. Therefore, expression of all AQPs initially decreased during water stress imposition, but then largely increased to or above control values as water stress intensified to g50. Subsequently, when plants were kept for a week at constant soil water deficit, regardless of its intensity, all expression values were decreased back to control values or even lower. Finally, re-watering resulted in maintenance or increase of expression levels depending on the specific AQP and the intensity of stress. Changes in the patterns of leaf AQP expression were not associated to the AQPs subfamilies ($P > 0.05$).

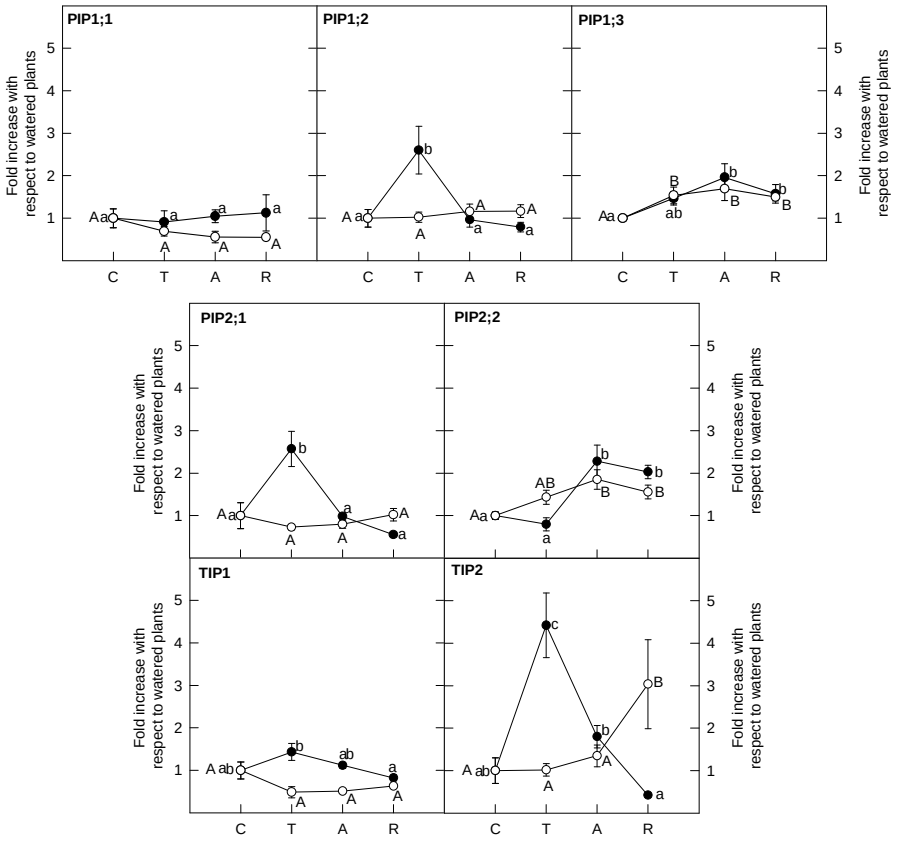

Figure 3. Expression of *Vitis* aquaporins in roots. Values represent the fold-increase expression for each aquaporin with respect to control plants. Filled symbols represent g150 treatment and open symbols g50 treatment. Data are average ± SE of 4-6 replicates. Measuring day abbreviations: C, control; T: first day under the correspondent treatment; A: acclimation after 7 days under the correspondent treatment; R: 3 days after re-watering. Different letters denote statistically significant differences by a Duncan's multiple comparison test ($P < 0.05$) within each treatment.

In roots, changes in AQP expression profile throughout the experiment were of similar magnitude to those reported in leaves (Fig. 3). Contrary to AQP expression in leaves, no general trend was observed in the evolution of AQP transcript abundance in roots. In addition, when significant changes occurred, they were only observed for plants subjected to moderate water deficit. Hence, only PIP1.2, PIP2.1 and TIP2 increased under moderate stress (Fig. 3). Nevertheless, this enhanced expression was reversed when plants were kept at constant soil water deficit for a week. Prolonged stress only resulted in significant increases in the expression of PIP2.2 in moderately stressed plants. Three days after re-watering, all genes maintained their expression levels in root constant, excepting TIP2 in moderately stressed plants, which further decreased (Fig. 3).

Table 2 relates the expression of AQPs in roots and leaves. Under well-watered conditions, only TIP1 and especially PIP1.1 were expressed more in roots than in leaves. In fact, these two AQPs were the only ones showing higher expression levels in roots than in leaves whatever the water deficit intensity and sampling day. As a result of the generalized AQP expression pattern in leaves, the evolution of the root to leaf expression profile was also found to be common for all the AQPs. The root to leaf expression ratio significantly increased ($P < 0.05$) when exposing plants to moderate stress.

Table 2. Expression of *Vitis* aquaporins in roots as compared to leaves. Values represents the fold-increase expression for each aquaporin in roots with respect to that observed in leaves under the same water stress treatment and measuring day. Asterisks denote statistical differences between root and leaf expression at $P < 0.05$ by Duncan Analysis. Measuring day abbreviations: C, control; T: first day under the correspondent treatment; A: acclimation after 7 days under the correspondent treatment; R: 3 days after re-watering.

Treatment	**Measuring day**	**PIP1-1**	**PIP1-2**	**PIP1-3**	**PIP2-1**	**PIP2-2**	**TIP1**	**TIP2**
Control	C	24.3 *	0.2 *	0.3 *	0.2 *	0.4 *	2.4 *	0.5
g150	T	127.4 *	5.0 *	1.4	2.8 *	1.5	18.8 *	12.2 *
	A	62.1 *	0.7	1.2	0.4 *	2.1 *	14.1 *	1.8 *
	R	51.1 *	0.3 *	0.4 *	0.1 *	0.6 *	3.5 *	0.3 *
g50	T	14.2 *	0.3 *	0.2 *	0.1 *	0.4 *	1.3	0.3 *
	A	32.3 *	1.1	0.7	0.3 *	1.5	5.9 *	1.5
	R	8.8 *	0.3 *	0.6	0.1 *	1.0	2.7 *	1.4

Then, prolonged water stress generally decreased the ratio of leaf to root transcript abundance to some extent, followed by further decreases after re-watering. Changes in the ratio of root to leaf expression for severely stressed plants were less evident but, contrary to moderately stressed plants, the highest ratio for all the AQPs analysed was observed after keeping the plants at constant soil water deficit for a week.

DISCUSSION

Few data exist concerning specific responses of plants to long-term water stress, including the phenomenon of acclimation (Conroy et al. 1988; Flexas et al. 2006a). In the present work, plants kept under constant water deficit for seven days did not show any significant sign of acclimation by modification in physiological parameters, either in g_s, ABA content, leaf water potential (Ψ) or hydraulic conductivity (Fig. 1). Either the plants can support the levels of constant water stress imposed without acclimation, or acclimation took place during the few days needed to establish the chosen stress intensities. Both possibilities would be consistent with the reputation of *Vitis* R-110 as a strongly drought-tolerant genotype.

Stomata close under water stress in response either to soil-to-leaf chemical signals (Tardieu and Simmoneau 1998), to Ψ (Comstock and Mencuccini 1998) or to leaf specific hydraulic conductivity (Schultz 2003). While correlations of g_s with Ψ or PLC (%) were non-significant ($P > 0.05$), g_s was significantly correlated with the ABA content in the xylem ($R^2 = 0.792$, $P < 0.01$). This fact supports the idea that g_s variations during water stress are mostly determined by ABA content in grapevines, as previously suggested (Lovisolo et al. 2002). However, it is remarkable that in plants maintained for a week at severe water stress g_s partially recovered three days after re-watering, while such recovery was not observed in plants maintained under moderate stress (Fig. 1). Since ABA contents were decreased in all plants, these results suggest that internal factors other than ABA regulate stomatal opening after a water stress period.

AQPs in plants often show a tissue/organ-specific expression (reviewed by Tyerman et al. 2002). In the present study, relative abundance of transcripts in roots and leaves strongly depended on which AQP and the treatment given. In non-stressed

plants, PIP1.1 was more abundantly expressed in the roots compared to leaves, consistent with the observations described for other PIPs in other species (Weig et al. 1997; Jang et al. 2004). Roots also presented higher TIP1 transcripts than leaves, in contrast to the results found in *Arabidopsis* TIPs (Alexandersson et al. 2005). On the other hand, the remaining five AQP genes showed higher transcript abundance in leaves than in roots, also contrasting with the results reported for *Arabidopsis* (Jang et al. 2004) and olive (Secchi et al. 2006).

Water stress intensity, prolongation of water stress, and re-watering, all affected the ratio of root to leaf transcript abundance (Table 2). Plants subjected to short-term water stress showed an enhanced ratio of root to leaf AQP expression, particularly for the moderate stress treatment. This is perhaps an indication that, under acute water stress, the function of these aquaporins is more important in roots than in leaves, which would be consistent with the idea that the do facilitate water transport (Luu and Maurel 2005). Seven days of maintenance at soil water stress and re-watering resulted in decreases of this ratio back to values close to those observed under control conditions.

These patterns of AQP gene expression result from specific expression profiles in leaves and roots under different water stress intensities. The role of AQPs in regulating plant water status under water stress is a complex issue, because the expression of different AQP genes may be stimulated, decreased, or unchanged under abiotic stress (Yamaguchi et al. 1992; Maurel 1997; Kirch et al. 2000; Kawasaki et al. 2001). In this study, changes in the expression profile in leaves have been observed to follow a common pattern (Fig. 2). First, expression levels were strongly decreased under short-term moderate water stress and maintained or increased under short-term severe water stress. Second, plants maintained for seven days under water stress presented approximately 50% transcript abundance of well-watered plants, independently of the intensity of water stress. Finally, AQP expression tended to increase after re-watering. Although the described pattern presented some particular trends for specific AQPs, a number of different AQP genes have been shown to respond to stress in a similar way. Furthermore, here we increase experimental evidence for the conclusion that the regulation of AQP gene expression is dual in

response to water status: a moderate decrease in water availability results in down-regulation, but the more drastic decrease results in up-regulation. The initial down-regulation and the subsequent up-regulation of AQP gene expression has been observed using microarrays in two rice cultivars (Kawasaki et al. 2001) and in *Arabidopsis* (Maathuis et al. 2003). Down-regulation of AQP gene expression by drought stress may result in reduced membrane water permeability, and so promote cellular water conservation during initial periods of dehydration stress (Li et al. 2000; Smart et al. 2001), therefore preserving leaf cellular turgor. This is reinforced by the analysis of transgenic tobacco plants over-expressing the *Arabidopsis* PIP1.2, which has been shown to have a negative effect during drought (Aharon et al. 2003). Up-regulation is thought to increase membrane permeability to water transport when water is less available (Yamada et al. 1997). The changes in AQP expression in response to stress were accompanied by no significant change in leaf water status.

Remarkably, this general pattern of expression in leaves was not observed in roots, which showed a more diverse pattern of transcripts expression under the different treatments, as usually reported (Yamada et al. 1997; Jang et al. 2004). The results imply that the responsiveness of each AQP to water stress was different in roots, and that the regulation of AQP expression in roots is perceived to be complex and to involve integration of different signals. Yamada et al. (1997) concluded that differential regulation in gene expression and the multiplicity of PIP genes may allow plants to respond to environmental changes differently, maintaining their basic water status.

In addition, in some cases, AQP gene expression profile in response to water stress in roots was observed to follow the opposite trend reported in leaves. This is the case of PIP1.2, PIP2.1 and TIP2 expression in plants kept for a week under moderate water stress. Jang et al. (2004) reported down-regulation of some PIP genes by drought in the aerial parts, but up-regulation by salt in the roots, and attributed this differential expression profile to diverse functionality of these PIPs in leaves and roots. In our study, for instance, while the expression of *Vitis* PIP1.2 gene in the aerial parts decreased under moderate water stress to limit water loss and keep water content constant during periods of dehydration stress, the same AQP gene increased

in the roots to enhance water uptake and to maintain reasonable water status (Figs. 2 and 3). In conclusion, the complex expression pattern of AQP genes suggests that maintenance of a proper water status under water deficit requires both increased water transport via AQPs in some cells and reduced water transport via AQPs in other cells and tissues.

The next important question is to verify whether these changes in transcript levels of each AQPs are related to improved physiological status of plants under stress. Down-regulation of AQP genes is commonly associated with losses in hydraulic conductance of plant organs, such as roots (Henzler et al. 1999; Clarkson et al. 2000; North et al. 2004). Decrease in AQP transcripts have been also correlated with the time course of turgor transition (Yamada et al. 1997). Nevertheless, in the present study changes in AQP gene expression did not significantly correlate with any of the physiological parameters considered. Therefore, rather than a simple correlation between AQP transcripts and physiological measurements, these results suggest that plants subjected to water stress modulate the expression profile of AQPs, to allow plants to maintain water homeostasis under stress conditions. For instance, an initial decrease in expression when stress is moderate may help to reduce water transport and hence to decrease stomatal conductance to keep plant water status (Li et al. 2000; Smart et al. 2001). At more severe stress, when stomatal conductance is already low, and soil water availability is strongly reduced, an increased expression of AQPs may help to increase membrane permeability to water transport to optimize plant water status (Yamada et al. 1997). The fact that these changes are not reflected in large changes in PLC could be due to the fact that aquaporins are mostly involved in symplastic water transport (Ranathunge et al. 2004; Luu and Maurel 2005), while PLC as measured in the present study deals essentially with flow in the axial pathway, which is apoplastic.

Alternatively, the lack of correlation between the expression patterns of PIP genes under stress conditions and physiological parameters could be due to the fact that regulation of AQP activity is not only restricted to the transcriptional level. The water permeability of specific AQPs is also likely regulated post-transcriptionally via phosphorylation at one or multiple sites. Johansson et al. (1996, 1998) demonstrated

that in spinach leaves the AQP PM28A was inactivated through dephosphorylation under drought conditions. Similarly, changes in cytosolic pH also influence AQP activity (Tournaire-Roux et al. 2003). Even more, a TIP isoform in ice plant has been shown to suffer re-localisation between cellular membranes due to glycosylation (Vera-Estrella et al. 2004). Finally, AQPs are not the unique way to control physiological parameters considered in the current paper. For instance, hydraulic conductivity of tissues is regulated by three different pathways of water flow; symplastic movement of water via plasmodesmata, transcellular movement across cell membranes, and apoplastic flow through the cell walls (Steudle et al. 1993; Steudle 1994). The relative contribution of each pathway to flow across tissues and organs varies depending on the exact circumstances, which provides a useful mechanism by which the plant can respond to changing environmental conditions (Steudle 1994; Steudle and Peterson 1998).

The use of transgenic *Vitis* plants, with either over- or reduced expression of specific AQPs, as available for many other species, would permit a more precise insight into the physiological roles of these proteins in *Vitis* and their real importance in drought tolerance mechanisms, in terms of both water relations and photosynthetic response. This should be a research priority for the near future.

In conclusion, the expression patterns of seven AQP genes were different in leaves and roots of Richter-110 plants under different water stress intensities. In leaves, the results obtained suggest that the regulation of AQP gene expression is dual in response to water status: a moderate decrease in water availability results in down-regulation, but the more drastic decrease results in up-regulation. However, in roots, AQP expression showed more diverse patterns, with no generality among different AQPs. AQP gene expression patterns were not related to changes in any water relations parameter measured concurrently in the same plants, such as the leaf water potential and relative water content, ABA content, stomatal conductance or shoot and root hydraulic conductivity. This lack of correlation between AQP transcript abundance and physiological measurements suggests that AQP expression is modulated by water stress to permit plants to maintain homeostasis in their water status.

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4.7. HYDRAULIC CONDUCTIVITY DYNAMICS IN CHARDONNAY UNDER WATER STRESS AND RE-WATERING AND THE RELATIONSHIP OF AQUAPORIN EXPRESSION

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ABSTRACT

Stomatal closure is one of the first processes that occurs in leaves in response to soil water stress, but less is known of how aquaporins may respond in the leaf and which may be important in regulation of leaf water balance. *Vitis vinnifera* L. cv Chardonnay grapevines were subjected to water stress (WS) such that stomatal conductance (g_s) declined to 50 mmol H_2O $m^{-2}s^{-1}$. This was followed by re-watering after WS acclimation (ACCL) of 7 days with periodic watering to maintain g_s near 50 mmol H_2O $m^{-2}s^{-1}$. To assess the extent of water flow through aquaporins in leaf membranes of control, recovered (REC) and WS plants, leaf hydraulic conductivity (Kh_{leaf}) was measured with and without the addition of 100 μM $HgCl_2$ to the perfusate entering petioles of detached leaves. Other potential aquaporin blockers were tested (H_2O_2 and K-acetate at low pH) but only $HgCl_2$ caused a significant reduction in Kh_{leaf} (22%) with only a small but insignificant effect on g_s. In the first stage of WS the inhibition by $HgCl_2$ was higher (40%) and then declined. During the first stages of recovery there was no significant inhibition of Kh_{leaf} by $HgCl_2$ until after Kh_{leaf} returned to control levels after 7 days since re-watering when $HgCl_2$ inhibition was largest. Expression of aquaporins (*VvPIP1;1, VvPIP2;1, VvPIP2;2, VvPIP2;3, VvTIP1;1, VvTIP2;1*) was examined during WS, ACCL and recovery. The expression of these aquaporins was also determined in response to 3 hours of perfusion with KNO_3 +/- $HgCl_2$ three days after re-watering. Treatment with $HgCl_2$

did change transcript abundance for *PIP2;2* (for non water stressed leaves) and *PIP2;3* (for water stressed and recovery leaves). A principal component analysis was performed on the physiological variables and aquaporin transcript abundance. There was a close association between Kh_{leaf} and the expression of *PIP2;3* and to a lesser extent with *PIP2;1 and TIP2;1*. Kh_{leaf} was correlated with gs and with the transcript abundance of *PIP 2;3, PIP2;1* and *TIP 2;1*. The proportion of apoplastic versus symplastic pathways of water movement into the leaf was assessed using HPTS (apoplastic tracer) and acetate-HPTS (symplastic tracer) in the perfusate. There was an apparent increase in apoplastic tracer accumulated under water stress than in control suggesting that there may be more flow via the apoplast in grapevine leaves under WS, or that the membrane permeability to acetate-HPTS declined under WS. This change in proportion of the accumulation of tracer corresponded with lower Kh_{leaf} and less inhibition by $HgCl_2$ of Kh_{leaf}.

INTRODUCTION

Typically, water loss is modulated by stomata in order to prevent desiccation of the plant in dry conditions. Many physiological variables are known to be implicated in stomatal regulation; some of these variables are involved in the mechanics of stomatal movements, others in the signals triggering these movements (Zeiger et al., 1987). A number of studies have confirmed a functional relationship between the hydraulic properties of plants and stomatal conductance, photosynthetic capacity and growth rate (Meinzer and Grantz 1990; Saliendra et al. 1995; Comstock 2000; Nardini and Salleo 2003; Brodribb et al. 2002, 2007, Santiago et al. 2004, Woodruff et al. 2007). Indeed, it has been suggested that stomata may respond to drought-induced changes in the hydraulic characteristics of the sap pathway, such as whole-plant hydraulic resistance (R_{plant}) (Meinzer and Grantz, 1990; Cochard et al., 1996a; Lu et al., 1996), soil resistance (R_{soil}), root resistance (R_{root}) (Meinzer and Grantz, 1990; Cochard et al., 2000), shoot resistance (R_{shoot}) (Salleo et al., 1992; Sperry et al., 1993), and more recently, leaf resistance (R_{leaf}; Nardini and Salleo 2003, 2005; Brodribb et al. 2005; Brodribb and Jordan 2008). When plants are exposed to water limited situations g_s usually declines and adjustments in hydraulic architecture

may occur in order to maintain hydraulic compatibility between plant and environment (Addington et al., 2006). In the long term, plants might respond by adjustments of their hydraulic architecture, mostly through altered root and shoot growth and differentiation, but in the short term, plant responses rely on stomatal regulation together with rapid changes in hydraulic conductivities of the root (Kh_{root}) and the leaf (Kh_{leaf}) (Kuppers 1984; Meinzer and Grantz 1990; Nardini and Salleo 2000; Asamaa et al. 2001; Brodribb and Holbrook 2004; Brodribb et al. 2005). In that sense, plants adjust their hydraulic efficiency by offering a series of resistances in different organs along the soil-plant-atmosphere continuum (Tyree and Zimmermann, 2002). It's important to note that leaves seem to contribute 50% of the hydraulic resistance of the aerial part of plants, which represents about 30% of the whole plant resistance (Becker et al. 1999; Nardini and Pitt 1999; Nardini and Tyree 1999; Nardini and Salleo 2000; Tsuda and Tyree 2000; Nardini 2001; Brodribb et al. 2002; Sack et al. 2003; Sack and Holbrook 2006), but this share would vary if leaf resistance is itself variable (Sack and Holbrook, 2006). In that sense, rapid adjustments to water scarcity can occur through active changes in properties of the water transport system, mainly from cell-to-cell through plasmodesmata (symplastic path) or membranes (transcellular path) (Canny 1988, 1995; Biela et al. 1999; Luu and Maurel, 2005; Aroca et al., 2006; Uehlein et al. 2003; Vandeleur et al. 2009), but also across cell walls (apoplastic path) (Gilliham et al., 2011).

Aquaporins appear to account for the major portion of the hydraulic conductivity of the plasma membrane and tonoplast (Maurel et al., 1997; Tyerman et al., 1999, 2002; Maurel, 2007), but not always the major proportion of the tissue hydraulic conductivity (Bramley et al., 2009; Voicu et al., 2009). Variations of hydraulic conductance in the short term are explained by physicochemical processes, such as cavitation (Salleo et al., 2001), wall collapse (Cochard et al., 2004), changes in water viscosity with temperature (Cochard et al., 2000), or changes of wall permeability with sap chemical composition (Zwieniecki et al., 2001). Evidence linking hydraulic conductance to plasma membrane aquaporins (plasma intrinsic proteins [PIPs]), which facilitate water transport through cell membranes, has been well documented for roots (Javot and Maurel, 2002; Tyerman et al., 2002; Vandeleur

et al. 2009), and for leaves (Sack and Holbrook, 2006; Heinen et al., 2009), where they might mediate the transport of liquid water in inner leaf tissues, from the veins to the stomatal chamber. In addition, a specific role for aquaporins during recovery in xylem conduit re-filling after drought-induced embolism has been proposed (Holbrok and Zwieniecki 1999; Tyree et al. 1999; Martre et al. 2002; Trifilò et al. 2003). The recovery of Kh in plants may be associated with an increase in the activity or the abundance of aquaporins in the plasma membranes of leaf cells. On the other hand, the opposite may happen during water stress: aquaporins may be down-regulated or inactivated (Alexandersson et al. 2005, Galmés et al. 2007; Secchi et al. 2007).

Besides water stress, the inhibition of water permeability by $HgCl_2$ is also suggested. The use of mercurial sulfhydril reagents as non-specific water channel inhibitors has been widely reported (Maggio and Joly 1995; Javot and Maurel 2002; Lovisolo et al. 2006), and this inhibition has permitted measurements of the proportion of water transported by mercury-sensitive aquaporins of treated cells or tissues (Caravajal et al. 1999; Amodeo et al. 1999; Martre et al. 2001). However, mercurials are also inhibitory of other cellular processes and may not directly inhibit aquaporins (Zhang and Tyerman, 1999), nevertheless they can still allow an estimate of aquaporin activity. Such studies indicate that aquaporins can account for up to 35 to 80% of root hydraulic conductance. Moreover, using mercury as an inhibitor of the activity of some aquaporins, it has been suggested that aquaporins are involved in the recovery after water stress of shoot (Lovisolo and Schubert 2006) and root (Lovisolo et al. 2008) hydraulic conductivity. The ability of the root to modulate its hydraulic conductance in response to a variety of stimuli has been shown to be correlated with the accumulation pattern of PIPs (Henzler et al. 1999; Lopez et al. 2003; Martínez-Ballesta et al. 2003). However, the mercury sensitivity of the water-transport activities differs among the aquaporin isoforms and the water-transport activity of the aquaporins is probably regulated by phosphorylation in response to the cellular water balance and metabolic activity. Suga and Maeshima (2004) measured aquaporin activity using the membrane vesicles of the yeast cells expressing radish plasma membrane aquaporins, and demonstrated that PIP2s generally have a much higher water transport activity than PIP1s. As the vesicles were incubated with 5mM $HgCl_2$,

the activities of PIP2s were severely inhibited while those of PIP1s were relatively insensitive to the mercury treatments.

Alteration of AQP expression by these approaches has been also shown to affect leaf cell water permeability (Aasamaa and Sober, 2005; Nardini et al. 2005; Voicu et al. 2008; Postaire et al. 2010) and, in some cases, other physiological parameters, such as water potential, water loss rate, and stomatal conductances, indicating the importance of AQP for leaf function. The hypothesis that rapid changes in Kh_{leaf} involve aquaporin regulation was further substantiated in a study in walnut trees (Cochard et al., 2007) were a very good kinetic correlation during a transition from dark to high light, between the increase in Kh_{leaf} and the increase in PIP2 aquaporin expression was obtained. However, a comprehensive understanding of how distinct cell layers and individual aquaporin isoforms contribute to the overall water transport capacity of the leaves and to its dynamic regulation is still being developed.

Another way to find out whether aquaporins play a role in water transport in the plant is to estimate apoplastic/simplastic water flux using two different fluorescent tracer dyes. Switching of water pathways involving apoplastic and symplastic transport may allow for some flexibility in the response of plants to water shortage. Further investigations on how apoplastic and symplastic pathways could be involved were elucidating in a number of early studies using fluorescent dyes such as trisodium 3-hydroxy-5,8,10- pyrenetrisulphonate (HPTS) frequently used as an apoplastic marker (Peterson et al. 1981; Hanson, Sucoff and Markhart 1985; Moon et al 1986; Wright et al. 1996; Kamaluddin and Zwiazek 2001) and acetate, 8-acetoxypyrene-l,3,6, trisulphonic acid, trisodium salt (HPTS-acetate) retained within the symplast (Wright et al. 1996). Based on such knowledge, this work aims to identify pathways through the leaf available to bypass water and solutes and to quantify the relation between both during irrigation and during water stress.

Here we report the expression profiles of six AQPs, belonging to two different subfamilies during water stress, and during re-watering from leaves of *Vitis vinifera* cv Chardonnay. The selection of AQPs, was based on previous reported analysis by Schelden et al. (2009) of the most highly expressed issoforms in Cabernet Sauvignon. The main objectives were i) identify the relevant hydraulic responses associated with

stomatal regulation during water stress and during recovery, ii) assess whether plasma membrane aquaporin expression changes with drought progression and during re-watering, ii) attempt to relate such changes with leaf hydraulic conductance by combining physiological and molecular approaches and iii) elucidating whether apoplastic or symplastic pathways for water and salts were traced from the vein endings to their sites of exits by placing leaves from well watered and water stressed plants with their cut petioles in solution containing one of two fluorochromes chosen to trace the transpiration stream.

Comparison of Kh_{leaf} and aquaporin expression patterns suggests that PIP2 members contribute to water flow in leaves mainly during recovery. Results are discussed in regard to the contribution of aquaporins to leaf water flow and we re-examined the importance of the cellular pathway under the use of $HgCl_2$, a well known AQP inhibitor.

MATERIAL AND METHODS

Plant Material

Two batches of experiments were performed from February to March of 2009 at South Australia (Adelaide). Initial experiments were performed to test the role/effects of different perfusion solutions on Kh_{leaf} responses in *V. vinifera* cv. Chardonnay from the Coombe vineyard (Waite Campus, Adelaide, South Australia). Shoots with mature leaves from the most exposed branches were collected from 5 to 10 plants the night before measurements. Immediately after cutting, branches were placed with their cut ends in distilled water and taken to the laboratory where the shoots were re-cut under water and rehydrated over-night in full darkness and covered with plastic bags until leaves were assigned to perfusing solutions. Leaves were excised from the apex, between nodes 2 to 4 of each branch.

For the second batch of experiments 14 one-year-old *Vitis vinifera* L. cv. Chardonnay were planted in 7.5 L pots containing a mixture of sand and peat moss (2:1), 50 g of calcium hydroxide, 90 g of calcium carbonate, and 100 g of Nitrophoska (12:5:1, N:P:K plus trace elements) per 100 L at pH 6.8. Pots were placed in a temperature controlled greenhouse and watered to field capacity every 2

days. Night/day temperatures were maintained at approximately 19/24ºC. Plants were trained to a two vertical shoots and all lateral shoots and fruit were removed during development.

Water was withheld and a subsequent recovery was applied on 7 plants, while another 7 plants were treated as controls (irrigated daily). Water stress was established according to a defined value of leaf maximum daily stomatal conductance (g_s) of about 50 mmol H_2O m^{-2} s^{-1}, as suggested by Flexas et al. (2002). Irrigation was stopped until the desired g_s was achieved, and then, plants were maintained at constant g_s for a week by daily replacing the amount of water consumed, as determined by weighing potted plants every evening. After one week at the established soil water deficit, all plants were re-watered to field capacity and recovery was followed for several days.

Gas-exchange measurements were taken daily, while the rest of the physiological measurements were performed on six specific sampling days: the day the desired stomatal conductance was first achieved (day 0), three and seven days after sustaining the plants at constant soil moisture (day 3 is 2 days after water stress was applied, and day 7, just before re-watering), and then 1, 3 and 7 days after re-watering, that is days 8, 10 and 15, respectively (Fig. 1).

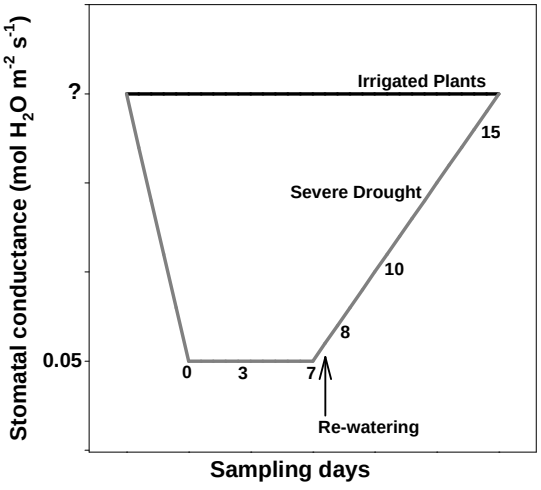

Figure 1. Experimental design, showing the expected time-courses and the target levels of g_s for each treatment. Numbers indicate sampling days (only those for severe stressed plants are showed for clarity).

Xylem sap perfusing solutions

Between 15 and 18 shoots of Chardonnay plants from the local vineyard were collected from the field and over night rehydrated in full darkness. Leaves were detached from rehydrated shoots, and immediately exposed to different filtered (0.45 μm), degassed solutions (15 mM KNO_3, 100 μM $HgCl_2$, 15 mM K-Acetate pH 5.5 and 2mM H_2O_2) by immersing the petiole inside 5 ml containers and placed under light for 3 hours. Light was provided by lamps delivering approximately 500 μmol $m^{-2}s^{-1}$ (PAR) at leaf level as measured by a Delta T AP4 porometer (Delta T Devices; Cambridge UK).

For grapevines grown in the glasshouse just two solutions were perfused into detached leaves as described above: $HgCl_2$ (as maximum Kh_{leaf} decreases were observed when $HgCl_2$ was applied in field plants experiments) and KNO_3 (as maximum Kh_{leaf} increases were observed when KNO_3 was applied in field plants experiments). The effects of $HgCl_2$ perfusion on a detached leaf was always compared over the nearest detached leaf treated with KNO_3 perfusion (considered as control solution). Each sampling day, leaves were harvested every 30-40 minutes under water from the shoots bent in water, between 10 AM and 13 PM from three different plants per treatment. From each plant 2 leaves were selected that were nearby, of similar area (fully expanded) and exposed to the same light intensity. The excised leaves were treated with either 100 μM $HgCl_2$ or 15 mM KNO_3 for 3 hours acclimation under light by immersing the petiole in one of these two solutions.

Evaporative flux density (i.e. transpiration) and leaf hydraulic conductance measurements were performed individually afterwards, in each excised leaf.

Gas exchange and evaporative flux density measurements

For the field vineyard plants, stomatal conductance (g_s) was measured, using a porometer (Delta T AP4) every hour during a 3 hours treatment in excised leaves, each one treated with different perfusion solution.

For glasshouse plants, g_s measurements were performed daily on two leaves per plant, between 11:00 and 13:00. Furthermore, each sampling day, leaves used for Kh_{leaf} measurements, were equally measured before they were excised from plants.

For each excised leaf, evaporative flux density (i.e. transpiration) was determined by quantifying weight decline on a 3 hours acclimation. Leaves with their petioles immersed inside specified solutions were weighted every hour and their weight difference was considered as the total evaporative flux density. A superficial layer of parafilm was added in each pot wrapping petioles to avoid evaporation. After 3 hours acclimation period, those leaves were removed from their respective solutions and were attached to the XYL'EM apparatus for Kh_{leaf} measurements (see below).

Amount of water available in the substrate

For indoor plants, stomatal conductance was measured every day in the glasshouse (Fig. 6C). The amount of water available in the substrate or soil-water content (AWA) (Fig. 6A) was calculated as: (Pot Weight – Minimum Pot Weight / (Saturated Pot Weight – Minimum Pot Weight) × 100. Minimum pot weight was considered at the wilting point. All plants were weighted every evening, before irrigation. Daily water lost was obtained from the weight differences between two consecutive days.

Whole-leaf hydraulic conductivity

Leaf hydraulic conductance on a surface area basis (Kh_{leaf}, mmol s^{-1} m^{-2} MPa^{-1}) was measured with the XYL'EM apparatus (Instrutec Company, H. Cochard and T. Ameglio, INRA-PIAF Laboratory, Clermont-Ferrand, France), based on a high-resolution liquid mass flowmeter (Liquiflow; Bronkhorst). The principle was to measure the water flow (F; mmol s^{-1}) entering the petiole of a cut leaf when exposed to a hydrostatic pressure gradient of 0.1 MPa (P; MPa). Upon steady state (typically, after 0.5h, when flow reached a stable value), Kh_{leaf} was computed as:

$$Kh_{leaf} = F/(P * LA)$$

where LA is the total leaf area (m^2). The XYL'EM was interfaced with a computer to log data automatically.

Previously treated leaves (either with $HgCl_2$ or KNO3) were attached to a XYL'EM apparatus through the petiole using compression fittings and the flow

entering each leaf was measured by using high pressure flow meter. Leaves were supplied with degassed and filtered (0.1μm) 15 mmol KCl solution from the XYL'EM 2-L captive air tank. A 15 mmol KCl solution is used to control for possible extra ionic effects on pit membrane conductance during Kh_{leaf} measurements.

During measurements, the leaves were submerged in an inner compartment filled with tap water in order to stop transpiration and to maintain a constant leaf temperature (±0.5ºC). The temperature in this compartment was adjusted with a regulated bath (Ministat; Huber) to 20ºC and continuously aerated. Also the XYL'EM system measures bath temperature and corrects for the effect of temperature on the viscosity of water so that all conductances are with respect to 20^oC. At the end of the experiment, the projected leaf area (LA; m^2) were determined using scanning images from each leaf processed in Matlab Softare (Matlab 7.0, MathWorks, Inc.).

Estimation of symplastic/apoplastic flow ratio

Relative changes in symplastic/apoplastic water flux were estimated in leaves from irrigated and water stressed Chardonnay plants using two different fluorescent tracer dyes, trisodium 3-hydroxy-5,8,10- pyrenetrisulphonate (HPTS) frequently used as an apoplastic marker (Peterson et al. 1981; Hanson, Sucoff and Markhart 1985; Moon et al 1986; Wright et al. 1996; Kamaluddin and Zwiazek 2001) and 8-acetoxypyrene-l,3,6, trisulphonic acid, trisodium salt (HPTS-acetate) retained within the symplast (Wright et al. 1996). Both fluorocroms were used as a solution of 5 mg ml^{-1} dissolved in 15 mM KCl.

Two detached leaves (from the same branch and matched approximately in the same size and position) were cut under water and immediately placed with their cut petioles in vials containing 3 mL of one of either of the dye solutions chosen to trace the transpiration for a previously tested time of 2.5-3 h. A superficial layer of parafilm was added in each pot wrapping petioles to avoid evaporation. After perfusion was completed, all the fluid was evaporated from the vials through leaf transpiration, so the total solution that was loaded and absorbed for each measured

leaf was the same. To explore either apoplastic or symplistic pathways from the leaf surface inwards, HPTS and Acetate-HPTS concentrations were measured respectively, with a Molecular Imager-ProPLus (Bio-Rad) fluorescence spectrophotmetry (fluorimeter) using an excitation wavelength of 405 nm and an emission wavelength of 515 nm. The recorded counts were normalized to the perfusion time and leaf area as counts m^{-2} h^{-1}.

The proportion of the apparent symplastic/apoplastic flow was estimated by dividing the Acetate-HPTS concentration in the symplastic and transcellular pathways in one leaf by the HPTS concentration in the xylem sap of the adjacent leaf. Between 2 and 5 leaves per treatment (irrigation and water stress) and dye solution, were used to complete the experiment, and from each leaf two images were taken one from the upper-side and one from under-side.

Quantitative real-time polymerase chain reaction (qPCR)

Two sets of Chardonnay leaves from glasshouse grown plants were collected for subsequent analysis of aquaporin transcript abundance by Real-Time Quantitative Reverse Transcription (RT)-PCR. For the first set, three replicates from well watered plants and three from water stressed plants were harvested at 11 a.m. each sampling day, and immediately frozen in liquid nitrogen and kept at -80 ºC until analysis. For the second set of experiments, treated leaves (previously perfused with KNO_3 and $HgCl_2$ solutions), were rapidly disconnected from the XYL'EM apparatus after Kh measurements and immediately immersed in liquid nitrogen. Samples were also stored at -80 ºC until analysis.

Total RNA from powdered leaf tissue was isolated using Spectrum Plant Total RNA extraction Kit (Sigma-Aldrich) including a special step for genomic DNA degradation. Quantification of total RNA in the processed samples was done by spectrophotometrical measurement at λ=260 nm using a Nanodrop Spectrophotometer ND-1000 (Bio-lab Ltd, Australia) and the integrity of the RNA by visual inspection of rRNA banding, following 2% agarose gel electrophoresis.

For the RNA analysis, first strand cDNA was synthesized from 1 μg of total RNA by reverse transcription using iScript cDNA synthesis Kit (Bio-rad, CA, USA) according

to the manufacturer's instructions. The reactions were incubated at 25 ºC for 5 min, 42ºC for 30 min, and 85ºC for 5 min.

Real-time polymerase chain reaction (PCR) reactions were performed in a 20 μL mixture containing 1 μL of diluted cDNA, 10 μL iQ SYBR Green Reaction-Mix (Bio-Rad), and 0.6 μM of each primer in the Bio-Rad iCycler iQ system (Bio-Rad Laboratories) using the following PCR cycle profile: one cycle of 30 s at 95ºC followed by 40 cycles of 20 s at 95ºC, 20 s at 59ºC, and 20 s at 72ºC. The fluorescence threshold value (Ct) was calculated using the iCycle iQ system software (Bio-Rad). Overall, a mean Ct value was calculated from three independent biological replicates, each with three PCR replicates. Relative gene expression numbers were calculated as relatives of control plants, using $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Data was normalized against Ubiquitin as reference gene. The absence of nonspecific products was confirmed by both the analysis of the melt curves and by electrophoresis in 2% (w/v) agarose gel of the PCR product. The sequences of the primers used for the amplifications were designed by Vandeleur et al. (2009) based on published sequences of aquaporins found in grapevine (Table I).

Table 1. Gene-specific primer pairs used in the semi-quantitative real-time PCR analysis

***Vitis* Gene**	**Primer**
PIP1.1	Forward; 5'-TGGTGCGGGTGTAGTGAAGG-3'
	Reverse; 5'-AGACAGTGTAGACAAGGACGAAGG-3'
PIP2.1	Forward; 5'-CCTCCTCCTGAATCTGGATTG-3'
	Reverse; 5'- TCATGCCCTCATACATATCAATAAC-3'
PIP2.2	Forward; 5'-AAAGTTTGGGACGACCAGTG-3'
	Reverse; 5'- TTTTTAGTTGGTGGGGTTGC-3'
PIP2.3	Forward; 5'- GCCATTGCAGCATTCTATCA-3'
	Reverse; 5'-TCCTACAGGGCCACAAATTC-3'
TIP1.1	Forward; 5'-CATTGCCGCCATCATCTAC-3'
	Reverse; 5'- AGAAATCTCAACCCCACCAG-3'
TIP2.1	Forward; 5'-GGAGGAAGAGCAAGTTGTGC-3'
	Reverse; 5'-GCACATCACCAACCTCATTC-3'
Ubiquitin	Forward; 5'-GTGCTGTCAACTGCAGGAAA-3'
	Reverse; 5'-GTAGCCATGGCACATCCAAT-3'

Data analysis

Regression coefficients and correlations were calculated with 8.0 Sigma Plot software package (SPSS; Chicago, IL, USA). Differences between means were assessed by Duncan analyses ($P < 0.05$), performed with SPSS 18.0 software packages. We tested whether the Kh_{leaf} and E responses were significantly different between treatments using t-tests. Furthermore, to evaluate an ordered behaviour of the data collected on Chardonnay along the experiment, a principal component analysis (PCA) was performed in order to identify the main sorting parameters and the possible cases gradients, using as variables: stomatal conductance (g_s, mmol H_2O m^{-2} s^{-1}), transpiration (E, mmol H_2O m^{-2} s^{-1}), hydraulic conductivity (mmol m^{-2} s^{-1} MPa^{-1}), and different AQPs expressions.

RESULTS

Characterization of physiological responses to different solutions

Different perfusion solutions applied to excised leaves for 3 h under light, by immersion of the petiole, resulted in different leaf hydraulic conductivities (Kh_{leaf}) and stomatal conductance (g_s) (Fig. 2A, C). However there were no significant differences in E between perfusates (Fig 2 B). Each solution was designed to test the role of AQPs since K-acetate at low pH (5.5) will acidify the cytoplasm and close PIP aquaporins while hydrogen peroxide has been shown to be a strong blocker of aquaporins in roots (Aroca et al. 2005). Kh_{leaf} was significantly less (9.8 mmol MPa^{-1} m^{-2} s^{-1}) with $HgCl_2$ perfusion than other treatments which showed Kh_{leaf} values about 14.8 mmol MPa^{-1} m^{-2} s^{-1} (Fig. 2A).

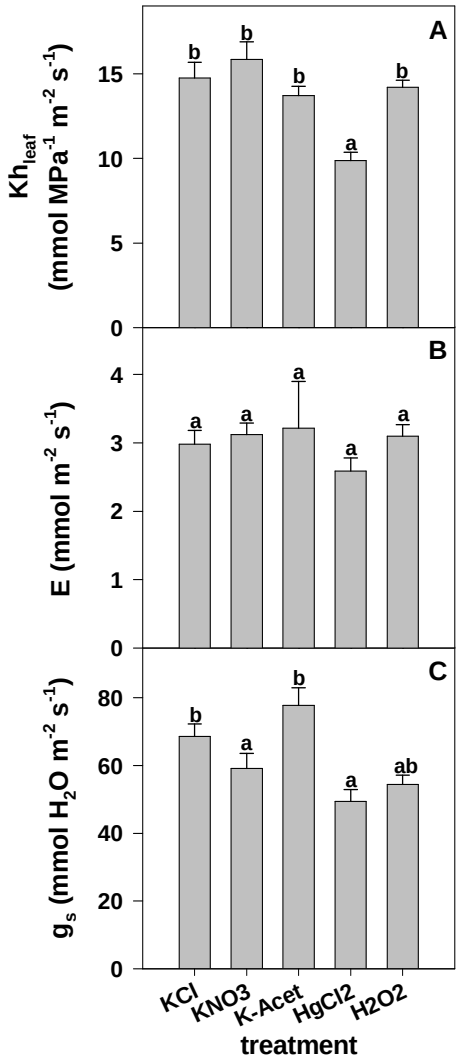

Figure 2. Response of (A) stomatal conductance; (B) evaporative flux density (i.e. transpiration) and (C) Leaf hydraulic conductivity of Chardonnay excised leaves to different chemicals. The values are means ± SD of 13 to 18 leaves after three hours acclimated in each treated solution. Columns with different letters are significantly different ($P < 0.05$).

There was no significant difference between the other perfusates, however the tendency showed that KNO_3 allowed higher Kh_{leaf} values than others. For g_s (Fig. 2C), K-acetate perfusion was higher than treatment with KNO_3 and $HgCl_2$, but there was otherwise no significant difference between KCl and H_2O_2. KNO_3 was subsequently used as the most suitable control perfusate since it gave the highest Kh, while $HgCl_2$ was used as a blocker solution since out of the potential AQP inhibitors

tested it was the most effective. These solutions were subsequently used to preload leaves before Kh was measured in the water stress and recovery experiments.

Plant water status

Irrigation was halted on potted plants until g_s reaching the desired value of 50 mmol H_2O m^{-2} s^{-1} (Fig 3). Under the conditions imposed this occurred within 3 days. Low AWA and g_s values were kept constant during the deficit irrigation period. After re-watering, AWA of stressed plants were fully recovered in one day. In contrast, g_s showed a slow recovery after irrigation, reaching almost full recovery 5 days after re-watering.

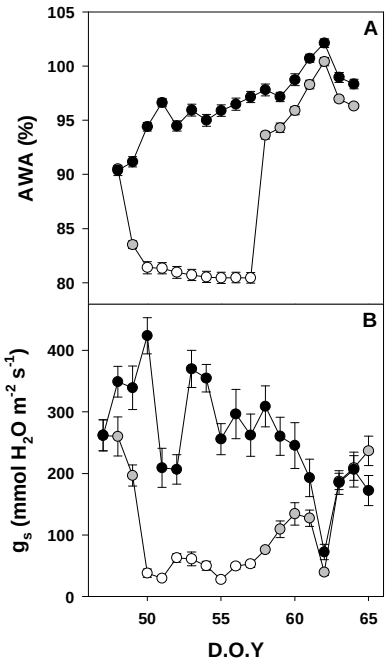

Figure 3. Changes from February 16, 2009 to March 6, 2009 in (A) the amount of water available in the substrate (AWA) and (B) stomatal conductance. Measuring days are expressed as DOY. Closed black symbols represents irrigated (i.e. control) plants, grey and white opened circles represent water stressed plants, being the white ones the measurements during the acclimation period. Values are means ± SD of 7 replicates.

Ψ_{MD} displayed similar pattern to that of AWA (Table 2). The minimum value achieved during water stress was -1.2 MPa. During acclimation to water stress, Ψ_{MD} started to recover, reaching values up to -0.9 MPa, and was fully recovered within a day after re-watering to -0.36 MPa.

Table 2. Changes of midday leaf water potential during the experimental time course. Values are means ± SD; n is the number of replicates measured for each treatment along the experiment. For each treatment, different letters denote statistically significant differences by a Duncan's multiple comparison test ($P < 0.05$).

Treatment	Ψ_{MD} (MPa)	n
Control	$-0.3^{a} \pm 0.02$	12
Water stress	$-1.16^{c} \pm 0.003$	3
Acclimation	$-0.9^{b} \pm 0.13$	6
Re-watering	$-0.36^{a} \pm 0.03$	9

Leaf hydraulic conductivity and leaf transpiration in water stress and during recovery

Control excised leaves (i.e. treated with 15 mM KNO_3) from irrigated plants showed Kh_{leaf} values of 8.8 mmol MPa^{-1} m^{-2} s^{-1}. However, a significant ($P < 0.05$) decrease in Kh_{leaf} was found when plants were exposed to sustained water stress. There was a significant reduction in Kh_{leaf} ($P < 0.05$), declining by 17.9 and 33.5% in the first (WS) and the seventh day (ACCL) after water stress imposition, respectively, compared with CONTR (Fig. 4A).

After 3h of 100 μL $HgCl_2$ treatment, Kh_{leaf} of control plants was reduced by 22.5%. In water stressed plants, the degree of inhibition by $HgCl_2$s of Kh_{leaf} was larger (33.5%) than controls (though not statistically significant), and then declined to a similar degree of inhibition to controls during the acclimation period (Fig. 4A).

Upon re-watering, Kh_{leaf} from control excised leaves increased to control values within 7 days (REC3) and in $HgCl_2$ inhibition was not significant until the 7th day after re-watering. In REC3 the degree of $HgCl_2$ inhibition was the largest (38.7%) (Fig. 4A).

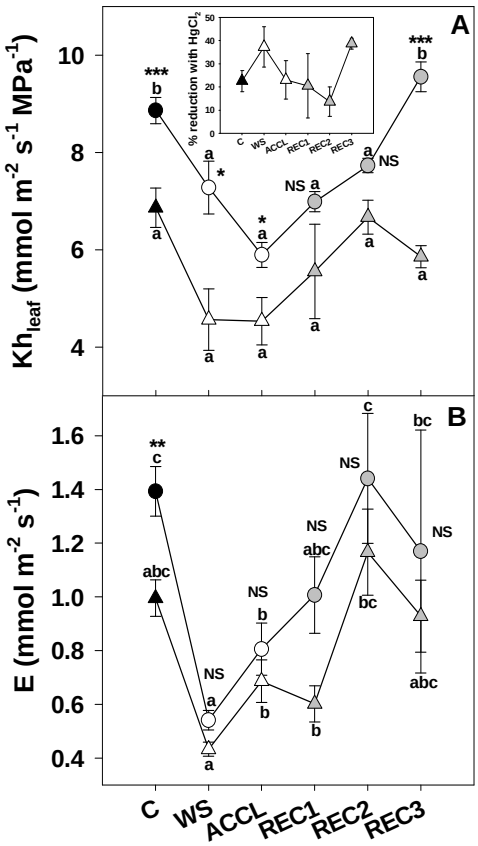

Figure 4. Changes on (A) leaf hydraulic conductivity and (B) leaf transpiration of Chardonnay pretreated leaves along the experimental time course. The inset graph represent the percentage of reduction with HgCl2-treated leaves respect non-treated leaves. Circles are leaves exposed to KNO_3 for 3h; and triangles are leaves exposed to $HgCl_2$ for 3h. Measuring day abbreviations means: CONTROL represent an average of all values from irrigated plants during the entire experiment; WATER STRESS is the day the desired stomatal conductance was first achieved; ACCLIMATION is the average of values from 3 and 7 days after sustaining the plants at constant soil moisture; RECOVERY 1, 2 and 3 are 1, 3 and 7 days after re-watering, that is days 8, 10, and 15 of the experiment, respectively. Colours represent the same as in figure 3. Values are means ± SD (n = 15 for control and n = 3 to 6 for water stressed plants). Different letters denote statistically significant differences by a Duncan's multiple comparison test ($P < 0.05$) within each treatment. Differences between treatments were assessed using t-tests: *$0.05 > P \geq 0.01$; **$0.01 > P \geq 0.001$; ***$P < 0.001$; NS, $P > 0.05$.

Evaporative flux declined as would be predicted during water stress, and then recovered after re-watering (Fig. 4B). There was only a small and not significant effect of $HgCl_2$ perfusion on leaf evaporative flux density consistent with the results shown in Fig. 2B. Consequently, a non-significant relationship between Kh_{leaf} and E was observed (Fig. 5B).

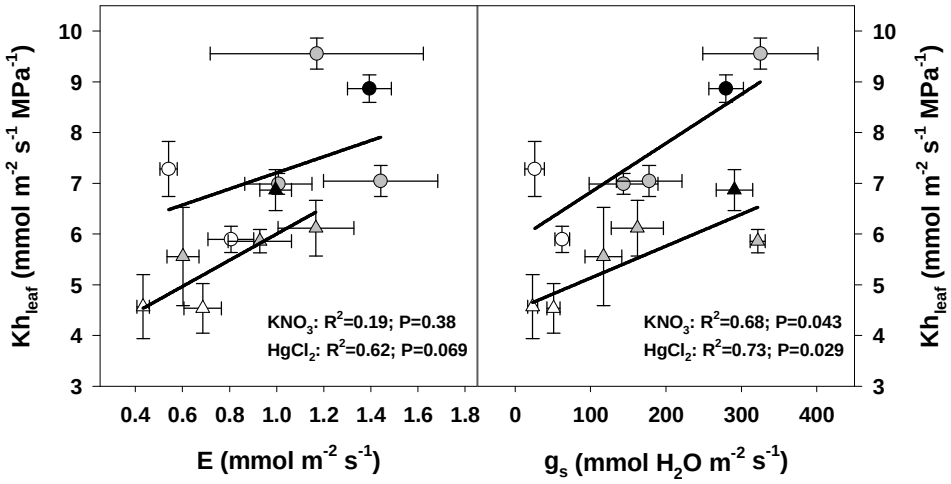

Figure 5. The relationship between Kh_{leaf} and A) leaf transpiration (E) and B) stomatal conductance (g_s). Represented symbols and colours are the same as in Figure 3. Values are means ± SD (n = 15 for control and n = 3 to 6 for water stressed plants).

We examined the potential correlations between Kh_{leaf} and g_s and E. Only gs and Kh_{leaf} were significantly positively correlated (Fig. 5). This also serves to demonstrate the much larger effect of $HgCl_2$ on Kh_{leaf} compared to g_s, i.e. the best fit line is shifted downward along the Kh_{leaf} axis, here plotted on the ordinate.

Water stress-induced changes in expression profile of AQPs

Real-time PCR was used to examine the expression of the *VvPIP1;1, VvPIP;1, VvPIP2;2, VvPIP2;3, VvTIP1;1* and *VvTIP2;1* genes in Chardonnay leaves during water stress and throughout the recovery process (along 2 weeks-period in total) within 6 different sampling days (Fig. 6).

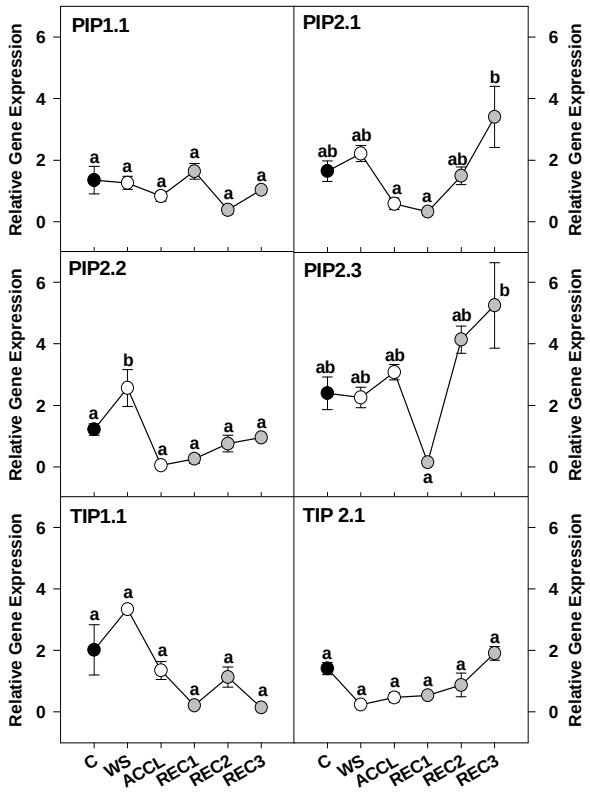

Figure 6. Relative gene expression of *Vitis* aquaporins in leaf during the experiment. Values represent the fold-increase expression for each aquaporin in leaves with respect to control plants. Data was normalized against Ubiquitin as reference gene. Represented symbols and colours are the same as in Figure 3. Values for C are means ± S.E. of 15 replicates, values for ACCL are means ± S.E. of 6 replicates, and the others are means ± S.E. of 3 replicates. Different letters denote statistically significant differences by a Duncan's multiple comparison test ($P < 0.05$) within sampling days.

For this, we used gene-specific primer pairs and quantified the abundance of aquaporins transcripts in three independent biological replicates. Water stress and recovery resulted in significant variations in AQP gene expression over time (Fig. 6). There were some similarities in expression patterns between isoforms with the PIP2s showing a similar pattern, particularly in respect of the increase in expression after re-watering, an initial increases during the first day under water stress and a minima in expression at REC1. *VvTIP2;1* also showed an increase in expression after re-watering. *VvTIP1;1* showed the opposite pattern to *VvTIP2;1*. *VvPIP1.1* transcript levels were relatively stable within the whole experiment as compared to control plants, only showing small (but not significant) increase between ACCL and REC1 (Fig. 6A).

The increase in PIP2 transcripts following re-watering after water stress may account for the increase in Kh_{leaf} during the recovery process and in particular the larger degree of HgCl2 inhibition observed at full recovery. A principal component analysis was performed on the physiological variables and aquaporin transcript abundance. There was a close association between Kh_{leaf} and the expression of *PIP2;3* and to a lesser extent with *PIP2;1 and TIP2;1* (Fig. 7). Kh_{leaf} was correlated with g_s and with the transcript abundance of *PIP 2;3*, *PIP2;1* and *TIP 2;1*.

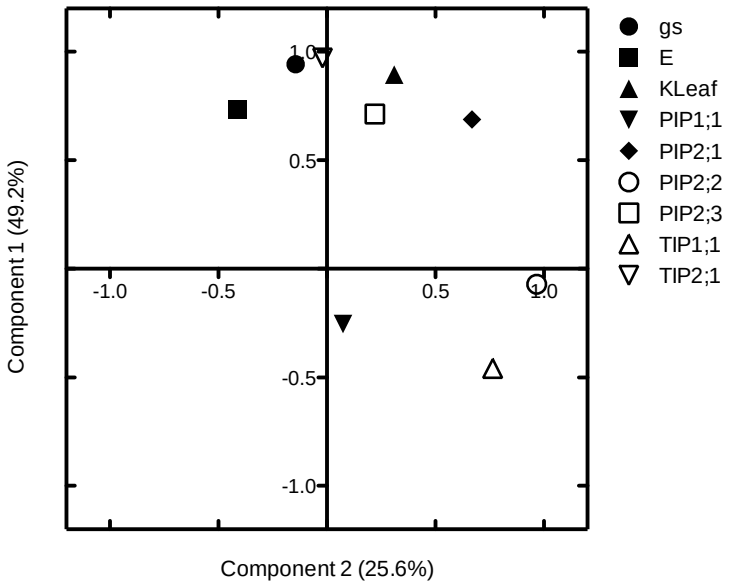

Figure 7. Distribution of physiological and molecular constituents as a function of components 1 (stomatal conductance; gs) and 2 (transpiration; E), resulting from performed Principal Component Analyses.

$HgCl_2$-effects in expression profile of Aquaporins

To investigate further the effects of adding $HgCl_2$ (a common aquaporin inhibitor) on leaf aquaporin function, we monitored the expression of all 6 previous tested genes in $HgCl_2$-treated leaves during recovery from control and re-watered plants, thus, the effect of the previous WS was also assessed in this analysis (Fig. 8). When comparing aquaporin expression between non-treated leaves (directly sampled from the plant) (Fig. 6) and treated leaves (3 hours acclimated under light either with KNO_3 or $HgCl_2$) (Fig. 8) in REC 2 (3 days after re-watering), similar expression patterns where observed from different aquaporins. However, percentages of

expressions are in general higher in leaves sampled directly from plants, than in leaves previously treated with either KNO_3 or $HgCl_2$. This might be because of leaf manipulation.

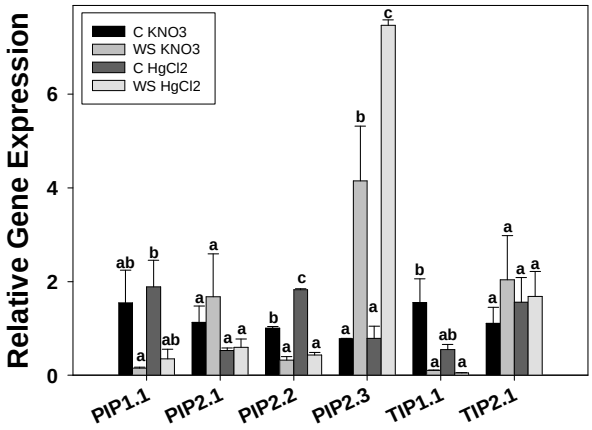

Figure 8. Effects in relative gene expression in leaves sampled from C and REC2 plants, previously immersed either in $HgCl_2$ or in KNO_3 for 3h. Values represent the fold-increase expression for each aquaporin in leaves with respect to control plants. Data was normalized against Ubiquitin as reference gene. Previous water stress and $HgCl_2$ effects are represented. Data are means ± SE of 3 replicates. Columns with different letters are significantly different ($P < 0.05$).

The assessed analysis during recovery of aquaporin expression on $HgCl_2$-treated leaves resulted in different responses of those aquaporins (Fig. 7). Thus, *VvPIP1;1* was clearly affected by previous water stress, but not by $HgCl_2$. However, some $HgCl_2$-sensitive aquaporin isoforms were observed (*VvPIP2;1*, *VvPIP2;2*, *VvPIP2;3* and *VvTIP1;1*) although their transcript levels were differently changing (some were increased by $HgCl_2$ and some were decreased by $HgCl_2$). In that sense, levels of *VvPIP2;1* showed an approximately 0.8 and 0.6-fold decrease in transcript abundance of water stressed and well watered leaves with $HgCl_2$. Without mercury, *VvPIP2;1* expression in water stressed leaves, recovered same levels as control leaves from irrigated plants. Transcript changes were opposite for *VvPIP2;2* and *VvPIP2;3*; for *VvPIP2;2* mercury treatment in leaves from control plants resulted in a 2 fold increase in transcript while for WS, transcript levels were reduced. On the other hand *PIP2;3* displayed an almost 6.5 fold increase in transcript with mercury treatment on of previously water stressed leaves and a reduction in transcript of treated leaves from control plants.

Transcript responses between the two TIPs were also different. In *VvTIP1;1* transcript levels were inhibited by both water stress and $HgCl_2$, while protein levels of *TIP2;1* were enhanced under all treatments.

Symplastic/Apoplastic water flow ratio

After labeling leaves for 3h with either HPTS-acetate (symplastic tracer) or HPTS (apoplastic tracer), fluorescence probes were observed in the vascular traces penetrating the tissue. Incubation for 3 h resulted in the whole tissue being permeated by either HPTS-acetate or HPTS.

Table 3. Symplastic/Apoplastic flow ratio calculated as the ratio between Acetate-HPTS and HPTS concentration (CNT mm^{-2}) on irrigated and water stressed plants. Values are means ± SD; n is the number of replicates measured for each treatment. Different letters denote statistically significant differences by a Duncan's multiple comparison test ($P < 0.05$).

	Symplastic dye Acetate-HPTS (m^{-2} h^{-1})	n	Apoplastic dye HPTS (m^{-2} h^{-1})	n	Acetate-HPTS/HPTS
Control	$0.34^{a} \pm 0.07$	6	$0.39^{a} \pm 0.08$	10	0.87
Water Stress	$0.59^{a} \pm 0.1$	4	$0.93^{b} \pm 0.1$	4	0.64

A comparison of leaves treated with those dyes demonstrated that the transfer of HPTS was slightly larger than that of Acetate-HPTS for control leaves. However, when comparing the ratio between both dyes, water stressed plants resulted in a much higher proportion of apoplastic tracer rather than symplastic relative to well watered plants, were higher ratios symplastic/apoplastic were observed (Table 3). Water stress appeared to enhance accessibility of the apoplastic tracer relative to that of the symplast tracer.in their movement into leaf tissues and may indicate a greater proportion of apoplastic water flow in WS leaves.

DISCUSSION

Water stress (WS) has an important effect on the hydraulic construction of leaves. This, in turn, might prove to be a crucial factor in plant–water relationships and gas exchange.

The aim of the present study was to check whether severe water stress had an impact on water permeability and how changes in this permeability influenced leaf

hydraulics. We investigated the possible role of PIP and TIP aquaporins to account for the regulation of water transport in the tonoplast and plasma membrane, respectively under WS and during recovery and which are the main pathways were water flows throughout the leaf lamina during WS. The results of this study provide evidences that: (i) water stress decreased Kh_{leaf} consistently; (ii) changes in the hydraulic efficiency of the leaf system were coordinated with leaf gas exchange rates during water stress being the stomatal conductance crucial for water fluxes regulation iii) although during re-watering, PIP2 aquaporins could play a major role in controlling radial water uptake leading to recovery of Kh_{leaf}; iv) and finally, under severe water stress, water flow across grapevine leaves preferentially following an apoplastic pathway instead going cell-to-cell and hence is not as sensitive to increases/decreases in aquaporin abundance.

Physiological responses to water stress and re-watering

Stomatal closure is one of the first processes that occurs in the leaves in response to soil water stress. Using the daily maximum value of g_s as an indicator of water stress, allows the regulation in grapevines subjected to progressive soil water stress on the basis of the main causes of photosynthesis A_N decline (Flexas *et al.*, 2002; Medrano *et al.*, 2002). In the present study, Chardonnay plants were subjected to severe water stress and as a consequence, decreases in $\Psi_{leaf,}$ Kh_{leaf} and E occurred when plants were water stressed. Kh_{leaf} was examined to determine whether this component of the water transport pathway plays a role in governing leaf phenology of Chardonnay. Because Kh_{leaf} was found to be highly correlated with g_s (Fig. 5) as has also been previously reported by several authors (Nardini and Salleo, 2003; Brodribb *et al.* 2002, 2003, 2005; Brodribb and Jordan, 2008; Franks, 2006), it can be concluded that during water stress, rates of leaf hydraulic conductance were highly associated with leaf gas exchange values. Moreover the combination of falling Ψ_{leaf} (Table 2), g_s (Fig. 3B) and soil drying (Fig. 3A) during the onset of the dry treatment and possibly increased xylem vulnerability (Schultz and Matthews, 1988; Hacke *et al.* 2001; Johnson *et al.*, 2009) are likely to have a big effect on plant water status,

resulting in significantly large reductions in Kh_{leaf} during water withholding (Fig 4A) in accordance with several previous studies (Nardini *et al.*, 2001; Schultz, 2003).

After seven days keeping plants under constant water deficit, we didn't observe any significant sign of acclimation by increasing Kh_{leaf}, while modifications in other physiological parameters (g_s, E and Ψ_{leaf}) showed increased their values. These results suggest that internal factors other than g_s, E and Ψ_{leaf} may regulate Kh_{leaf} after water stress period.

Relatively few studies have addressed the rate and limiting factors for the recovery of grapevine leaves after water stress (Flexas *et al.*, 2006; Pou *et al.*, 2008). The rehydration treatment following drought stress caused an increase in these parameters to values similar to those observed in irrigated controls. In this study, g_s was completely recovered after subjected plants to severe water stress, but this recovery was achieved after 6 days the irrigation was applied. Similar results were also obtained in the hybrid Richter-110 subjected to water withholding, reaching recovered g_s values in almost 2 weeks (Pou *et al.*, 2008). In contrast, several cultivars and rootstocks including Cabernet Sauvignon showed almost complete recovery 2 days after re-watering of severe water stressed plants (Guan *et al.*, 2004).

Effect of mercuric ions on hydraulic conductance under water stress and re-watering and the role of AQP mediated water transport

After 3 hours of imbibition leaves in 100 μM $HgCl_2$, Kh_{leaf} of C plants was reduced by almost 22.5% compared with values from KNO_3-treated leaves of same irrigated plants and further reductions (37%) on Kh_{leaf} were observed during WS (Fig. 4A) which indicates the possibility that $HgCl_2$ decreases cell-to-cell water transport by inhibiting water channel activity (Chaumont *et al.*, 2000; Javot and Maurel, 2002). This reduction in Kh_{leaf} by $HgCl_2$ is also comparable to that found by Lovisolo and Schubert (2006) in shoots of grapevines, which was interpreted as an inhibition of the water channels. Consistent with this, we found that initial increases of VvPIP2s and VvTIP1.1 were assessed the first day under WS (Fig. 6) and thus, initial up-regulation might thought to increase membrane permeability to water transport when water is less available (Yamada *et al.*, 1997).

After seven days of acclimation, even though significant differences in Kh_{leaf} absolute values were obtained between treated and non-treated leaves, water withheld decreased Kh_{leaf} while $HgCl_2$-treatment did not result in further inhibition thus resulting in similar Kh_{leaf} final reductions in both, treated and non-treated leaves (33.9 and 33.5%, respectively). So here, leading decreases of Kh_{leaf} by the exposure of leaves to a bathing solution containing 100 µM $HgCl_2$ was eliminated by water stress, indicting that the gain in leaf hydraulic conductivity in response to $HgCl_2$ may mostly be due to physiological process neither than aquaporin related. Similar results were observed by Miyazawa *et al.*, (2008) in tobacco plants were water permeability of the leaf tissues and g_i (internal conductance) in drought-acclimated plants were insensitive to $HgCl_2$ treatments suggesting that deactivation of aquaporins is responsible for the significant reduction in g_i observed in plants growing under long-term drought. In that sense *VvPIP2;1* and *VvPIP2;2* and mRNA levels in Chardonnay leaves were significantly down-regulated in ACCL.

After 7 days of re-watering, when some of the analysed aquaporins (mainly *VvPIP2;1*, *VvPIP2;3* whose transcripts levels rapidly increased their levels up to 3.5 and 5 fold, respectively) showed their maximum expressions levels, differences in Kh_{leaf} between $HgCl_2$-treated leaves and control leaves were higher. Therefore, this lack of recovery in $HgCl_2$-treated leaves could be caused by an inhibition in recovery of some AQPs, as shown by the fact that the protein expression levels of mercury-sensitive aquaporin isoforms (PIP2s) in control leaves from rehydrated plants were increased at the end of recovery (Fig. 6) thus showing a high water transport activity, as previously described Chaumont *et al.* (2002). Also in walnut (*Juglans regia* L.), PIP2 aquaporins, localized in xylem vessel parenchyma cells, were activated during spring embolism recovery (Sakr *et al.*, 2003). However, the nature of the mechanism responsible for maintaining a balance between open and closed aquaporins is not well known.

Finally, the expression of *VvPIP1;1* maintained unchanged values along the experiment. This is consistent with the fact that regulation of plasma membrane aquaporins is complex and occurs at multiple levels (Luu and Maurel, 2005).

In addition, the fact that the leaf transpiration of non-treated leaves was reduced more rapidly during WS, and that differ less from the $HgCl_2$-treated leaves (Fig. 4B), suggests that in these treated leaves, there must be a signal moving water throughout them. Therefore, the control of leaf homeostasis through a combined regulation of Kh_{leaf} and stomatal exchanges may be dependent on aquaporin regulation.

Responses of leaf AQPs to $HgCl_2$

Among the many internal or external factors that can affect AQP activity, mercury chloride ($HgCl_2$) has been shown to be an effective inhibitor of AQPs (Maurel et al. 1997; Niemietz and Tyerman 1997; Javot and Maurel 2002; Lovisolo and Schubert 2006). In tomato (*Lycopersicon esculentm*), $HgCl_2$ addition to a root bathing solution results in a drop in the pressure induced water flux with consequent reductions of whole-root system hydraulic conductivity by 57% (Maggio and Joly 1995). Similarly, in our experiment, the expression profile of some AQPs in leaf was affected by $HgCl_2$ treatment inducing changes in four of the studied aquaporins (*VvPIP2;1*, *VvPIP2;2*, *VvPIP2;3* and *VvTIP1;1*) either in water stressed or in well-watered plants (Fig. 7). However *VvPIP;1* and *VvTIP2;2* were unaffected; *VvPIP1;1* was clearly affected by previous water stress, but not by $HgCl_2$. It has recently been shown that the mercury sensitivity of the water-transport activities differs among aquaporin isoforms. In that sense, Suga and Maeshima (2004) measured aquaporin activity and demonstrated that PIP2s generally have a much higher water transport activity than PIP1s, and as the vesicles were incubated with 5mM $HgCl_2$, the activities of PIP2s were severely inhibited while those of PIP1s were relatively insensitive to the mercury treatments.

The assessed analysis during re-watering (REC2) on $HgCl_2$-treated leaves resulted in AQPs transcripts levels differently changing leading to mean inhibition of Kh_{leaf} from $HgCl_2$ treated leaves by 13.7% in respect to control leaves (Fig 4A). The fact that there was no total inhibition of water uptake (Kh_{leaf}) by the presence of $HgCl_2$ across the plasma membrane (mainly during water withheld) could be due to the lack of active aquaporins or the fact that not all aquaporins are Hg-sensitive

(Maurel *et al.*, 1997; Niemietz and Tyerman, 1997). Moreover, the slightly recovery of Kh_{leaf} of $HgCl_2$-treated leaves during irrigation indicates that not all water transport was channel mediated.

The role of AQPs in regulating the pathway of water flow in leaves

The role of AQPs in flow of water through leaves has been comprehensively reviewed recently by Heinen et al. (2009), but distribution through leaves was not considered.

From the combination of facts, we propose that most water transport through leaves might follow an apoplastic route (Table 3) as has been previously observed by Sack and Holbrook (2006). Due to its low resistance to water flux, the apoplastic path is believed to be the main route during transpiration. However other several studies had been attributed an important role to the cell-to-cell paths (Chrispeels and Maurel, 1994; Cochard *et al.*, 2007; Ye *et al.*, 2008) because such high resistances to water flow in the plasma membrane.

Using HPTS (frequently used as an apoplastic tracer dye) and HPTS-acetate (retained within the symplast) we observed that apoplastic and symplastic pathways were co-existing into the abaxial epidermis in leaves (being the first higher than the second), but their contribution varied depending on plant water status, being the ratio symplastic/apoplastic higher under WS and lower under C. This implies that under WS, symplastic water transport (via aquaporins) becomes less important than apoplastic water transport. It is important to notice that this is in accordance with previous results were we assumed Kh_{leaf} decreases due to AQP inhibition during WS (Fig 4A). Thus, the results from the present study might allow supporting previous studies in Grenache by Vandeleur *et al.* (2009) were drought-induced root abscisic acid (ABA) biosynthesis increases apoplastic concentration because of increased suberisation of apoplastic barriers caused a reduction of water conductivity which is not compensated by aquaporin-mediated water transport. However during re-watering and corresponded to a high Kh_{leaf} value (Fig. 4A), the conductance of the recovery-induced pathway might be highly modulated to strong accumulation of PIP2 aquaporin transcripts. Similarly results were observed by using mercury as an

inhibitor of the activity of some aquaporins were it has been suggested that aquaporins are involved in the recovery after water stress of shoot (Lovisolo and Schubert 2006) and root (Lovisolo *et al.*, 2008) hydraulic conductivity.

In summary, we demonstrated that $HgCl_2$ induced a rapid and significant decrease in Chardonnay Kh_{leaf} which may be interpreted as an inhibition of the water channels. However, treatments of severe WS that cause general metabolic inhibition also reduce Kh_{leaf} to a similar extent to that caused by $HgCl_2$ treatment afterwards in ACCL and declining the degree of $HgCl_2$ inhibition. Furthermore, as shown in this study, there was no additional effect of $HgCl_2$ treatment on the Kh_{leaf} of cells already metabolically compromised by WS treatment. This indicates that $HgCl_2$ could reduce Kh_{leaf} via general metabolic inhibition that may affect various water flow pathways, rather than by a direct block of water channels.

Finally, the presence of functional water channels in leaf cells could be of highly importance in the regulation of water flow during re-watering and an inhibition of AQP expression by $HgCl_2$ in this period could be of major importance hindering the recovery of Kh_{leaf}.

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Chapter 5

GENERAL DISCUSSION

The hypothesis of the present work was that the regulation of grapevine acclimation responses to water stress and to recovery could present important differences among both and differ from simple progressive water stress responses. In particular, it has been suggested that mechanisms regulating plant hydraulics and CO_2 transport inside leaves can exert a crucial role under these conditions. Thus, studying water and carbon fluxes and their associated mechanisms during these two conditions (acclimation to water stress and recovery following re-watering) should lead to a better understanding of grapevine performance under semi-arid conditions. From the obtained results we could show different aspects of the regulation of these fluxes and the involved conductances, as well as their important effects on the leaf water use efficiency. In depth discussions on the specific data are given inside the reported publications.

This general discussion chapter aims at a global discussion of all the results, in order to explicitly present the important achievements of the specific objectives and to advance on the major conclusions of this work.

5.1. REGULATION OF WATER FLOWS AND ITS EFFECTS ON WATER-USE-EFFICIENCY

The first Specific Objective of this thesis was to analyze how water relations, transpiration and stomatal conductance are regulated under water stress and re-watering and how this regulation is mediated by plant and leaf hydraulics and how it affects leaf WUE.

When exposed to water stress, plants reduce the rate of water flow by decreasing their hydraulic conductance (Jones, 1998; Steudle, 2001) and by closing their stomata in response to chemical signals (Jackson, 1997; Soar *et al.*, 2006). In grapevines, there is strong evidence for hormonal control of stomatal closure mainly mediated by abscisic acid (ABA) (Correia *et al.*, 1995; Lovisolo *et al.*, 2002; Pou *et al.*, 2008). The latter is commonly assumed as the most important factor regulating stomatal aperture and WUE in grapevines under water stress (Loveys, 1984; Stoll *et al.*, 2000; Davies *et al.*, 2000), perhaps because a role for ABA in stomatal closure was first described in grapevines (Kriedemann *et al.*, 1972; Loveys and Kriedemann,

1974). On the other hand, it has been suggested that the iso- or anisohydric behaviour of different varieties may be related to differences in ABA concentration/ sensitivity as well as to differences in the regulation of plant and/or of leaf hydraulics (Schultz, 2003; Soar *et al.*, 2006; Vandeleur *et al.*, 2009). However, this different physiological behaviour in response to water deficits may not be just cultivar-related. As has been previously shown by several authors (Medrano *et al.*, 2003; Lovisolo *et al.*, 2010; Chaves *et al.*, 2010), the same variety could behave iso- or anisohydrically, depending on complex interactions between the variety itself, the rootstock, the climatic conditions (e.g. VPD, temperature), or the intensity and duration of water deficits.

Most of these described mechanisms arose from studies in which grapevines were subjected to short-term water stress, but much less is known about acclimation responses as well as the regulation of water fluxes after re-watering.

The results obtained in the present Thesis confirm the current knowledge on water stress-induced stomatal regulation, but they emphasize that the controlling factors determining grapevine responses appeared likely to be a combination of differences in hydraulic conductivity (Kh_{plant} and Kh_{leaf}) and a higher potential to control stomatal aperture through enhanced regulation of ABA in the xylem, since highly significant relationships between g_s and either hydraulic conductivity and [ABA] were observed for data corresponding to irrigated and water-stressed plants, already shown in other studies with grapevines (Liu *et al.*, 1978; Stoll *et al.*, 2000; Lovisolo *et al.*, 2002; 2010). These correlations were obtained including data from the early days of water stress imposition pooled with data from the acclimation period. However, this correlation weakened during re-watering, since leaf xylem ABA levels were fully restored to control values, while g_s remained relatively low, in association with a low Kh_{plant}. Thus, there is strong evidence for hydraulic control, which seems dominant under persistent stomatal closure, such as under long-term water stress and during recovery after re-watering. Although there is increasing evidence that xylem embolism triggers stomatal closure (Salleo *et al.*, 2000; Hubbard *et al.*, 2001), a significant correlation was found between g_s and either Kh_{plant} or Kh_{leaf} for leaf water potentials above those known to induce cavitation in grapevines

(Winkel and Rambal, 1990; Schultz, 2003; Choat *et al.*, 2010). In our experiments, this was observed even in Chardonnay, a variety that displayed anisohydric behaviour. These results contradict the idea that fine stomatal regulation resulting in isohydric behaviour occurs when plant hydraulic system is operating close to its capacity, i.e., when water potential is near its cavitation threshold (Salleo *et al.*, 2000; Buckley, 2005). Instead, these results suggest that reduced hydraulic conductivity may act as a trigger for decreased g_s in order to keep Ψ_{MD} almost constant even when being relatively far from the cavitation threshold.

Concerning the effects of this regulation on intrinsic water use efficiency (WUE_i), although the varieties that displayed near-isohydric behaviour increased their A_N/g_s earlier after the imposition of water stress, the differences within the aniso-hydric variety were generally low. Therefore, the present results do not support the idea that a pronounced and steady increase of WUE_i is more typical of an isohydric strategy, as proposed by Poni *et al.* (2007). Moreover, Schultz and Stoll (2010) recently showed that A_N/g_s and A_N/E can frequently change to opposite directions. Since $E = g_s$ x LAVPD, and reduced g_s results in increased LAVPD due to leaf heating, A_N/E could be maintained or even reduced while A_N/g_s is increased. The results obtained in this Thesis confirm that in Chardonnay (i.e. the variety which showed anhisohydric behaviour) a lower control over g_s allowed a higher evapotranspiration at the leaf surface resulting in lower leaf temperatures and, hence, LAVPD. So, although a somewhat lower WUE_i was recorded for this variety as compared with more isohydric-behaving varieties, its WUE_{inst} was even higher.

Finally, in the three studied varieties of *Vitis vinifera* as well as in the hybrid Richter-110, water stress did not substantially change stomatal responsiveness to LAVPD. That is, on the one hand g_s decreased at high LAVPD, while on the other hand the evaporative demand was higher, resulting in a similar E and WUE_{inst} at different LAVPDs. Consequently, WUE_{inst} was mostly determined by water stress acclimation and recovery-induced hydraulic regulations on g_s, rather than by the genotype or the environmental conditions (LAVPD).

In summary, the results of the present Thesis suggest that even though ABA exerts a control of water flow during early water stress and acclimation, there is a

very strong link between plant and leaf hydraulic conductivity, stomatal conductance and WUE in grapevines. This link is kept under conditions of early water stress, acclimation and re-watering, and is largely independent of LAVPD, while it differs little among genotypes.

5.2. REGULATION OF CARBON FLOW AND ITS EFFECTS ON WATER-USE-EFFICIENCY

As for the second objective of this thesis, the interdependence of the two CO_2 diffusion limitations (g_s and g_m) on photosynthesis under acclimation to water stress and recovery were analysed.

Net photosynthesis is also progressively reduced by plant water stress, partly as a result of stomatal closure but also through direct effects of tissue dehydration on the photosynthetic system (Slatyer, 1967; Lawlor and Cornic, 2002; Flexas *et al.*, 2004). Water stress-induced stomatal closure has been shown to act as the initial and most prominent limitation to CO_2 assimilation, as diffusion of CO_2 from the atmosphere to the sites of carboxylation in the chloroplast is impaired. However, the impairment of photosynthetic CO_2 assimilation during water stress may not be exclusively explained by stomatal resistance to CO_2 diffusion. Another important limitation of CO_2 assimilation results from leaf-internal resistances, consisting of the CO_2 pathway from the intercellular airspaces to the mesophyll cells, to the chloroplasts and the sites of carboxylation (Flexas *et al.*, 2008). In grapevines, a decreased mesophyll conductance (g_m) under water stress has been shown to exert a limitation to photosynthesis of similar magnitude as that imposed by stomatal closure (Flexas *et al.*, 2002).

The study of photosynthesis limitations during prolonged water stress and subsequent re-watering confirmed that, besides $g_{s,}$, the leaf-internal diffusion of CO_2 (i.e. g_m) contributes to the limitation of photosynthesis mainly during sustained water stress, but its role is strongly dependent on the impact of additional environmental factors such as cumulative irradiance. Thus, it appears, that the response of g_m to water stress may depend on the prevailing light conditions, similar to what was demonstrated for Rubisco activity and photochemistry (Zhou *et al.*, 2007), while g_s

appears to be more independent of environmental conditions, except for *VPD*. Consequently, and contrary to the early report by Flexas *et al.*, (2002), the present results did not show a strict co-regulation between g_s and g_m.

The contribution of g_m to photosynthesis limitation was largely reduced during recovery after re-watering due to a rapid and complete recovery of g_m to control values. Under these conditions, stomatal limitations were the most important in delaying photosynthesis recovery after re-watering. This is in agreement with reports by Gallé and Feller (2007) and Gallé *et al*. (2007), who showed in several tree species a sustained reduction of g_s lasting for several days or weeks after re-watering.

Because g_s affects both CO_2 and water flow, whereas g_m affects CO_2 flow only, the observed lack of interdependence between the two conductances allows for a high degree of regulation of WUE. Indeed, Flexas *et al*. (2010) showed that WUE_i in grapevines is strongly and positively related to the ratio g_m/g_s. The results of this Thesis confirmed this relationship, and showed that under water stress WUE_i increased in particular days that concurred with the reversion of g_m inhibition, i.e. on days with lower incidence of excess irradiance. On the other hand, it has been shown that the rapid recovery of g_m after re-watering – in contrast to slow reversion of g_s – resulted in the maintenance of a largely increased WUE_i during many days after re-watering, even when photosynthesis rates were almost totally restored.

In summary and in contrast to progressively induced water stress, the regulations of carbon flux under sustained water stress and recovery are somewhat independent of water flow regulation. In particular, g_m shows a large degree of independency from g_s which results in important improvement of the WUE.

5.3. THE CO-REGULATION OF WATER AND CARBON FLOWS IN LEAVES

The third objective was to characterise the interconnection between diffusion pathways of water and CO_2 across the mesophyll (g_m), and to assess whether the isotopic analysis is a viable method of monitoring changes in the lamina hydraulic conductivity (Kh_{lamina}).

In the previous sections it has been shown that there is a strong link between K_h and g_s, while the link between g_s and g_m is weaker. However, it is still possible that K_h and g_m can be co-regulated to some extent. Clear evidences demonstrating an adaptive link between the gas-exchange capacity and hydraulic efficiency comes from previous studies on different species at the leaf level (Nardini and Salleo, 2000; Aasamaa *et al.*, 2001; Sack *et al.* 2003b; Brodribb *et al.*, 2005), stem level (Brodribb and Field, 2000; Santiago *et al.*, 2004), root level (Becker *et al.*, 1999) and the whole plant level (Meinzer and Grantz, 1990; Sperry, 2000; Meinzer, 2002). In all these studies significant correlations were observed between photosynthesis (A_N) and parameters indicative of hydraulic conductivity, although the mechanistic links between these two variables have not been analyzed. In the present Thesis it was hypothesised that the main link comes from the fact that water and CO_2 share at least a part of their respective pathways inside leaves (for instance, the sub-stomatal cavity path) and they also share the mechanisms facilitating their respective flows along such pathways (for instance, aquaporins).

To assess this issue we tried to characterize the variability of the 'scaled effective path length' (L, in m), as determined by leaf water isotope enrichment with both drought progression and vein severing of leaf lamina, coupled with simultaneous estimates of lamina hydraulic conductivity (Kh_{lamina}) and mesophyll conductance for CO_2 (g_m). Like this we could establish a link between L and measurable physiological variables that are likely to respond to similar processes in the mesophyll. It was found that a strong effective relationship between L and both Kh_{lamina} and g_m exists, suggesting the idea that water and CO_2 are sharing an important part of their diffusion pathways through the mesophyll, and that the regulation of such pathway by water stress (and leaf severing) affects similarly to water and CO_2 diffusion. This is in accordance with recent findings by Evans *et al.* (2009) and Terashima *et al.*, (2011), who showed that water and CO_2 diffusion are sharing at least in part, common diffusion pathways in the mesophyll, including the transcellular pathway, in which the transport of both water and CO_2 may be facilitated by aquaporins (Uehlein *et al.*, 2003; Flexas *et al.*, 2006b; Kaldenhoff *et al.*, 2008; Heinen *et al.*, 2009; Otto *et al.*

2010). This observation led us to formulate the last objective of the present Thesis, in which is to explore the role of aquaporins in the observed responses of *Kh* and g_m.

5.4. THE ROLE OF AQUAPORINS IN THE CONTROL OF WATER FLOW

The fourth and final specific objective was to understand the relative contributions of leaf xylem and extra-xylem (the latter involving aquaporins) hydraulic resistances to the leaf water flow, and assess to what extent Kh_{leaf} is related to aquaporin expression.

Aquaporins are responsible for the fine regulation of water flow across plant membranes (Tyerman *et al.*, 1999; Kjellbom *et al.*, 1999) and therefore may be responsible for regulating flow across the whole leaf. However, the role of AQPs in regulating plant water flow is a complex issue, because different subfamilies and subclasses may be up- or down-regulated or remain unchanged depending on the degree of water deficit and/or the time during the stress period. In that sense, expression patterns in AQP genes may not necessarily correlate with physiological parameters, such as hydraulic conductance, because the complexity of responses on the metabolic, cellular, organ or whole plant level may mask such correlations.

Despite of that, we first made the attempt to search for a link between aquaporin expression and water stress in grapevines, as observed in other species (reviewed by Tyerman *et al.*, 2002). For such purpose, AQP gene expression was assessed during drought and recovery in the drought-adapted *V. berlandieri* × *V. rupestris* R-110 plants. The results confirmed that several PIP and TIP aquaporins are up-regulated at early stages of water stress in roots, while they are mostly down regulated in leaves. This may be an indication that the function of some AQP is more important in roots than in leaves, which would be consistent with the idea that they facilitate water transport (Luu and Maurel, 2005), but perhaps it could also indicate that in leaves fluxes are decreased by means of down-regulated AQPs to preserve leaf water status. Interestingly and in contrast to water stress, most aquaporins were up-regulated in leaves but not in roots after re-watering the stressed plants. This differential behaviour illustrates the different function of aquaporins in leaves and roots, and their complex nature of regulation (Kaldenhoff *et al.*, 2008). Furthermore,

changes in transcript levels of each AQP were related to an improved physiological status of plants under stress and it was found that AQP gene expression did not significantly correlate with any of the physiological parameters considered (stomatal conductance, leaf water potential, xylem abscisic acid (ABA) content and root and stem hydraulic conductivity). This lack of correlation suggests that AQP expression is modulated by water stress to enable plants to maintain homeostasis in their water status. For instance, an initial decrease in expression under moderate stress may help to reduce water transport and hence to decrease stomatal conductance to maintain plant water status (Smart *et al.*, 2001). At more severe stress, when stomatal conductance is already low, and soil water availability is strongly reduced, an increased expression of AQPs may help to increase membrane permeability to water transport to optimize plant water status (Yamada *et al.*, 1997).

On the other hand, when studying a different genotype (Chardonnay), which contrary to R-110 displayed anisohydric behaviour, leaf hydraulic conductance was up-regulated under water deficit in correspondence with up-regulation of the expression of *VvPIP2s* and *VvTIP1.1*. Noticeably, the hydraulic conductance of $HgCl_2$-treated leaves was lowered by 20-40%, suggesting an important involvement of AQPs in the regulation of *Kh* as suggested for grapevines (Vandeleur *et al.*, 2009) as well as for other species like tomato (Sade *et al.*, 2009).

Using mercury as an inhibitor of the activity of some aquaporins, it was revealed that these proteins are especially involved in the recovery of leaf hydraulic conductivity after water stress when differences in Kh_{leaf} between $HgCl_2$-treated leaves and control leaves were highest. Consistently, some of the analysed aquaporins (mainly *VvPIP2.1*, *VvPIP2.3*) showed their maximum expressions levels during re-watering.

These results largely support the idea that AQPs could help regulating Kh_{leaf}, and thus g_s and, hence, optimizing water-use efficiency in plants, in particular during recovery from water stress.

5.5. GENERAL OVERVIEW

The results of the present Thesis have shown that grapevine responds to water stress and re-watering in a way that somehow differs from its response to progressive water stress imposition. A number of physiological and molecular mechanisms have been shown to act in relation to each other, inducing a fine regulation of stomatal closure, photosynthesis and water-use-efficiency. In particular, the importance of plant and leaf hydraulics as well as of mesophyll conductance to CO_2 has been highlighted, which are related to the activity of aquaporins. These results allowed us to build up an outline diagram of plant responses to each of these conditions: water stress imposition, water stress acclimation, and recovery upon re-watering.

A theoretic diagram is presented below (Fig. 5.1) to show some response routes in grapevine subjected to A) water stress; B) acclimation and C) re-watering. The direction of inset arrows indicates the sense (i.e. up- or down- regulation) and intensity (number of arrows) of the response of each of the parameters represented inside the ovals. Red colour means differences between genotypes. Specific genotype responses during the acclimation period are represented in the inset table, were equal sign means a lack of variation and arrows in both directions means oscillations between days. Outside arrows indicated the proposed mechanistic link between different parameters, and their thickness represents the importance of the relationship under each condition. Dotted arrows indicate hypothesized – not confirmed – links.

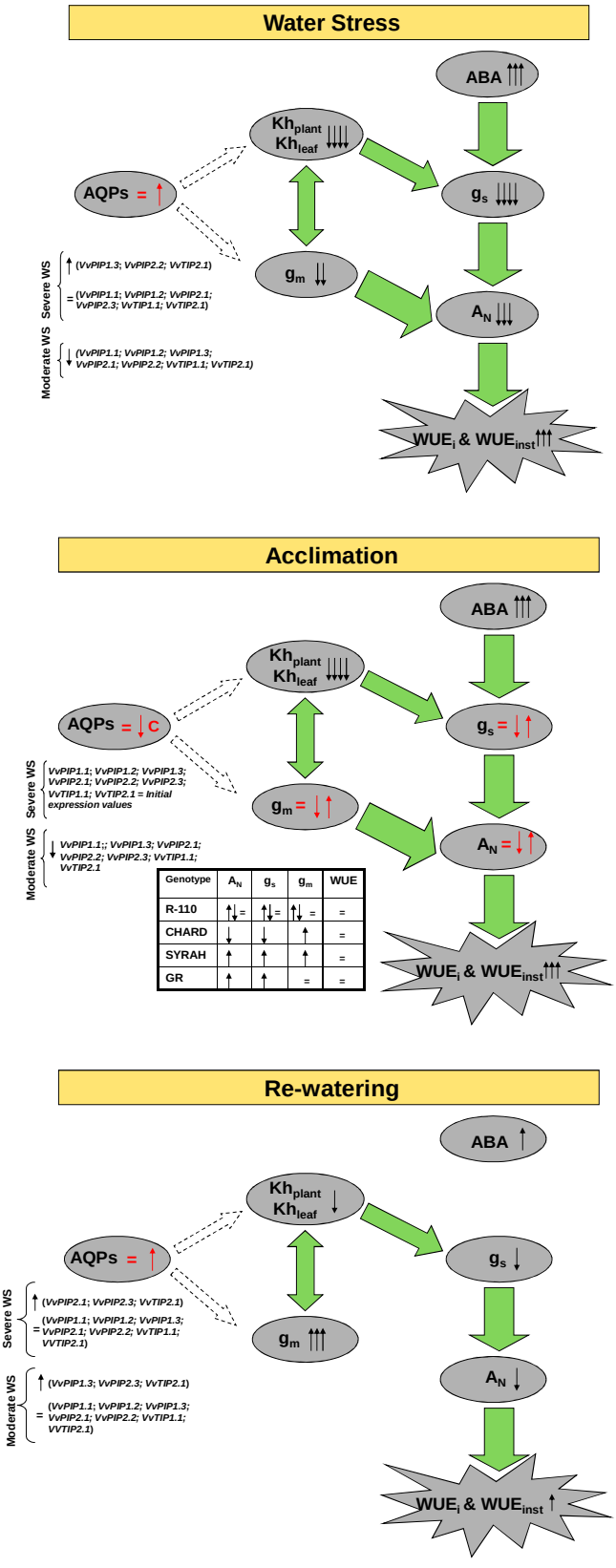

Figure 5.1. Theorethical diagram of grapevine responses to A) water stress, B) acclimation and C) re-watering.

Under **water stress**, photosynthesis and stomatal aperture are strongly reduced, and this seems regulated by both hydraulic and chemical signals. Under these conditions, down-regulation of stomatal conductance is strongly related with elevated levels of ABA in the xylem, although leaf hydraulics are also down-regulated, possibly influencing the observed decreases in g_s. In addition to stomatal closure, a concomitantly decreased mesophyll conductance to CO_2 is also responsible for decreased photosynthesis. The fact that g_s is generally further reduced than g_m under water stress allows for increased leaf-level WUE under these conditions. During the imposition of water stress, some AQP`s are up-regulated in sever water stressed plants (e.g. *VvPIP1.3*, *VvPIP2.2* and *VvTIP2.1*) while some others are down-regulated in moderate water stressed plants (e.g. *VvPIP1.1*, *VvPIP1.2*, *VvPIP2.1*, *VvPIP2.2* and *VvTIP1.1*). We hypothesize that these changes are involved in the fine tuning of Kh_{plant}, Kh_{leaf} and g_m, although the direct link between AQPs and these processes remains still unknown (dotted arrows).

During **acclimation**, a persistent decrease of the stomatal conductance limits net photosynthesis and water transpiration, leading to increased WUE. However during this period MCL eventually increases, being of similar magnitude or even higher than SL, while g_s and A_N suffer adjustments of different sensitivity and magnitude depending on the species and study conditions. Overall, the regulation of g_s and WUE under acclimation does not differ strongly from that during the imposition of water stress.

Upon **re-watering**, the extent and velocity of recovery strongly depend on pre-drought intensity and duration. A persistent decrease in stomatal regulation in late recovery after irrigation is mostly determined by whole-plant and leaf hydraulic conductivity, since ABA levels are rapidly restored to control levels. During this period, photosynthetic limitations were mostly related to SL, because SL lasted for at least one week after re-watering, while MCL was rapidly reversed and disappeared completely in a few days. Moreover, an up-regulation of the expression of some aquaporins may play an important role during water stress and could be involved in the rapidly recovery of leaf hydraulic conductivity after re-watering.

In summary, the results of the present Thesis reveal that grapevines subject to water stress, acclimation and recovery develop some physiological tight regulations to limit leaf transpiration while enhancing water use efficiency, in which plant and leaf hydraulics play an important role. In addition to fine stomatal regulation, mesophyll conductance reacts co-ordinately to optimize WUE. Aquaporins may play a role in both of these mechanisms, thus representing a potential link between *Kh* and g_m. So, a combination of different strategies allows plants to react in a changing, restrictive environment, to minimize water losses and maximize WUE.

Chapter 6

CONCLUSIONS

From the results of the present Thesis, a series of conclusions can be reported.

1. The stomatal conductance declines not only during the imposition of water stress, but also during acclimation to water stress, and it is persistently decreased many days during recovery after re-watering. This results in enhanced WUE under all of these three conditions.

2. Increases in the xylem sap abscisic acid (ABA) concentration strongly promote stomatal closure during drought, however, it is also evident that additional hydraulic signals are involved in this stomatal response, and that Kh_{plant} and not ABA is fully responsible for delayed g_s recovery after re-watering.

3. These persistent reductions of Kh_{leaf} triggering decreased g_s allow Ψ_{MD} to remain within a narrow range even when being relatively far from the cavitation threshold.

4. This pattern was common for all the studied genotypes regardless of their behaviour as iso - or anysohydric varieties.

5. Contrary to what has been previously described for responses to water stress, under acclimation and recovery the g_m is largely uncoupled from g_s, although it still contributes largely to the limitations of photosynthesis.

6. Mesophyll conductance appears highly responsive to the prevailing light conditions, while stomatal conductance is more independent of environmental conditions except VPD.

7. During the imposition of water stress the main limitation to photosynthesis was stomatal closure. However, during acclimation, reductions in stomatal and mesophyll conductance alternate as the main limitations depending on the day to day variations of environmental conditions. However, under re-watering, stomatal limitation was the only constraint to photosynthesis recovery.

8. Mesophyll conductance and hydraulic conductivity decreased while the scaled effective path length' (*L*) increased in response to both water stress and leaf severing.

9. The strong interdependence between these three variables strongly supports that water and CO_2 share at least a part of their diffusion pathways trough the mesophyll.

10. The aquaporin expression under moderate water stress was increased for roots and decreased for leaves. The opposite pattern was observed under severe water stress.

11. In leaves the expression of some aquaporins decreased under moderate water stress, while under severe water stressed it increased. Nevertheless, during stress-acclimation the expression of all aquaporin returned to initial values. After re-watering most of the leaf aquaporins tended to increase their expression. These complex regulations might have contributed to the maintenance of hydraulic homeostasis in leaves.

12. The predominant role of aquaporins during recovery as suggested by the highest AQP expression was confirmed by using $HgCl_2$. This inhibitor of aquaporin activity also showed a maximum effect under recovery.

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