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Physiological mechanisms involved in water use efficiency in grapevines

PhD Thesis

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CERTIFIQUEM :

Que el present treball titulat *"Physiological mechanisms involved in water use efficiency in grapevines"*, presentat per Magdalena Tomás 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.

Revisat el present treball, autoritzem la seva presentació per què pugui ésser jutjada pel tribunal corresponent.

Palma de Mallorca, 23 d'Abril del 2012

Director	Director	Autora
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Als meus pares,

Joan i Malena

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SYMBOLS AND ABBREVIATIONS LIST

Symbols	**Meaning**
ς	diffusion path tortuosity
α	leaf absorptance
ABA	abcisic acid
A_N	net CO_2 assimilation rate
AOP	alternative oxidase pathway
ATP	adenosine triphosphate
β	fraction of absorbed light that reaches photosystem II
C_a	atmospheric CO_2 concentration
C_c	chloroplastic CO_2 concentration
C_e	CO_2 concentration of air entering in the gas exchange chamber
C_i	sub-stomatal CO_2 concentration
C_i*	apparent CO_2 photocompensation point
C_o	CO_2 concentration of air leaving the gas exchange chamber
COP	cytochrome oxidase pathway
C_s	leaf surface CO_2 concentration
cw	cell wall
$\Delta^{13}C$	carbon isotope discrimination
Δ_i	carbon isotope discrimination predicted
ΔL_i	diffusion path length
ΔL_{ias}	half of mesophyll thickness
Δ_{obs}	carbon isotope discrimination observed
D_a	diffusion coefficient for CO_2 in the gas-phase
D_L	leaf density
D_w	aqueous phase diffusion coefficient for CO_2
E	leaf transpiration rate
E_{night}	leaf night transpiration
ET_0	Evapotranspiration
ETR (J_{flu})	electron transport rate
Φ_{CO2}	apparent quantum efficiency of CO_2 fixation
Φ_{PSII}	maximum quantum efficiency of the PSII photochemistry
F	curvature correction factor
F'_m	maximum fluorescence in light-adapted state
F_0	basal fluorescence of a dark adapted leaf
f_{ias}	volume fraction of intercellular air spaces
F_m	maximum fluorescence in dark-adapted state
F_o	basal fluorescence of the dark adapted leaf
F_t	steady state fluorescence emission
F_v/F_m	maximum quantum efficiency of PSII photochemistry
Γ*	CO_2 compensation point in the absence of mitochondrial respiration
g_b	boundary layer conductance
g_{ct}	cytosol conductance
g_{cut}	cuticular conductance
g_{cw}	cell wall conductance
g_{env}	chloroplast envelope conductance
g_{ias}	intercellular air space conductance to CO_2/ gas phase conductance
g_{lip}	lipid phase conductance
g_{liq}	liquid phase conductance
g_m	mesophyll conductance

Symbols	**Meaning**
g_{night}	night conductance
g_{pl}	plasmalemma conductance
g_s	stomatal conductance to H_2O
g_{sc}	stomatal conductance to CO_2
g_{str}	chloroplast stroma conductance
g_{tot}	total conductance
IBA	indolbutyric acid
J_a	linear electron transport rate by gas exchange
J_f	linear electron transport rate by chlorophyll fluorescence
J_{max}	Maximum photosynthetic electron transport rate
K_c	Michaelis constant for the carboxylation activity of Rubisco
K_{cat}	in vivo specific activity of Rubisco per active sites
K_o	Michaelis constant for the oxygenation activity of Rubisco
LA	leaf area
LAI	leaf are index
l_b	biochemical limitation
L_{chl}	length of chloroplasts exposed to intercellular air spaces
l_i	liquid and lipid phase limitation
l_{ias}	gas phase limitation
L_m	length of mesophyll cells exposed to intercellular airspaces
l_m	mesophyll limitation
l_s	stomatal limitation
M_A	leaf mass per area
NPQ	non-photochemical quenching
O_i	leaf internal oxygen concentration
PAR	photosynthetic active radiation
PB	plant biomass
PCB	whole plant carbon balance
PEPC	phosphoenolpyruvate carboxylase
p_i	effective porosity
PPFD	photosynthetic photon flux density
PRD	partial root drying
Q_A	quinone A
R_d	leaf dark respiration
RDI	regulated deficit irrigation
RGR	relative growth rate
R_L	leaf light respiration
R_{leaf}	leaf respiratioin
ROS	reactive oxygen species
R_{root}	root respiration
R_{stem}	stem respiration
Rub	Rubisco
S_c/S	chloroplast surface area exposed to intercellulare air spaces
S_m/S	mesophyll surface area exposed to intercellulare air spaces
S_s	cross sectional area of mesophyll cells
SWC	soil water content
T	Rubisco specificity factor
T_{cw}	cell wall thickness
T_{cyt}	cytoplasm thickness
T_L	leaf thickness
t_{mes}	mesophyll thickness
TPU	triose phosphate
T_{soil}	soil temperature
v_c	velocities of carboxylation of Rubisco

Symbols	**Meaning**
$V_{c,max}$	maximum rates for the carboxylation activity of Rubisco
v_o	velocities of oxygenation of Rubisco
$V_{o,max}$	maximum rates for the oxygenation activity of Rubisco
VPD	vapour pressure deficit
V_{TPU}	velocity of triose-phosphate utilization rate
w	width of leaf anatomical section
WUE	water use efficiency
WUE_c	crop water use efficiency
WUE_i (A_N/g_s)	intrinsic water use efficiency
WUE_{inst} (A_N/E)	instantaneous water use efficiency
WUE_l	leaf water use efficiency
WUE_{WP}	whole plant water use efficiency
WUE_y	yield water use efficiency
$\delta^{13}C$	carbon isotope composition
δ_e	isotopic composition of CO_2 entering in gas exchange chamber
δ_o	isotopic composition of CO_2 leaving gas exchange chamber
Ψ_{MD}	midday leaf water potential
Ψ_{PD}	predawn leaf water potential

CONTENTS

Chapter 1

INTRODUCTION

1.1. WATER STRESS AND MEDITERRANEAN CLIMATE

1.1.1. The importance of water stress in plants

Water is an essential component for the plants life owing to the crucial role that water plays in all physiological processes. Water comprises 80 to 95% of the fresh weight of most herbaceous plants and 50% of woody plants. The physical and chemical properties of water make it essential for many functions in plants such as: a constituent of most parts of plants, a solvent providing a good medium for biochemical reactions and transport, a reactant in different processes such as photosynthesis, respiration and other enzyme-mediated processes; and in the maintenance of turgidity in plants.

Plant physiological processes are directly affected by the amount of water that plants can extract from the soil. Water stress limits plant growth and crop production more than any other single environmental factor (Boyer, 1982). Stomatal closure, together with leaf growth inhibition, are among the first processes affected in response to drought, protecting plants from extensive water losses and reducing the source of assimilates (photosynthetic rate) and, consequently, the resulting biomass. Therefore, 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 the ecosystem productivity worldwide (Passioura, 1996). Furthermore, water is a key element determining vegetation type, plant distribution and its survival which strongly depend on their ability to adjust to environmental variations. Therefore, different genotypes have adapted to a lack of water in the environment avoiding or tolerating drought. These strategies can be observed in Mediterranean type vegetation where plants with predominant drought-avoidance strategies, which die when they run out of water, coexist with drought-tolerant sclerophylls. As a result, large variations among species in photosynthetic response to water stress can be observed (Sullivan *et al.*, 1974), affecting the distribution of the species (Wuenscher & Kozlowski, 1971; Chaves *et al.*, 2007).

1.1.2. The special interest of Mediterranean climate areas

There are five regions over the world that present Mediterranean climate. They are located between 32º- 40º North and South of the Equator, in the west

side of the continents (Aschmann, 1973). The Mediterranean Basin, surrounding the Mediterranean Sea, is the largest area where this climate type is found. It also prevails in coast of California, parts of Western and South Australia, the southwest of South Africa, sections of Central Asia, and parts of the central coast of Chile. All these areas combined represent between 1% and 4% of the land surface (di Castri, 1981; Mooney, 1982). They are characterized by hot and dry summers and cool and wet winters (Köppen, 1923). Winter temperatures are mild (7-13ºC) with infrequent frosts and snow, whereas summers are hot with mean temperatures of 14-25ºC (Paskoff, 1973). On the other hand, the annual precipitation ranges from 275 to 900 mm/year (Conacher, 1995; Conacher & Sala, 1998). Most rainfall falls during winter months, and this is one of the key elements that differentiate Mediterranean, Temperate and Subtropical climates. According to Aschmann (1973) more than 65% of the annual rainfall generally falls in winter, although in some areas this may variate (Köppen, 1923) with important spring and autumn rains (Southern California and Southern Europe – Miller, 1983). During summer, a drought period is present with variable length and intensity between years. 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 plants, including crops 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. Consequently, the most important characteristic of this type of climate is the absence of a period with both favourable temperatures and high soil water availability.

Most of the present Thesis research was developed in the Balearic Islands, located in the Mediterranean Basin. This area is characterized by summer periods with high irradiance, air temperature and high evaporative demand combined with drought, limiting plant growth and productivity (Chaves *et al.*, 2007). Moreover, according to climate change predictions (IPCC 2007), soil moisture content could decrease even more. It is expected that soil water availability could decrease from 20-30% for most of the Mediterranean areas and it can be down to 70% in the Iberian Peninsula and the Balearic Islands, caused by increasing temperature induced by a two-fold increase of the current levels of CO_2 concentration

expected during the next century (Schultz, 2000). This situation would imply the need of irrigation and a more efficient use of water in agronomical practices, as well as selecting plants more adapted to drought-prone areas.

A large part of the agricultural production in the Mediterranean Basin comes from irrigated areas. Hence, agriculture consumes about 80% of water in the region (Rijsberman, 2004). Increasing growing population and aridity in Mediterranean areas, as suggested by climate change predictions, will convert water into a scarce commodity. Thus, how to reduce agricultural water use and make water resources more sustainable is an increasingly urgent need. Solving it requires combined agronomic, physiological, biotechnological/genetic and engineering approaches to save water.

1.2. GRAPEVINE: A CLASSIC MEDITERRANEAN CROP

Grapevines are one of the oldest cultivated plants that, along with the process of making wine, have resulted in a rich geographical and cultural heritage (Johnson, 1985; Penning-Roswell, 1989; Unwin, 1991). Grapevine is one of the major horticultural crops produced on a worldwide basis and is mainly located in Mediterranean areas (Mullins *et al*., 1992).

Vines can be cultivated from Temperate (from 30-50º North or South) to Tropical climates, but most vineyards are planted in Temperate zones with high variability of environmental conditions. Considering its large cultivation area, the most significant grape-growing region is Europe, with 4.4 million hectares. Among all countries, Spain accounts for the largest grape-producing areas (1.082.000 Ha) with a prolific economy (1.200-1.900 M€), representing more than 10% of its total agricultural production, a socioeconomic profit (with about 400.000 wine producers) and an environmental-landscape profit (OIV, Organisation Internationale de la Vigne et du Vin, 2011)

The range and magnitude of environmental factors (e.g., solar radiation, heat accumulation, extreme temperatures, precipitation, wind, and extreme weather events such as hail) differ considerably from region to region and are the principal environmental constraints for grape production and wine quality. Water shortage is probably the most dominant environmental constraint within the production areas (Williams & Matthews, 1990). The combined effect of drought

with periods of high air temperature and high evaporative demand during the grapevine growing season limit grapevine yield as well as berry and wine quality (Escalona *et al.*, 1999a; Chaves *et al.*, 2007; Costa *et al.*, 2007). In addition, the combined effect of these stress conditions may promote dramatic reductions in plant carbon assimilation due to a severe decline of photosynthesis, 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; Santos *et al.*, 2007).

In general, the grapevine crop areas in Spain are semi-arid (i.e Mediterranean) and, consequently, although it is a typical dry land crop, irrigation practices have been progressively adopted in this crop and they have become common in modern Mediterranean viticulture. Moreover, it has been demonstrated in different conditions that soil water availability regulates crop load and fruit quality. Excess of water can reduce colour and sugar content of fruit and produce imbalanced acidity wine (Matthews *et al.*, 1990; Medrano *et al.*, 2003; Romero *et al.*, 2010), while a drastic reduction of water will reduce crop yield and frequently fruit quality. Therefore, the heterogeneity in grapevine responses to soil water deficit and the difficulty to establish the optimum water dosage to be used in a given environment and cultivar explain the need to study the physiological responses to water stress in grapevines (Medrano *et al*., 2002, 2003; Flexas *et al.* 2002, 2006a).

1.3. WATER USE EFFICIENCY

1.3.1. Concepts of water use efficiency

The term *WUE* reflects the balance between gains (kg of biomass produced or mols of CO_2 assimilated) and costs (m^3 of water used or mols of water transpired). This balance can be measured at different space scales (Medrano *et al*., 2010) (Fig. 1.1) from leaves (the ratio of net assimilation *vs* leaf transpiration) to whole plant or crop. Furthermore, this term can be studied at different time scales from months (ie. biomass accumulation or yield) to minutes (instantaneous exchange of water vapour for carbon dioxide).

At the leaf level, *WUE* can be measured at short (instantaneous gas exchange measurements) or long term (carbon isotope ratio of leaf dry matter). The carbon isotope ratio of leaf dry matter ($\delta^{13}C$) is determined by the gradient

between CO_2 concentration in the substomatal cavity (C_i) and the atmosphere (C_a), established by the level of discrimination against ^{13}C ($\Delta^{13}C$), and is often regarded as an integrative estimation of leaf WUE (WUE_l) over the growth period (Farquhar & Richards 1984). At shorter term, it is common to use single leaf gas exchange measurements, relating net CO_2 assimilation rate (A_N) either to stomatal conductance (g_s), called intrinsic water use efficiency (WUE_i), or A_N to leaf transpiration rate (E), defined as instantaneous water use efficiency (WUE_{inst}) (Fischer & Turner, 1978) (Fig. 1.1). WUE_{inst} (A_N/E) is influenced by the environmental conditions, because E depends on the degree of stomatal opening and vapour pressure deficit (VPD) of the atmosphere surrounding the leaf ($E = g_s \cdot VPD$). On the other hand, WUE_i (A_N/g_s) excludes the effects of changing evaporative demand on water flux out of the leaf depending only on the stomatal opening (Bierhuizen & Slatyer, 1965). Therefore, it is of particular interest to determine intrinsic differences in water use efficiency independent of specific environmental conditions (Bota *et al*., 2001; Souza *et al*., 2005; Anyia *et al*., 2007; Barbour *et al*., 2010; Gómez-Alonso *et al*., 2010; Galmés *et al*., 2011; Zegada-Lizarazu *et al*., 2011).

WUE_{inst} (A_N/E) can also be used as daily estimation of WUE using the daily integrals of A_N and E (i.e., the accumulated carbon gain and water loss during a whole day by a given leaf) (Medrano *et al.* 2003). In addition, when leaf area is included in this daily estimation, WUE_l could be scaled up to whole plant WUE (WUE_{WP}) (Medrano *et al.* 2012).

Whole plant WUE (WUE_{WP}) is the balance between total plant dry matter production and total water consumption by plant. In grapevines in particular, transpired water depends on canopy structure and growth (leaf angle in relation to the incoming irradiance and shoot positioning) determining the light interception by the plant or the energy load by transpiration (Escalona *et al.*, 2003; Medrano *et al.*, 2012) (Fig.1.1). Moreover, WUE_{WP} depends on water losses during non-assimilatory periods (cuticular and night transpiration) (Caird *et al.* 2007), and it is also dependent on respiration in leaves, stems and roots during whole day. These processes are not considered in the leaf gas exchange measurements to estimate WUE_l, therefore, these physiological processes could decrease WUE_{WP} and not influence WUE_l estimates.

For agronomic purposes it is more common to study *WUE* at yield level (WUE_y). Yield *WUE* (WUE_y) is defined as the balance between the total production of harvested yield, and total water consumption during the season by the crop. In grapevines, bunches are the most active sink for photosynthates. Grapes constitute only 20-30% of total plant dry biomass but up to 80-90% of total photosynthesis assimilates obtained in one growing season are transported to the fruits (Bota *et al.* 2004). Therefore, for practical purposes, in grapevines it is a convenient procedure to take into account WUE_y as an optimum target to improve *WUE*.

At the crop level, *WUE* is defined as the amount of yield produced per unit of water used (WUE_c). This concept depends on crop water consumption during the growing season. This is the sum of the amount of water lost not being used by the plants (runoff, percolation and direct soil evaporation) plus the water transpired by the plant (Fig. 1.1). The losses of water that are not directly consumed by the crop can be avoided with agronomical practices (cover crops, deficit irrigation, drip irrigation, night irrigation, etc.). Total irrigation joint with yield can provide a rough estimate of WUE_c and can help to develop a sustainable crop.

The present Thesis focuses mainly in *WUE* at leaf and whole plant level.

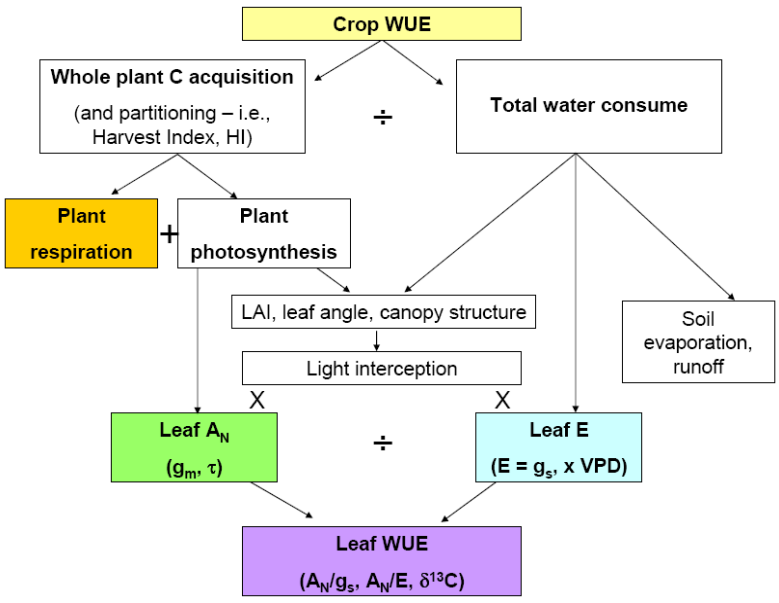

Figure 1.1. Theoretical diagram of the components of *WUE* showing the dependency of crop water use efficiency (WUE_c) on different processes and its links with leaf *WUE* (WUE_l). *LAI*, leaf are index; *VPD*, vapour pressure deficit; g_s, stomatal conductance; A_N, net photosynthesis rate; *E*, transpiration rate; g_m, mesophyll conductance; τ, Rubisco specificity for CO_2. From Flexas *et al.* (2010)

1.3.2. Methodologies to determine water use efficiency

The methodologies for *WUE* measurements can be described on the basis of time/space scale of its determinations; from instantaneous leaf measurements to surrogate parameters such as $\delta^{13}C$ for the whole growth season in a plant (see Medrano *et al.*, 2010) (Fig. 1.2).

As stated above, leaf *WUE* (WUE_l) can be easily determined as the ratio between net CO_2 assimilation rate (A_N) and transpiration rate (E) or stomatal conductance (g_s). A_N/E and A_N/g_s are measured by gas exchange systems near steady-state conditions in the field obtaining an instantaneous and non destructive measurement that reflects the leaf capacity for WUE_l. In addition, WUE_l can be measured by carbon isotope ratio of leaf dry matter ($\delta^{13}C$), often used as an indicator of long-term *WUE* (Farquhar & Richards, 1984). Stable isotope discrimination can be measured on-line to give instantaneous measurements of *WUE* (Condon *et al.*, 1990) or short-term *WUE* (1-2 days) by measuring the isotopic composition of leaf soluble sugars (Brugnoli *et al.*, 1988). However, the major impact of the use of stable isotopes has been in the analysis of dry matter to provide a simple and rapid screening method that integrates *WUE* over time, given the fact that carbon isotope ratio of tissue carbohydrates depends on the processes involved for their synthesis. This technique is based on the differential use of carbon isotopes by the enzymes that fix CO_2, RUBP carboxylase (Rubisco) and PEP carboxylase, as well as on the physical differences of the isotopes that induce different diffusivities (O'Leary *et al.*, 1992). Carbon isotope discrimination has generally been related to C_i/C_a according to the equation developed by Farquhar *et al.* (1982a):

$$\Delta = a + (b - a)\, C_i/C_a$$

where a is the fractionation occurring due to diffusion in air (4.4‰), b is the net fractionation caused by $RuBP_2$ and PEP carboxylation (between 27‰ – 30‰), and C_i/C_a is the ratio between CO_2 concentration in intercellular spaces (C_i) and ambient air (C_a). CO_2 diffuses into leaves through stomata. During diffusion, the heavier $^{13}CO_2$ molecules diffuse more slowly. Thus, the air inside the leaf pore space (C_i) is depleted in $^{13}CO_2$. The concentration of CO_2 inside the leaf depends on the rate of photosynthesis and the opening of the stomatal pores, which in turn influences isotopic discrimination ($\Delta^{13}C$) – higher C_i/C_a results in greater $\Delta^{13}C$, while lower C_i/C_a reduces $\Delta^{13}C$ –. This implies that changes in A_N

and g_s modify C_i/C_a ratio and, therefore, the carbon isotope composition of plant tissues ($\delta^{13}C$) and *WUE*.

To determine WUE_{WP}, soil water evaporation, percolation and runoff must be minimized. Thus, to use gravimetric methods (or individual lysimeters) in potted plants to measure plant water transpiration is a reasonable approach. In addition, this method allows determining accurately the biomass production. However, canopy of potted plants does not match properly with real canopies in the crop, and the roots development is constrained which could modify plant development. As an alternative for gravimetric estimates, the use of lysimeters or sap flow meters (in the case of woody plants) are optional techniques to obtain good measurements of plant water consumption in the field (Kostner *et al.*, 1998; Escalona *et al.*, 2002; Netzer *et al.*, 2005). However, these methods present other limitations. In relation to sap flow meters, the accuracy of the method depends on stem characteristics and fluxes rates of the plant studied. On the other hand, lysimeters involve building a complex and expensive equipment in the middle of a standard crop. Therefore, in spite of the limitations described for the gravimetric method in potted plants, it seems to be the most adequate method to compare environmental and genetic effects on WUE_{WP}.

Crop *WUE* depends on the crop characteristics and environmental variability. Canopy crop, water losses from the soil profile and the unknown root system (length and depth) need to be considered to determine WUE_c. Eddy covariance has been proposed as the more suited technique to derive global carbon and water fluxes at the local scale (Valentini *et al.*, 2000; Trambouze *et al.*, 1998; Spano *et al.*, 2004). Thus, it is a promising approach to provide gross estimates of WUE_c.

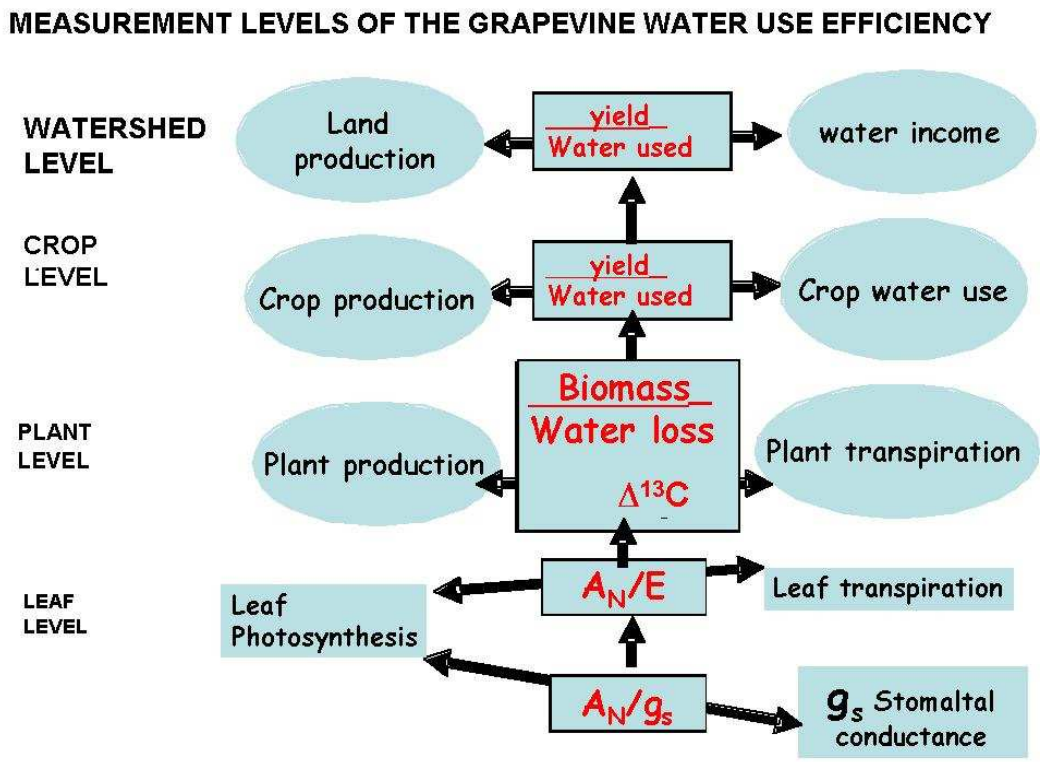

Figure 1.2. Different levels for water use efficiency measurement. From leaf to watershed, (instantaneous to growth season or yearly basis) there is a progressive integration of different crop production processes and water expenses with different measurement techniques implicated to determine the final *WUE* balance (Medrano *et al*., 2010).

1.3.3. Improvement of water use efficiency

1.3.3.1. Agronomical management practices

Proper crop management practices are a key procedure to optimize crop production with limited water supplies. Among agronomical practices, canopy management is the most common technique used. In grapevines in particular, canopy management consist of several practices which can be addressed to increase soil water availability, regulating the plant water necessities and reducing watering volumes. These practices have an effect on soil, microclimate, plant growth, yield, grape composition, wine quality and also on light absorption by the canopy and, consequently, in photosynthetic activity and water losses (Smart *et al.*, 1990; Hunter *et al.*, 1995, 2000; Williams & Ayars, 2005). Moreover, there are other techniques related to vineyard management such as the use of mulching (Gregory *et al.*, 2004) and cover crops (Pou *et al.*, 2011; Monteiro & Lopes, 2007), to control the vegetative vigour and reduce transpiration losses. However, the most effective way to increase water use efficiency in the vineyard is the

precise control of irrigation. Several systems to regulate irrigation have been developed such as regulated deficit irrigation (RDI) and partial root drying (PRD), which are often supported by plant-based water status indicators to adjust the irrigation protocol (trunk diameter sensors, sap flow meters, infrared thermometry, etc.). Under RDI method, water is supplied at levels below full crop evaporation during the growing season optimizing the number of fruits, fruit size and quality, and keeping grapevine vigour in balance with potential production (Souza *et al.*, 2005; Romero *et al.*, 2010). PRD method involves the exposure of about half of the roots in a drying state while the remaining root system is irrigated, altering the irrigated roots in cycles of 2-3 weeks. Root zone exposed to soil drying induces a root-to-shoot signalling mechanism, such as abcisic acid (ABA) synthesis, that causes partial stomatal closure maintaining a good plant water status due to the watered root side (Stoll *et al.*, 2000; Santos *et al.*, 2003; De la Hera *et al.*, 2007).

1.3.3.2. Genetic variability of water use efficiency

Inter-specific natural variations in *WUE* have been observed in many species (Anyia *et al*., 2007; Medrano *et al*., 2009; Barbour *et al*., 2010; Shao *et al*., 2010; Galmés *et al*., 2011; Daymond *et al*., 2011; Lelièvre *et al*., 2011) including grapevines (Bota *et al*., 2001; Gaudillère *et al*., 2002; Schultz, 2003; Souza *et al*., 2005; Pou *et al*., 2008; Koundouras *et al*., 2008; Zsofi *et al*., 2009; Gómez-Alonso *et al*., 2010; Prieto *et al*., 2010; Rogiers, 2011; Costa *et al.*, 2012). Hence, besides the agronomical practices previously described the genetic variability in *WUE* observed in many species has to be taken into account before considering plant breeding and genetic engineering to improve leaf and whole plant *WUE* (Condon *et al*., 2004). In grapevines in particular, it has been demonstrated that there are significant differences in *WUE* among cultivars by direct measurements of A_N/g_s, ranging from 25 to 100 mmol CO_2 mol^{-1} H_2O in irrigated plants, and between 100 and 200 mmol CO_2 mol^{-1} H_2O under drought conditions (Flexas *et al.* 2010). Moreover, interesting variability has also been described in longer term measurements, $\delta^{13}C$, which values ranged between -31‰ and -27‰ under well-watered conditions and from -26‰ to -24.7‰ under water-stress. However, less data are available in WUE_{WP} in grapevines described in only one study comparing 19 cultivars, where the values ranged between 2.5 and 3.2 g dry matter/ kg H_2O transpired (Gibberd *et al.*, 2001). This variability observed at

different levels indicates that genotype selection could be an effective way to gain significant improvements in WUE_l and WUE_{WP}.

1.3.3.3. Potential physiological targets for plant breeding and biotechnology

The physiological level is relevant to understand and improve leaf and whole plant *WUE*. Intrinsic water use efficiency (A_N/g_s) is a good basis to summarise the potential targets for genetic improvement of WUE_l (Parry *et al.*, 2005). Figure 1.3 represents two main ways to improve *WUE* on the basis of the relationship between stomatal conductance (water expenses) and photosynthesis (carbon gain) (Medrano *et al*., 2003). According to the curvilinear relationship between A_N and g_s, an increment in WUE_i is usually accompanied by decreases in A_N (Flexas *et al.*, 2004). This situation is illustrated in figure 1.3 where a given genotype (G1) can move along the curve "A" decreasing g_s and A_N – for instance, under water stress – and increasing WUE_i but, as an undesireable consequence, decreasing productivity (G1 to G2). However, a given genotype can also increase A_N with different strategies, such as irrigation, maintaining or even increasing yield but decreasing WUE_i (G1 to G3 in curve A). Clearly, an improvement in WUE_i without modifications in productivity could only be achieved when genotype G1 is converted in genotype G4 with higher rates of A_N for the same level of g_s (Fig. 1.3) In that case, it could be possible to improve carbon balance without modifications in water losses. Therefore, genetic manipulation efforts should be directed to convert genotype G1 into G4.

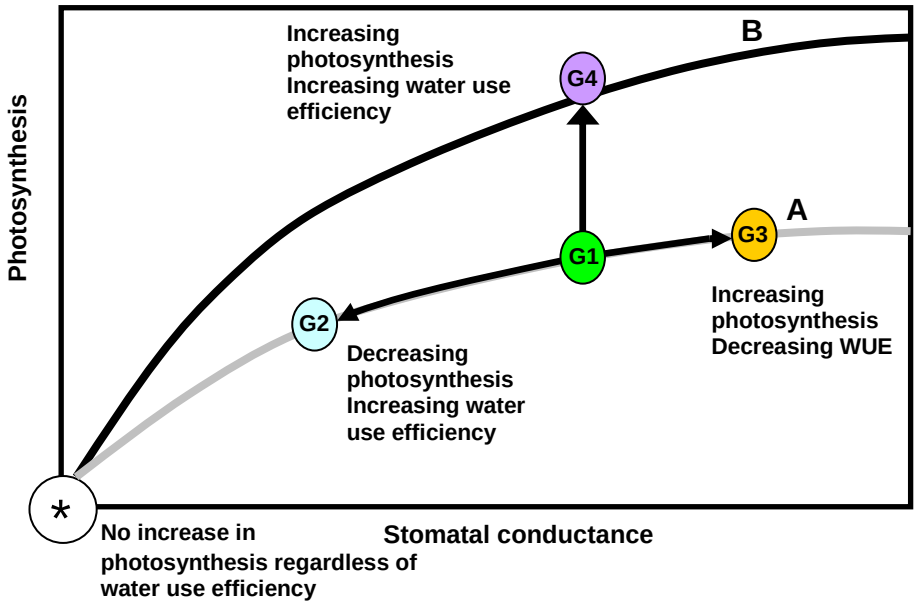

Figure 1.3. The relationship between photosynthesis and stomatal conductance in plants. This figure illustrates how WUE could be improved maintaining or even increasing yield at a given value of g_s. From Parry *et al.* (2005).

To increase leaf photosynthetic activity at any given g_s seems to be the best option to improve WUE_l. The genotypic modifications in A_N-g_s relationship to improve leaf photosynthetic activity and convert the situation G1 to G4 could be possible to achieve by different physiological mechanisms. These include improving carboxylation efficiency, and/or improving CO_2 diffusion in the mesophyll increasing the CO_2 concentration at the site of carboxylation inducing C_4 photosynthetic metabolism in C_3 plants (Long *et al.*, 2006), or increasing the CO_2 diffusion from sub-stomatal cavities to chloroplasts (i.e. mesophyll conductance – g_m) (Flexas *et al.*, 2008).

Improvements in carboxylation efficiency could be achieved by increasing the Ribulose-1,5- bisphosphate carboxylase/oxygenase (Rubisco) catalytic rate (Parry *et al.*, 2007) and/or specificity factor for CO_2 (Galmés *et al.*, 2005), reducing or bypassing photorespiration (Long *et al.*, 2006,; Kebeish *et al.*, 2007), increasing the capacity for RuBP regeneration (Feng *et al.*, 2007; Peterhansel *et al.*, 2008) or reducing the photoprotective state upon high-to-low light transitions (Long *et al.*, 2006). Grapevine photosynthesis normally operates in the Rubisco-limited region, particularly under drought conditions when stomatal and mesophyll conductance (g_s and g_m) decrease and the CO_2 availability in the chloroplast is reduced (C_c) (Flexas *et al.*, 2002, 2006a). Therefore, increasing Rubisco specificity for CO_2 (τ) could be more effective to improve *WUE* under

Mediterranean conditions than increasing Rubisco concentration, catalytic coefficient or activation state. Rubisco specificity for CO_2 (τ) has been determined in two grapevine cultivars, Tempranillo and Manto Negro, obtaining identical values for both cultivars (100 mol mol^{-1}; Bota *et al.*, 2002). Although, τ does not present substantial variability within C_3 plants, higher values than those of grapevines have been found in other species such as the Mediterranean species *Limonium gibertii* (110.5 mol mol^{-1}; Galmés *et al.*, 2005), and the red algae *Galdieria partita* (240 mol mol^{-1}; Uemura *et al.*, 1997). These differences in τ have a clear genetic basis that could be used to improve the carboxylation efficiency, and as a result WUE_l.

Higher *WUE* has been found in C_4 plants as compared to C_3. The key trait of C_4 photosynthesis is the compartmentalization of activities into two specialized cell and chloroplast types (Hatch, 1987). The C_4 pathway reduces photorespiration by elevating the CO_2 concentration at the site of Rubisco using a biochemical CO_2 pump. C_4 plants have two chloroplast types, each found in a specialized cell type, with different morphologies and functions (Kranz anatomy). Atmospheric CO_2 is fixed in the mesophyll cells by phosphoenolpyruvate carboxylase (PEPC) to form the four-carbon dicarboxylic acid oxaloacetate (hence the name C_4). Then, these C_4 acids are diffused into the bundle sheath cells where they are decarboxylated in the chloroplasts. The CO_2 produced is then refixed by Rubisco. In this way, CO_2 is concentrated in chloroplasts of bundle sheath where Rubisco is located, thus reducing oxygenase activity of Rubisco (photorespiration).

Another option to improve leaf photosynthetic activity is increasing the diffusion of CO_2 from sub-stomatal cavities to the carboxylation site through leaf mesophyll (Flexas *et al.*, 2008; Warren, 2008). The pathway of CO_2 from the atmosphere to the sub-stomatal cavities is regulated by stomatal conductance (g_s), which affects photosynthesis and transpiration. Then, the diffusion of CO_2 from sub-stomatal cavities to the carboxylation site is regulated by the so-called mesophyll conductance (g_m). Under water stressed conditions g_s and g_m decrease and, as a result, the CO_2 available in the chloroplasts (C_c) decreases as well. Increasing g_s increase water losses and decrease WUE_l. However, increasing g_m would increase C_c, and, hence, A_N without any effect of plant water status and, consequently, it would increase WUE_l displacing A_N-g_s relationship vertically (from G1 to G4, Fig. 1.3.).

The transfer of C_4 metabolism to C_3 plants and the improvement of τ could be achieved by site-directed mutagenesis. In that way, chloroplast transformation has been possible in tobacco plants replacing the native large Rubisco subunit by other higher plants versions (Kanevski *et al.*, 1999; Whitney & Andrews, 2001; Sharwood *et al.*, 2008; Whitney *et al.*, 2011). However, transformed plants presented low amount of Rubisco because of the lack of proper chaperons and other molecules needed for correct transcription, translation and assembling of the enzyme (Sharwood *et al.*, 2008). This might suggest that genetic engineering in grapevines could be an unachievable goal. Therefore, increasing the diffusion of CO_2 through the mesophyll needs to be explored, as has been suggested for several authors (Warren & Adams, 2006; Aranda *et al*., 2007).

At the whole plant level, photosynthesis and respiration are the physiological processes that directly affect to carbon balance, and transpiration to water losses, making them good candidates to improve WUE_{WP}. Photosynthesis could be improved by increasing total leaf area (Sharma-Natu & Ghildiyal, 2005; Long *et al.*, 2006). Escalona *et al.* (2003) showed that leaves at the inner canopy layers in grapevines, which represent 35-40% of total leaf area, contribute less than 5% to total net carbon gain with a higher percentage of total water losses. This suggests that increasing photosynthesis by increasing total leaf area would only increase shaded leaves, thus reducing total *WUE*. Therefore, respiration and transpiration remain as important physiological processes to be considered for improving WUE_{WP}. Substantial water losses that are not included in the opening of the stomata pores and daytime g_s can affect WUE_{WP}. Significant transport of water occurs through the cuticle and incomplete stomatal closure during night without any carbon gain (cuticular and night conductance). Therefore, reducing the night water losses by transpiration could be a way to improve WUE_{WP}. In addition, respiration occurs continuously in all plant organs through the entire plant life. It implies that 30% to 90% of carbon fixed in photosynthesis is used for growth and maintenance respiration (Amthor, 2000). Hence, reducing the percentage of carbon lost in respiration could increase whole plant carbon gain. Mitochondrial respiration can proceed via the alternative oxidase pathway (*AOP*) and the cytochrome oxidase pathway (*COP*). A significant part of maintenance respiration is sustained by *AOP* (Flórez-Sarasa *et al.* 2007). Considering *AOP* as a futile pathway, a reduction in *AOP* respiration may resemble an opportunity to

decrease overall respiration without significant consequences, improving carbon balance and *WUE*.

Among all these potential targets to improve *WUE*, the present Thesis focuses on three of them: (1) mesophyll conductance to CO_2, (2) cuticular and night conductance and (3) whole plant respiration.

1.4. PROMISING PHYSIOLOGICAL PARAMETERS TO IMPROVE WATER USE EFFICIENCY

As stated above, to improve *WUE* is an unavoidable goal for grapevine crop. Besides the agronomic methods, which could result in a more immediate reduction of water needs, there is a general consensus on the necessity to explore the capability to select grapevine cultivars with enhanced water use efficiency. The use of physiological parameters as putative selection criteria is of major interest for genetic improvement as well as for biotechnology approaches (Flexas *et al*., 2010) and, in that way, this work intends to explore the interest of mesophyll conductance, night transpiration and plant respiration for those purposes. In the present work, we evaluate the environmental and genetic induced variations on those parameters in order to offer a first assessment of their interest as surrogate parameters for *WUE* improvement.

1.4.1. Mesophyll conductance to CO_2

Photosynthesis depends on the diffusion of CO_2 from the atmosphere surrounding the leaf to the sub-stomatal internal cavities through the stomata, and from there to the carboxylation site located inside the stroma through the leaf mesophyll. Mesophyll is a complex structure that varies greatly between species and growth conditions (Evans *et al.*, 2009). The diffusion pathway of CO_2 through the mesophyll tissue can be separated in 3 phases, gas (intercellular air spaces), liquid (cell walls, cytosol and chloroplast stroma) and lipid phase (plasma membrane and chloroplast envelope membranes) (Fig. 1.4).

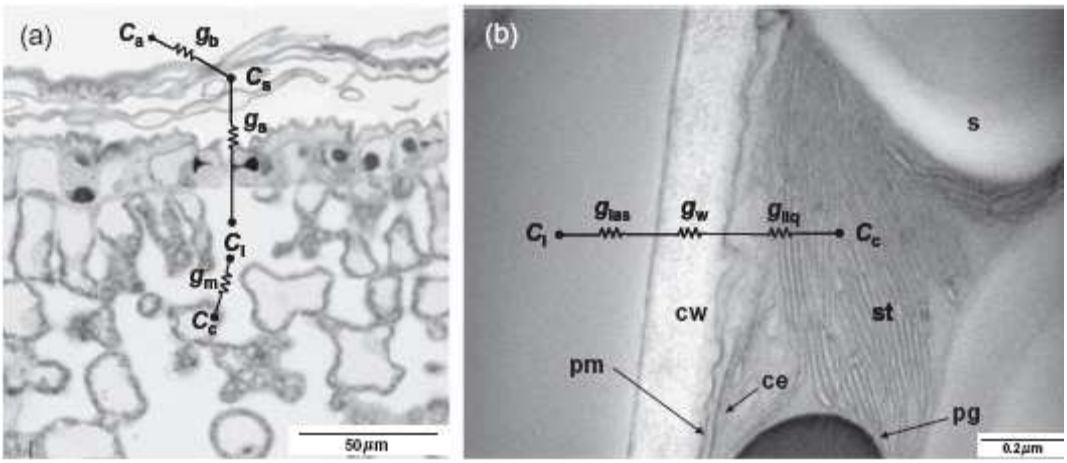

Figure 1.4. (a) Light micrograph of the abaxial surface of an olive leaf, where the stomata can be seen (bottom side up), as well as the pathway of CO_2 from ambient (C_a) through leaf surface (C_s) and intercellular air spaces (C_i) to the chloroplast (C_c). Boundary layer conductance (g_b), stomatal conductance (g_s) and mesophyll conductance (g_m) are indicated. **(b)** Electron micrograph of a grapevine leaf where cell wall (*cw*), plasma membrane (*pm*), chloroplast envelope (*ce*) and stroma thylakoid (*st*) can be observed. The pathway of CO_2 from C_i to chloroplastic CO_2 (C_c) is characterized by intercellular air space conductance to CO_2 (g_{ias}), through the liquid and lipid phase (g_{liq} and g_{lip}) from intercellular airspaces to inside the cell. A grain of starch (s) and a plastoglobule (pg) can be also observed in the picture. From Flexas *et al.* (2008).

From first Fick law of diffusion, the net photosynthetic flux at steady state (A_N) can be expressed as: $A_N = g_s (C_a - C_i) = g_m (C_i - C_c)$; where g_s and g_m are the stomatal and mesophyll conductance to CO_2 diffusion, and C_a, C_i and C_c are the CO_2 concentrations in the atmosphere, in the sub-stomatal internal cavity and in the chloroplast stroma, respectively (Long & Bernacchi, 2003). Then, decreases of CO_2 diffusion through the leaf depend on stomatal closure and mesophyll conductance.

In Gaastra's (1959) pioneer work on leaf photosynthesis, mesophyll conductance was defined as a diffusion component of the photosynthesis pathway, and it was confirmed by later works showing that g_m could be variable and could respond to environmental factors (Jones & Slatyer, 1972; Samsuddin & Impens, 1979). Thereafter, for decades, gas exchange studies assumed that C_i=C_c and therefore, g_m was constant and infinite. However, evidences that C_c was lower than C_i were repeatedly demonstrated. There were several confirmations by comparing online carbon isotope discrimination studies (Evans *et al.*, 1986), comparing the initial slope of A_N- C_i curves with the activity of ribulose 1·5-bisphosphate carboxylase/oxygenase (Rubisco) determined *in vitro* (Evans & Terashima, 1988) and by comparison of chlorophyll fluorescence with gas exchange measurements (Bongi & Loreto, 1989; Di Marco *et al.*, 1990). In

summary, there is now convincing evidence that g_m is finite and not constant, C_c is significantly less than C_i, and g_m is a significant limitation to photosynthesis.

Several methods to estimate g_m have been developed, which principles and procedures are explained in detail by Warren (2006). Briefly, the used methods to determine g_m include (1) online carbon isotope discrimination (Evans *et al.*, 1986; Sharkey *et al.*, 1991; von Caemmerer & Evans, 1991; Loreto *et al.*, 1992; Lloyd *et al.*, 1992), (2) the "constant and variable J" fluorescence method (Di Marco *et al.*, 1990; Harley *et al.*, 1992; Epron *et al.*, 1995), (4) "curve-fitting" (Ethier & Livinsgton, 2004; Sharkey *et al.*, 2007) and (5) anatomical analysis (Nobel, 1991; Evans *et al.*, 1994; Syversten *et al.*, 1995; Tholen *et al.*, 2011; Tosens *et al.*, 2011).

1.4.1.1. Variability of g_m among species and its response to environmental variables

Mesophyll conductance (g_m) is a limitation of A_N of similar magnitude than g_s (Evans & Loreto, 2000; Warren & Adams, 2006). Among species there is a positive relationship between A_N and g_m, but there is a wide variation in this relationship. For instance, for a given value of A_N (10 μmol CO_2 m^{-2} s^{-1}), great variations of g_m can be found (from 0.06 to 0.31 mol CO_2 m^{-2} s^{-1}). Data on g_m are available in the literature for more than 100 species, varieties and forms. Mean maximum values of g_m (i.e. in the absence of stress conditions) ranged from 0.4 mol CO_2 m^{-2} s^{-1} to values very close to zero (Flexas *et al.*, 2012; Niinemets *et al.*, 2009a) (Fig. 1.5). The vast majority of g_m estimates are made in Spermatophytes (angiosperms and gymnosperms) (Niinemets *et al.*, 2009a), with very few data for liverworts and hornwort (Meyer *et al.*, 2008), while there is no data available for groups such as mosses, lycophytes, equisetophytes and ferns (Flexas *et al.*, 2012). In general, herbaceous annual and biannual plants present the largest mean values of g_m (around 0.4 mol CO_2 m^{-2} s^{-1}) being the group that also present fast-growing strategies accompanied by high A_N. Perennial herbs and woody deciduous and semi-deciduous angiosperms show medium values (around 0.2 mol CO_2 m^{-2} s^{-1}). Woody evergreen plants (angiosperms and gymnosperms) and succulent CAM plants present lower values (below 0.1 mol CO_2 m^{-2} s^{-1}). On the other hand, hornworts and liverworts present estimates of g_m five orders of magnitude smaller than for Spermatophytes (on average, $2.5 \cdot 10^{-5}$ mol CO_2 m^{-2} s^{-1}), distinguished for

lack of stomata and a low degree of cuticularization. Moreover, a large variability of g_m also is present within groups, genus and species (Lauteri *et al.*, 1997; Centritto *et al.*, 2009; Barbour *et al*., 2010), suggesting that g_m is likely to be involved in the differences of A_N found among species and cultivars.

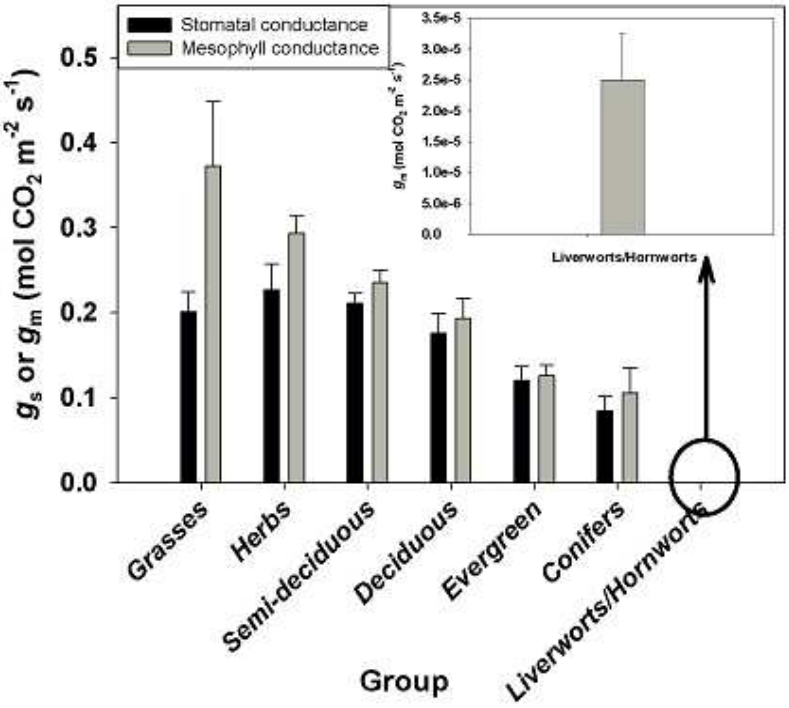

Figure 1.5. Average ± S.E. values for g_s and g_m in different pooled groups of plants. Data obtained from Meyer *et al.* (2008) and Niinemets *et al*. (2009). (From Flexas *et al.* 2012)

It is known that g_m is not only a constitutive property of leaf anatomy that differs between species. Mesophyll conductance (g_m) can change significantly in response to environmental variables, external factors (abscisic acid, virus infection, etc.) and internal factors (leaf ageing and development), in short (minutes) and long term (days or weeks) (Warren, 2008; Flexas *et al.*, 2008). Most g_m studies are focus on the variability of g_m in response to environmental factors, being mostly studied for water stress, salinity, temperature, CO_2 concentration and light availability. Among them, g_m can present long-term responses (acclimation) or in some cases rapid changes (within few minutes after applying the treatment), such as rapid leaf desiccation (cut petiole in air) or short-term exposure to different temperatures, CO_2 concentrations or light intensities. A large number of studies show how water stress and salinity reduce g_m in a similar magnitude than g_s (Scartazza *et al.*, 1998; Delfine *et al.*, 1999; Loreto *et al.*, 2003; Flexas *et al.*, 2004; Ripley *et al.*, 2007; Galmés *et al.*, 2011; Duan *et al*., 2011; Ferrio *et al.*, 2012) with responses that range from several days to weeks or

sometimes even in minutes in the case of leaf desiccation (Flexas *et al.*, 2006b). On the other hand, temperature is also a source of variation of g_m. In general, studies show that g_m increases from 10 to 20ºC while at temperatures over 20ºC changes of g_m differ among species with responses that can go from minutes (Warren & Dreyer, 2006; Scafaro *et al.*, 2011) to months (Yamori *et al.*, 2006; Díaz-Espejo *et al.*, 2007). Regarding to carbon dioxide concentration around leaves more controversial results have been obtained. Long exposures to elevated CO_2 concentrations (months to years) causes reductions of g_m in some species (Singsaas *et al.*, 2003) while in others it is unaffected (Eichelmann *et al*., 2004; Bernacchi *et al.*, 2005). Furthermore, there is still no consensus about rapid changes in g_m under short exposures to different CO_2 concentrations. While, at decreased CO_2 concentration around leaves g_m increases (Centritto *et al.*, 2003; Flexas *et al.*, 2007a), at higher concentrations of CO_2 g_m decreases (Düring, 2003; Flexas *et al*., 2007a; Hassiotou *et al*., 2009; Vrabl *et al*., 2009; Tazoe *et al*., 2011; Douthe *et al.*, 2011) or in other studies g_m is maintained stable (Von Caemmerer & Evans, 1991; Loreto *et al*., 1992; Tazoe *et al*., 2009). Concerning light availability, in general g_m responses are in the same direction as A_N and g_s, at higher light intensities, higher values of A_N, g_s and g_m are obtained (Piel *et al*., 2002; Laisk *et al.*, 2005; Warren *et al.*, 2007; Bunce *et al.*, 2010; Cano *et al.*, 2011). Few studies have tested rapid responses of g_m to irradiance, while in some studies g_m increases with increasing light availability (Gorton *et al.*, 2003; Flexas *et al*., 2007a), in others no relation between g_m and measuring light intensity was observed (Tazoe *et al*., 2009; Yamori *et al.*, 2011).

As for internal factors, g_m increases during leaf development up to the point of full leaf expansion, and then, g_m declines as leaf ages and /or senescences (Miyazawa & Terashima, 2001; Niinemets *et al.*, 2006; Flexas *et al.*, 2007b; Whitehead D. *et al.*, 2011). The causes of changes of g_m with leaf age have been associated with changes in anatomical traits (Hanba *et al.*, 2001).

The results obtained from all g_m studies present a large body of evidence to conclude that g_m is not finite, and it acclimates and responds to different factors including environmental variables such as, water availability, temperature, light and CO_2, that can be important in regulating A_N, especially in Mediterranean areas where these environmental factors could be intensified according to climate change predictions. Despite other external and internal factors have been studied

(i.e. low nitrogen availability, high O_3, high altitude, leaf porosity, etc.) the environmental ones explained in this section are the most widely studied.

1.4.1.2. Mechanisms that determine g_m

Large variability of g_m has been described among species, genotypes and for long-term and rapid responses to environmental factors. However, the mechanisms that regulate these changes remain unclear.

The variability of maximum values of g_m observed among species and genotypes involved in adaptative and acclimation responses have been associated to leaf structure and anatomical properties (Niinemets *et al.*, 2009a; Terashima *et al.*, 2011). Leaf dry mass per area (M_A), the simplest indicator of leaf structure composed by leaf thickness and density, has been proposed as a limiting factor of maximum g_m. It has been observed that g_m increases with leaf thickness, while it is reduced with leaf density (Niinemets *et al.*, 2009b; Poorter *et al.*, 2009; Hassiotou *et al.*, 2010). Hence, it has been shown that M_A strongly limits g_m in evergreen species (large M_A) with lower photosynthetic rates due to large CO_2 drawdown from sub-stomatal cavities (C_i) to chloroplasts (C_c) (Flexas *et al.*, 2008).

More specific leaf anatomical traits have been proposed as main determinants of the variability of g_m among species (Evans *et al.*, 2009; Terashima *et al.*, 2011). The CO_2 diffusion pathway consists of gas, liquid and lipid phase. The rate of diffusion through the composite segments of the diffusion pathway depends on the effective thickness and diffusivity of each anatomical component (Flexas *et al.*, 2012). Gas phase is assumed to have smaller effect on diffusion limitations than the components of liquid and lipid phases (Evans *et al.*, 2009). This was confirmed in several studies comparing CO_2 diffusion in air and helox – air where helium replaces nitrogen to increase diffusivity – showing that liquid and lipid conductance (g_{liq} and g_{lip}) are the most limiting g_m components (Genty *et al.*, 1998; Piel *et al.*, 2002). Among the components of g_{liq} and g_{lip}, cell walls and chloroplast membranes have been proposed as the most important factors affecting CO_2 diffusion inside leaves (Terashima *et al.*, 2011). Several reports have shown that the surface area of chloroplasts exposed to intercellular air spaces (S_c/S) correlates positively with g_m (Evans *et al.*, 1994; Evans & Loreto, 2000; Terashima *et al.*, 2006; Tosens *et al.*, 2011). In addition, differences in cell wall thickness (T_{cw}) have been shown to explain from 25% to 50% of the variability in

g_m (Evans *et al.*, 2009; Terashima *et al.*, 2011). Recent studies have presented negative correlations between g_m and T_{cw} when comparing Australian *Banksia* species (Hassiotou *et al.*, 2010), rice relatives (Scafaro *et al.*, 2011) and Mediterranean *Abies* species (Peguero-Pina *et al.*, 2012). Furthermore, Terashima *et al.* (2011) demonstrated, combining data from literature, a negative and highly significant relationship between the ratio $g_m/(S_c/S)$ and T_{cw}. Therefore, the variability of g_m among species and even varieties could be related to differences in T_{cw}. In addition, other traits have been proposed to affect g_m, including leaf porosity (Miyazawa & Terashima *et al.*, 2001; Peña-Rojas *et al.*, 2005), mesophyll surface area exposed to intercellular air spaces (S_m/S)(Evans & Loreto, 2000) and chloroplast arrangements (Tholen *et al.*, 2008) although not proven in all cases (Kogami *et al.*, 2001; Gorton *et al.*, 2003; Hanba *et al.*, 2004).

While anatomical traits correlate well with g_m, the latter can change more rapidly than leaf anatomy and morphology. The temperature dependency of g_m shows a Q_{10} of 2.2, suggesting that there must be an enzymatic or protein-facilitator mechanism for the diffusion control of g_m (Bernacchi *et al.* 2002). The most likely candidates proposed for the most dynamic g_m changes have been carbonic anhydrase and aquaporins, involved in membrane permeabilities and consequently in g_{liq} and g_{lip} variations.

Carbonic anhydrases catalyse the dilution of CO_2 in liquid phase, being it converted in HCO_3^-. Three different classes of carbonic anhydrases have been described in plants (α, β, γ) located in the plasma membrane, cytosol and chloroplasts, being β class the most abundant form (Fabre *et al.*, 2007). Several studies performed with modifications of carbonic anhydrase in transgenic plants shown little evidence of the involvement of the most abundant forms in g_m and A_N (Price *et al.*, 1994; Williams *et al.*, 1996). However, Gillon & Yakir (2000) showed that the contribution of carbonic anhydrase to g_m is species dependent and it may become more important when g_m is low as in sclerophyllous species.

Aquaporins are membrane intrinsic proteins that facilitate the movement of water, CO_2 and other small neutral solutes across cellular membranes. Aquaporins can be divided into five 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), the Small Intrinsic Proteins (SIPs) and X intrinsic proteins (XIPS) (Chamount *et*

al., 2001; Johanson *et al.*, 2001; Sakurai *et al*., 2005; Danielson & Johanson, 2008). The first evidence of the possible role of aquaporins in the diffusion of CO_2 was provided by Terashima and Ono (2002), showing that $HgCl_2$, a non-specific inhibitor of aquaporins, reduced g_m. Later, it was confirmed by Uehlein *et al.* (2003), who demonstrated that tobacco aquaporin NtAQP1 facilitates trans-membrane CO_2 transport by expression in *Xenopus* oocytes. Subsequent studies with transgenic plants, rice, tobacco and *Arabidopsis thaliana* have reported the role of some specific aquaporins (HvPIP2,1; NtAQP1 and AtPIP1,2 respectively) in the regulation of g_m (Hanba *et al.*, 2004; Flexas *et al.*, 2006c; Heckwolf *et al.* 2011). In addition, Uehlein *et al.* (2008) showed that NtAQP1 is also located in the inner chloroplast membranes and it considerably affects leaf CO_2 transport. On the other hand, Miyazawa *et al.* (2008) studied the role of aquaporins in g_m under drought conditions in tobacco plants, however, the variations in g_m were not clearly explained by the total plasma membrane aquaporin (all PIP). Although the regulation of aquaporin activity in the short term is not fully understood, several mechanisms have been proposed, including direct phosphorylation of aquaporins (Kjellbom *et al.*, 1999), an osmotically driven cohesion/tension mechanism (Ye *et al.*, 2004), pH-dependent gating of aquaporins (Tournaire-Roux *et al.*, 2003) and transcriptional regulation and protein stability (Eckert *et al.*, 1999).

1.4.1.3. Mesophyll conductance (g_m) in grapevines

Studies about g_m in grapevines are relatively scarce. Patakas *et al.* (2003) described that g_m in grapevines could have a genetic basis. In this study, important differences in leaf anatomy traits among three different cultivars affecting internal CO_2 gas phase conductance (g_{ias}) were described, although g_m was not determined. Regarding to changes of g_m in response to environmental conditions, Flexas *et al.* (2002) showed that g_m is progressively reduced under drought in the long term, under field conditions; and Düring *et al.* (2003) showed that g_m declined at high CO_2 supply in field-grown Riesling vines. Afterwards, several studies in grapevines confirmed that g_m decreases under increasing drought conditions (Moutinho-Pereira *et al.*, 2004; Ferrio *et al*., 2012), being restored after re-watering (Flexas *et al*., 2009; Pou *et al.*, 2012). In addition, Pérez-Martin *et al.* (2009) studied the interaction of soil water deficit and VPD on g_m, where drought dominated the response of g_s and g_m.

Altogether, data on g_m are available for seven cultivars of *Vitis*, which values range from 0.060 to 0.340 mol CO_2 m^{-2} s^{-1} under irrigation conditions, and from 0.010 to 0.200 mol CO_2 m^{-2} s^{-1} under stressful conditions. More studies are needed to extend our knowledge on genotypic variation of g_m and to evaluate whether this variability translate into variations of WUE_l and WUE_{WP} that could open new opportunities for *WUE* improvements.

1.4.2. Cuticular and night conductance

1.4.2.1. Variability and factors affecting night conductance (g_{night})

Transpiration is an unavoidable process occurring in parallel to photosynthesis. However, during the night when photosynthesis stops, significant transpiration can still occur uncoupled from any carbon gain, hence, contributing negatively to WUE_{WP}.

The amount of water transpired by leaves depends on cuticular and stomatal conductance. Cuticular conductance (g_{cut}) is not regulated by guard cells, however, it decreases WUE_l because this is almost totally impermeable to CO_2, and as a result no gain in photosynthesis is obtained while water losses are maintained (Boyer *et al.*, 1997). Generally, g_{cut} is lower than g_s, between 0.004 and 0.020 mol m^2 s^{-1} (Rawson & Clarke, 1988; Nobel, 1991; Kerstiens, 1995; Boyer *et al.*, 1997; Burghardt & Riederer, 2003; Howard & Donovan, 2007). Thus, most reported values of g_{night} are largely influenced by g_s.

Incomplete stomatal closure during the night is observed in a diverse range of C_3 and C_4 species. Stomatal conductance (g_s) and transpiration (E) are maintained during the nigh-time when no photosynthesis occurs and the need to cool leaves is reduced or absent, constituting a significant source of water losses and reduced WUE_l (Caird *et al.*, 2007). Several studies have demonstrated that E_{night} can be an important fraction of daytime transpiration, from 5% to 30% (Bucci *et al.*, 2005; Daley & Philips, 2006; Cavender-Bares *et al.*, 2007, Scholz *et al.*, 2007). Night conductance (g_{night}) has been studied in a large range of genera and life forms (annuals and perennials; monocots, herbaceous dicots, shrubs, and trees) native of different habitats (Fig. 1.6): e.g. wetland (Loftfield, 1921), desert (Donovan *et al.*, 2003; Snyder *et al.*, 2003; Ludwig *et al.*, 2006), neotropical savanna (Bucci *et al.*, 2004, 2005; Domec *et al.*, 2006; Scholz *et al.*, 2007),

temperate deciduous and evergreen forests (Benyon, 1999; Oren *et al.*, 2001; Barbour *et al.*, 2005; Daley & Phillips, 2006; Kavanagh *et al.*, 2007), and subalpine forest (Herzog *et al.*, 1998). Among g_{night} values described in many life forms and habitats, substantial g_{night} have been observed in many crops, such as *Distichlis spicata* (C_4; Snyder *et al.*, 2003), wheat (*Triticum aestivum*; C_3; Rawson & Clarke, 1988), tomato (*Lycopersicon esculentum*, C_3; Caird *et al.* 2007) and *Vicia faba* (C3; Ealson & Richards, 2009). In addition, high variability among species has been observed (Snyder *et al.*, 2003; Bucci *et al.*, 2004; Daley & Phillips, 2006; Kavanagh *et al.*, 2007), with values ranged from 0.003 mol H_2O m^{-2} s^{-1} in *Pinus ponderosa* to 0.450 mol H_2O m^{-2} s^{-1} in *Chrysothamnus nauseosus* (Caird *et al.*, 2007). However, the relationship of species differences to source environment or habitat remain unclear, therefore, more studies are required to determine whether species differences in g_{night} are adaptive or a mechanistic limitation (i.e inability to close at night). Among cultivars and accessions of single species substantial variations in g_{night} have also been described (Caird *et al.*, 2007), indicating that genetic factors could influence g_{night} in plants.

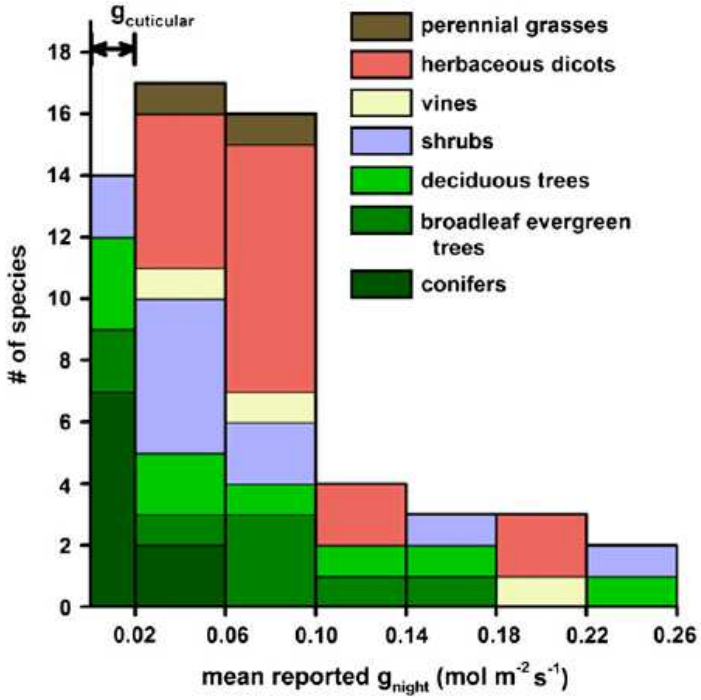

Figure 1.6. Histogram with reported g_{night} values in species among different plant functional groups. For each species, g_{night} was averaged from all reported values (field and greenhouse studies) with units mol m^{-2} s^{-1}. The black two-headed arrow at the top left of graph represents the range from reported cuticular conductance (g_{cut}) taken from many species, and reported g_{night} within this range may be largely due to cuticular conductance (g_{cut}) rather than stomatal conductance (g_s). (From Caird *et al.* 2007).

The variability of g_{night} observed among species and in some cultivars suggests that g_{night} is a regulated character with a genetic basis. Although, in addition, environmental conditions and, external and internal factors have been proposed as sources of variations in g_{night}. Stomatal density among abaxial and adaxial leaf surface and stomatal responses to different environmental conditions can contribute to variations in g_{night}. It has been shown in some species such as in cotton (*Gossypium hirsutum*, Sharpe, 1973) and fava bean (*Vicia faba*, Aben *et al.*, 1989), although further studies are needed to understand how these factors affect g_{night}. Moreover, g_{night} is not stable during the night, increasing towards pre-dawn. These changes during the night-time are associated to starch metabolism and circadian rhythm, two internal factors that can affect stomatal opening, as demonstrated in transformed *Arabidopsis thaliana* with disrupted circadian rhythm (Dodd *et al.*, 2004, 2005), and in starch deficient *Arabidopsis* mutants (Lasceve *et al.*, 1997).

Night conductance (g_{night}) responds to similar external factors as daytime g_s, including VPD, ABA, water stress and salinity (Chu *et al*., 2009; Ealson & Richards, 2009; Zeppel *et al.*, 2012). Different responses to VPD have been observed. While increased VPD correlated with greater night transpiration (E_{night}) in many tree species measured by sap flux (Herzog *et al.*, 1998; Benyon, 1999; Oren *et al.*, 2001; Daley & Phillips, 2006; Kavanagh *et al.*, 2007; Zeppel *et al.*, 2012), increasing VPD has been observed to lower g_{night} in some species (Muchow *et al*., 1980; Oren *et al.*, 2001; Bucci *et al.*, 2004; Barbour & Buckley, 2007), or to leave g_{night} unaffected (Barbour *et al.*, 2005). On the other hand, water stress and salinity reduces g_{night} in field and greenhouse experiments in many species, including *Hibiscus cannabinus* (Muchow *et al.*, 1980), *Pseudostuga menziesii* (Running, 1976; Blake & Ferrell, 1977), *Helianthus anomalus* (Ludwig *et al*., 2006), shrubs (Donovan *et al*., 2003), wheat (Rawson & Clarke, 1988), *Helianthus* species (Howard & Donovan, 2007) and in two evergreen temperate woodlands species (Zeppel *et al.*, 2010). Photoperiod length and light intensity can affect the speed and degree to which stomata close in the dark. Short-day induces incomplete stomatal closure as opposed to long-day photoperiod in *Chrysanthemum* and *Vicia faba* (Schwabe, 1952; Ealson & Richards, 2009). Furthermore, day time conditions (light intensity) also affect g_{night} (Lasceve *et al.*, 1997). High concentrations of exogenous ABA decrease g_{night} as well as ABA

content of leaves in response to water stress (Leymarie *et al*., 1999; Blake & Ferrell, 1977). On the other hand, the effects of mineral nutrition on g_{night} are unclear (Ludwig *et al*., 2006; Scholz *et al.*, 2007; Howard & Donovan, 2007).

The results obtained summarized in this section present a large evidence that g_{night} is variable and somehow regulated, in many ways similar to daytime stomatal regulation, however, the mechanisms that regulate g_{night} are poorly understood. Moreover, in some species, night transpiration can result a relatively important part of the total transpiration. Therefore, future research on plant regulation of g_{night} is needed to understand such regulations as well as the possible consequences on the leaf and plant *WUE*.

1.4.2.2. Cuticular and night conductance in grapevines

In spite of the variability described in g_{night} and E_{night} among species, data in grapevines are scarce. Cuticular conductance (g_{cut}) in grapevines is generally quite low, around 0.005 mol H_2O m^{-2} s^{-1} (Boyer *et al.*, 1997; Flexas *et al.*, 2009). Hence, stomatal conductance maintained during night may represent the most important source of water losses. Until now only two studies reported values of g_{night} and E_{night} in grapevines. Caird *et al.* (2007) showed contrasting values for g_{night} between 0.03 and 0.06 mol H_2O m^{-2} s^{-1} in *Vitis berlandieri* and *Vitis rupestris*, and values up to 0.205 mol H_2O m^{-2} s^{-1} in *Vitis riparia*. Rogiers *et al.* (2009) studied daytime and night-time g_s across a wide range of VPD and soil moistures in ten cultivars of grapevine. The results obtained showed the highest values for anhysohidric cultivars such as Semillon and Cabernet Sauvignon, between 0.015 and 0.025 mol H_2O m^{-2} s^{-1} for g_{night}, and between 0.20 and 0.30 mmol H_2O m^{-2} s^{-1} for E_{night}, respectively. On the other hand, the lowest values were observed in Grenache, classified as isohydric cultivar, which values were 0.005 mol H_2O m^{-2} s^{-1} for g_{night} (close to g_{cut} values) and 0.08 mmol H_2O m^{-2} s^{-1} for E_{night}.

The limited information in g_{night} and E_{night} in grapevines and the possible reductions of WUE_{WP} due to water losses during night, indicate that more efforts are needed to determine the importance of g_{night} and E_{night} on the variation of leaf and plant *WUE*.

1.4.3. Plant respiration

Plant respiration can be defined as the combination of metabolic reactions where reduced carbon compounds are oxidized to CO_2 and H_2O, and the energy released is used for the synthesis of adenosine triphosphate (ATP) and reducing equivalents. The energy conserved as ATP and reducing equivalents together with the intermediate carbon compounds produced during respiratory metabolism are used for growth and maintenance processes of plants. Therefore, respiration is crucial for growth and productivity and, together with photosynthesis, determines the carbon balance of the plants (Amthor, 1989).

Part of respiration can proceed via non-phosphorylating alternative pathway (*AOP*) that is cyanide-resistant and generates less ATP than cytochrome pathway (*COP*).The types and rates of plant respiration depend on a combination of energy demand, substrate availability and oxygen supply.

1.4.3.1. The importance of leaf respiration and its functions.

Carbon accumulation is the primary process for plant biomass production, and depends on photosynthesis and respiration. Rates of respiration are lower than photosynthesis rates; however, photosynthesis occurs only in leaves and during daytime, whereas respiration is a process that occurs in all organs and during whole day. This implies that 30% to 90% of the carbohydrates fixed in photosynthetic processes are respired (Amthor, 2000). The balance between photosynthesis and plant respiration is defined as the carbon balance (mols of CO_2 incorporated per mols of CO_2, O_2 or CH_2O consumed), and hence the capacity of plants to produce new biomass for growing and developing reproductive structures, as well as to maintain them (Poorter *et al.*, 1992).

The fraction of carbohydrates produced in photosynthesis and consumed by respiration is dependent on species (Galmés *et al.*, 2007) and plant organs (Laureano *et al.*, 2008; Vivin *et al.*, 2003). Root respiration accounts for 10% to 50% of the total carbon assimilated each day in photosynthesis. This percentage includes the respiration of symbionts that can be up to 25% of total carbohydrates (Lambers *et al.*, 1998), or mychorrizal fungi (Bryla & Eisenstat, 2005). Root respiration provides the driving energy for root maintenance and growth, ion absorption and transport into the xylem. The large variation described in root respiration is related to variations in nutrient supply and genotype (Lambers *et al.*,

2008). Leaf respiration provides the energy for leaf growth and maintenance. Respiration of other plant parts is largely associated with their growth, although maintenance components also play a role.

Plant respiration can be divided in two different physiological processes, growth and maintenance. This can be expressed mathematically as follows:

$$r = rG + rM = gR * RGR + mR$$

where r is the overall respiration rate per unit dry mass (mol 'X' g^{-1} s^{-1}), rG and rM are growth and maintenance respiration (mol 'X' g^{-1} s^{-1}). RGR is the relative growth rate (g_{growth} g^{-1} s^{-1}), gR represents the specific costs of tissue construction (mol 'X' g_{growth}^{-1}) and mR are the specific costs of tissue maintenance (mol 'X' g^{-1} s^{-1}), with 'X' being either ATP, O_2, CO_2 or CH_2O. In the case of roots, gR includes the costs of nutrient uptake (Amthor *et al.*, 2000; Bouma *et al.*, 2005).

Growth respiration is the respiratory energy required to convert non-structural carbohydrates into new plant constituents. In photosynthetically active leaves, metabolic energy (ATP and NAD(P)H) may come directly from photosynthesis, while, in heterotrophic tissues such as roots, stems, and leaves in the dark, respiration provides the required energy. Different subprocesses of growth that consume energy and/or C-skeletons have been identified: nitrate reduction, active uptake of minerals and organic substrates into growing cells, monomer synthesis from those substrates, polymerization, maintenance and phloem loading (Penning de Vries *et al.* 1974). For most vegetative plant tissues the construction costs or glucose requirements for growth are similar, approximately 1.5 g glucose g^{-1} biomass (Lambers *et al.* 2008). Tissues of fast-growing species have high investment in proteins (expensive constituents), minerals and organic acids (cheap constituents) (Poorter, 1994; Villar *et al.*, 2006), while evergreen leaves present higher investment in costly structural and defense compounds such as phenols or lignin (Miller & Stoner *et al.*, 1979). Despite the variability of composition structures between different species, the overall construction cost of vegetative plant biomass is quite constant. This similarity is mainly explained by the positive correlation between the concentration of expensive and inexpensive fractions in fast and slow growth plants (Poorter & Bergkotte, 1992; Villar & Merino, 2001). In contrast, seeds have higher carbon costs than other plant tissues owing as lipids are primarily stored energy (1.65 g glucose g^{-1} biomass).

Maintenance respiration accounts for 20-60% of photosynthates produced per day (3-6% of dry mass per day) in herbaceous (De Visser *et al.*, 1992) and woody species (Ryan *et al.*, 1994), higher values being associated with slow growth plants (Lambers *et al.*, 2008). This process is defined as the respiratory energy associated with protein turnover, maintenance of ion gradients across membranes and operation of metabolic processes involved in physiological adjustments to environmental changes. Protein turnover rates may vary among plant organs, species and growth conditions. For example, in higher plants from 2% to 20% of proteins can be replaced daily (Bouma *et al.*, 1994). Hence, the maintenance requirement of protein turnover is quite substantial, being its total cost from 3% to 5% of dry mass per day (Van der Werf *et al.*, 1992). Besides proteins, membranes and other macromolecules are also turned over, although their costs are lower or unknown (most macromolecules) (Steer, 1988; Matile *et al.*, 1999). Active ion transport to counteract membrane leaks, regulate pH or osmotic potential, are also an important maintenance process. Some estimates suggest that costs of maintaining ion gradients are up to 30% of the respiratory costs involved in ion uptake (Bouma & De Visser, 1993). Then, from experimental values compiled in the literature is inferred that protein turnover and ion gradients cause the largest costs of maintenance processes.

The costs of growth and maintenance respiration of plant organs are the major components of individual carbon balance and ecosystem production. Maintenance respiration is an essential process for plants; nevertheless, its costs could be reduced in favor of growth respiration obtaining an increase of crop production and WUE_{WP}. Cyanide-resistant alternative pathway (*AOP*) has been described as a constant process independent of the growth rate, and mostly associated with maintenance respiration (30-40% of total maintenance respiration) (Flórez-Sarasa *et al.*, 2007). The major characteristic of *AOP* is that it does not synthesize ATP, then this pathway consumes part of the carbon gain without obtaining any benefit for the plant and consequently reducing WUE_{WP}. However, several studies have suggested that *AOP* has an important role under stress situations by preventing over-reduction of the ubiquinone pool and the formation of reactive oxygen species (ROS) in mitochondria (Purvis & Shewfelt, 1993; Maxwell *et al*., 1999; Vanderberghe *et al*., 1995; Møller *et al*., 2001). Therefore, more studies are needed to determine the functions of *AOP* and whether it could

be a possible way to reduce the overall respiration and, consequently, improve WUE_{WP}.

1.4.3.2. Respiration in grapevines

Regarding respiration, most studies in grapevines are focused on leaf respiration (Escalona *et al.*, 1999b, 2003; Zufferey, 2000; Gómez-del-Campo *et al.*, 2004) although some studies about root respiration are also available (Comas *et al.*, 2000; Huang *et al.*, 2005; Franck *et al.*, 2011). Leaves and roots are the organs that present the highest construction costs in grapevines, and consequently, the highest respiration rates (Vivin *et al.*, 2003). For leaves, no significant differences have been observed between cultivars (Zufferey *et al.*, 2000), while no study about genetic variability in roots is available. Unlike photosynthesis, in general respiration in grapevines does not present large variations in response to changes of environmental conditions. Respiration rates are not as much affected by drought as photosynthesis, so that photosynthesis rates under stress conditions are low while respiration is maintained, thus, contributing to decrease WUE_l (Escalona *et al.*, 1999b, 2003). However, leaf and root respiration are enhanced in response to increases of leaf temperature (Zufferey *et al.*, 2000; Weyand *et al.*, 2006; Franck *et al.*, 2011).

Given that information about respiration in grapevines is limited and to this process could be a possible target to improve WUE_{WP}, further studies are needed to characterize respiration and the carbon balance in grapevines.

Chapter 2

OBJECTIVES AND OUTLINE

2.1. GENERAL OBJETIVES

As previously presented in the Introduction, drought is one of the major limitations to agriculture in general, and to grapevine growth and production in particular. The major viticulture regions are located in semi-arid areas with a seasonal drought that coincides with the grapevine growing season. This situation according to climate change predictions could be accentuated, and it would imply the need of irrigation. Therefore, to improve *WUE* in grapevines is necessary to make viticulture sustainable.

The general objectives of the present work were: **(1)** to analyze the mechanisms that could induce the variability of mesophyll conductance (g_m) and whether such variability can be linked to improvements of leaf *WUE* (A_N/g_s), **(2)** to establish links between leaf and whole plant *WUE*, **(3)** to determine what physiological processes could be a potential target to improve whole plant *WUE*.

These general aims are divided in several specific objectives

2.2. SPECIFIC OBJECTIVES

1. To study the genetic variability of g_m in grapevines under irrigation and water stress treatment.

2. To analyze the importance of different anatomical traits in the variability of g_m and leaf photosynthetic capacities between species with a wide range of foliage architectures, and between different cultivars of grapevine.

3. To understand the interrelation between changes of A_N/g_s (leaf *WUE*) and g_m.

4. To determine the genetic variability of WUE_{WP} in grapevines and whether leaf *WUE* estimates can be used as proxies for WUE_{WP}

5. To determine the importance of cuticular and night time transpiration for daily water losses and WUE_{WP} in grapevines.

6. To estimate the contribution of plant respiration (roots, leaves and stems) to whole plant carbon balance of grapevines.

2.3. OUTLINE OF THE THESIS

The Present Thesis is organised in 9 Chapters. Across these chapters, the physiological processes that could improve water use efficiency at leaf and whole plant level are presented. A study in different cultivars of grapevine (*Vitis vinifera* L.) grown under water stress treatments is detailed, which is preceded by a leaf anatomy study in different species characterised by contrasted leaf anatomical properties.

Chapter 1: INTRODUCTION

This Chapter introduces the background and sets the contexts for this Thesis. It includes a general overview of water use efficiency and the agronomical practices, genetic variability and physiological mechanisms at leaf and whole plant level that can be potential targets to improve water use efficiency.

Chapter 2: OBJECTIVES AND OUTLINE

In the present Chapter the general and specific objectives are presented as well as a brief outline of the Thesis. Finally, a list of publications derived from this Thesis is detailed.

Chapter 3: MATERIAL AND METHODS

This Chapter contains a detailed description of the plant material and methodology used, as well as a basic explanation of the parameters calculated.

Chapter 4: IMPROVEMENT OF WATER USE EFFICIENCY IN GRAPEVINES

This Chapter summarizes the general overview of *WUE* in grapevines at different levels (crop, whole plant and leaf) with special focus on physiological processes that can be considered to improve *WUE* at leaf and whole plant level.

Chapter 5: VARIABILITY OF MESOPHYLL CONDUCTANCE AND MECHANISTIC BASIS OF THESE VARIATIONS

This Chapter covers the variability of g_m as a potential tool to improve leaf *WUE*, and explores how such variability is related to key anatomical traits at the broad (between species, section 5.1) and narrow scales (between cultivars of grapevine, section 5.2 of genotypic variability).

Specific objectives 1, 2 and 3 are addressed in this chapter.

Chapter 6: WATER USE EFFICIENCY AT LEAF AND WHOLE PLANT LEVEL

In this Chapter genetic variability of WUE_{WP} between cultivars of grapevines is studied, as well as the correlation between leaf and whole plant *WUE*.

Specific objective 4

Chapter 7: IMPORTANCE OF PLANT RESPIRATION, CUTICULAR AND NIGHT TRANSPIRATION IN WHOLE PLANT WATER USE EFFICIENCY

This Chapter focuses on different physiological processes, cuticular and night transpiration (section 7.1) and plant respiration (section 7.2), that can affect whole plant carbon balance and consequently WUE_{WP}.

Specific objectives 5 and 6 are addressed in this chapter

Chapter 8: GENERAL DISCUSSION

This Chapter contains a general discussion and overview of all the results presented in Chapters 4, 5, 6 and 7.

Chapter 9: CONCLUSIONS

The last Chapter presents a list of the main conclusions derived from the present Thesis in relation to the general objectives described in Chapter 2.

2.4. PUBLICATIONS DERIVED FROM THE PRESENT THESIS

Chapter 4

Flexas J., Galmés J., Gallé A., Gulías J., Pou A., Ribas-Carbó M., **Tomás M**. & Medrano H. (2010) Improving water use efficiency in grapevines: potential physiological targets for biotechnological improvement. *Australian of Grape & Wine Research* 16, 106-121

Chapter 5

Section 5.1.

Tomás M., Flexas J., Copolovici L., Galmés J., Hallik L., Medrano H., Tosens T., Vislap V. & Niinemets Ü. (2012). Importance of leaf anatomy in determining mesophyll diffusion conductance to CO_2 across species: quantitative limitations and scaling up by models. *Plant, Cell & Environment* (submitted).

Section 5.2.

Tomás M., Medrano H., Martorell S., Pou A., Escalona J.M., Ribas-Carbó M. & Flexas J. (2012) Mesophyll conductance and leaf anatomy traits in different cultivars of grapevine (in preparation).

Chapter 6

Tomás M., Medrano H., Pou A., Escalona J.M., Martorell S., Ribas-Carbó M. and Flexas J. (2012) Water use efficiency in grapevine cultivars grown under controlled conditions: effects of water stress at the leaf and whole plant level. *Australian Journal of Grape & Wine Research* 18, 164-172

Chapter 7

Section 7.1.

Escalona JM., Fuentes S., **Tomás M**., Martorell S., Flexas J. & Medrano H. (2012) Responses of leaf night transpiration to water stress in *Vitis vinifera* L. *Annals of Botany* (submitted).

Section 7.2.

Escalona J.M.*, **Tomás M.***, Martorell S., Medrano H., Ribas-Carbó M. & Flexas J. (2012). Carbon balance in grapevines under different soil water supply: importance of whole plant respiration. *Australian Journal of Grape & Wine Research* (in press)

*These authors contributed equally to the present study.

Chapter 3

MATERIAL AND METHODS

3.1. PLANT MATERIAL

Experimentation of this Thesis is mainly focused on a single species (*Vitis vinifera* L.), except for the experiment of section 5.1 that consisted of a survey of 15 species with a wide range of leaf forms and ecological niches (Table 3.1).

Experiments described in chapters 5 to 7 were performed in eight different cultivars of *Vitis vinifera* L. Cabernet Sauvignon, Grenache, Pinot Noir, Tempranillo (cultivars of widespread use), Callet, Malvasia of Banyalbufar, Manto Negro, Escursac (Balearic cultivars) and one hybrid rootstock of *Vitis*, Richter-110 (*Vitis berlandieri x Vitis rupestris*). (Fig. 3.1).

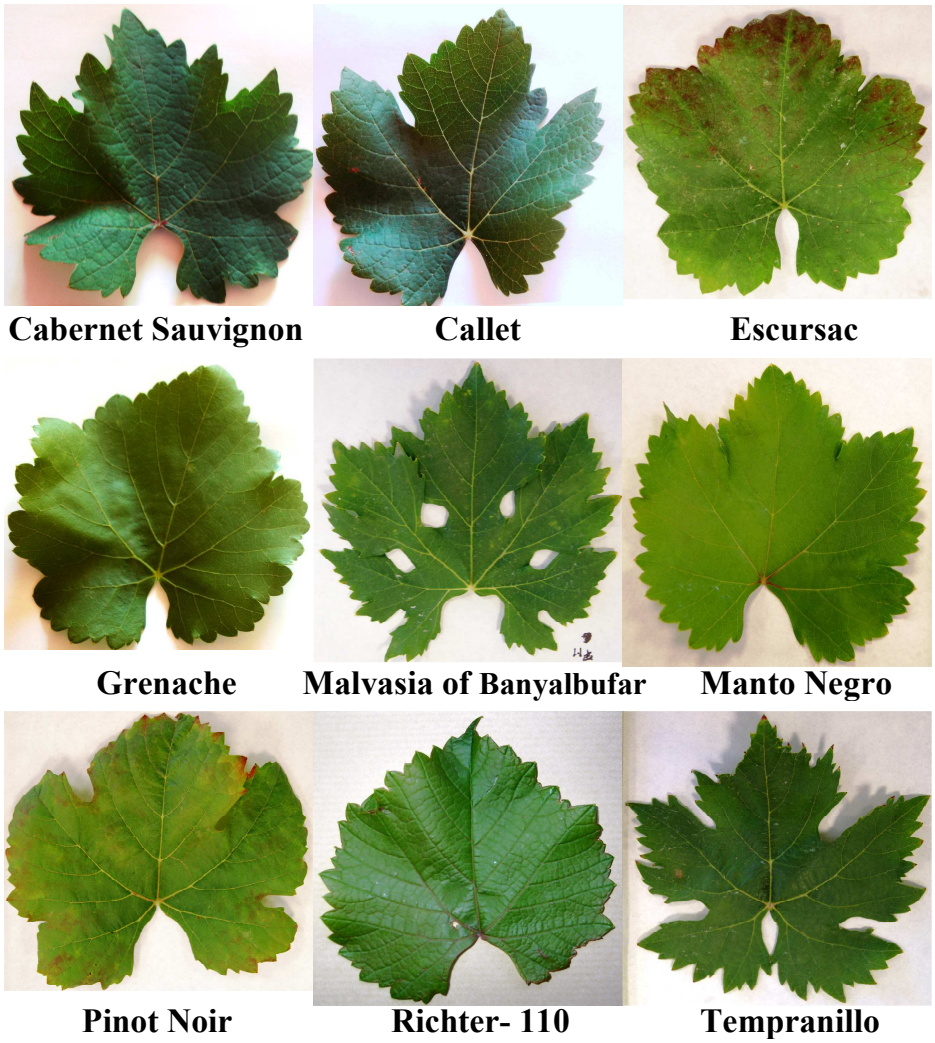

Figure 3.1. Grapevine cultivars used in this Thesis

Table 3.1. Species selected in the experiment of section 5.1.

Species	Family	Life form	Species origin (climate)
Capsicum annuum cv. *Super Chilli F1*	Solanaceae	Herb	Central America (tropics)
Helianthus annuus cv. *Simple Giant*	Asteraceae	Herb	Central America (subtropics to tropics)
Ocimum basilicum	Lamiaceae	Herb	South Asia (tropics to subtropics)
Phaseolus vulgaris cv. *Neckargold*	Fabaceae	Herb	Central to South America (subtropics to tropics)
Spinacea oleracea cv. *Matador*	Amaranthaceae	Herb	Central and Southwestern Asia (temperate to subtropics)
Acer negundo	Sapindaceae	Woody deciduous	North America (temperate)
Alnus subcordata	Betulaceae	Woody deciduous	West Asia and the Caucasus (warm temperate)
Betula pubescens	Betulaceae	Woody deciduous	Europe and Asia (temperate)
Catalpa speciosa	Bignoniaceae	Woody deciduous	North America (warm temperate)
Quercus brantii	Fagaceae	Woody semi-deciduous	West and South Asia (warm temperate)
Citrus reticulata	Rutaceae	Woody evergreen	South-East Asia (subtropics to tropics)
Ficus elastica	Moraceae	Woody evergreen	South Asia and South-East Asia (subtropics to tropics)
Pittosporum tobira	Pittosporaceae	Woody evergreen	East Asia (warm temperate)
Quercus ilex	Fagaceae	Woody evergreen	Europe (Mediterranean)
Washingtonia filifera	Arecaceae	Woody evergreen	North America (warm temperate)

3.2. GROWTH CONDITIONS

In the experiment from section 5.1, species were grown from seeds, either commercial or collected in the field (for more details see material and methods in section 5.1), except for *Ficus elastica* that was obtained from rooted cuttings. Plants were grown in pots of different sizes (1-5 L) depending on plant age and size, in a growth chamber at the Department of Plant Physiology (Estonian University of Life Sciences). Photoperiod was set to 10/14h with a constant photon flux density of 350 μmol m^{-2} s^{-1} at plant level provided by Philips HPI-T Plus 400 W metal halide lamps, corresponding to 30% of average daily light in completely open location during the growing season. Temperatures during the light and dark period were 24ºC and 18ºC, respectively. Air relative humidity was maintained above 60%. Growth substrate was a 1:1 mix of quartz sand and standard potting soil (Biolan Oy, Finland) including slow release NPK (3-1-2 ratio) fertilizer with microelements. Plants were irrigated daily to field capacity. Herbs were used 1 month after seed germination, while woody species were selected on the second growing year.

Plants of *Vitis vinifera* L. were obtained by direct rooting from 0.3 m shoots collected from an experimental farm near Palma (Conselleria d'Agricultura, Mediambient i Territori), except for the experiment of section 7.1. Cuttings were hydrated for 24 hours and soaked in 0.3% Captan solution for 3 hours. Rooting was induced in cuttings with indolbutyric acid (IBA, 2g L^{-1}) in a growth chamber under controlled conditions. Soil temperature was 26-28ºC, air temperature was 23ºC and air humidity about 80%. When cuttings presented 4-5 expanded leaves, they were transplanted into pots and grown outdoors in 15 L pots filled with organic substrate and perlite mixture (3:1). They were irrigated daily from May until the start of the experiment supplemented with organic-mineral fertilizer NPK containing (%): N, 5; P_2O_5, 8; K_2O, 15; MgO, 2; organic C, 17.4, humic acid, 5; SO_3, 15; Fe, 1; Zn $2x10^{-3}$; Mn $1x10^{-2}$. A thick layer of perlite was added to the surface of each pot to decrease soil evaporation. All plants were maintained with one or two shoots and without fruits to compare only the genetic variability of the physiological parameters. In the experiment of the section 7.1 ten-years-old grapevine plants (cv.Tempranillo) grafted in 110-Richter

rootstock were used. They were grown in 70 L containers filled with a mix of sand, horticultural substrate and perlite (1:1:1).

Experiments with *Vitis vinifera* L. were conducted outdoors. Climate conditions during the experiments were typical for Mediterranean regions with mean maximum and minimum temperatures of 32ºC and 17ºC respectively, integrated evapotranspiration (ET_0) between 5.3 and 5.6 L m^{-2} day^{-1}, and daily mean vapour pressure deficit (VPD) above 1 KPa.

3.3. WATER STRESS TREATMENTS

Plants of *Vitis vinifera* L. and the rootstock of *Vitis*, Richter-110, described in the experiments of chapters 5 to 7 were subjected to water withholding until leaf maximum daily g_s dropped to 0.085-0.1 mol H_2O m^{-2} s^{-1}, considered as moderate drought (Flexas *et al*. 2002). Once these values were achieved (typically 5-7 days after withholding water) pots were weighted daily in the evening and the amount of water consumed was replenished to maintain the same level of drought for three weeks. Control plants were maintained at field capacity throughout the experiments.

In the experiment of the section 7.1, two levels of water stress were imposed. Plants were subjected to water withholding during 4 and 7 days to achieve moderate and severe water stress, respectively.

3.4. MEASUREMENTS

3.4.1. Plant and soil water status

3.4.1.1. Leaf water potential

Predawn (Ψ_{PD}) and midday leaf water potential (Ψ_{MD}) were measured in fully expanded leaves with a Scholander pressure chamber (Soilmoisture Equipment Corp., Santa Barbara, CA). In this technique, a leaf is excised from the plant breaking the water column of the xylem under tension. The excision causes a negative pressure in cells that pulls water towards the cells from the xylem. A leaf is sealed in the chamber and an external pressure is added into the chamber until water between surrounding living cells and the xylem returns to its initial distribution. When the external pressure is raised to balance the tension, the xylem

solution appears at the cut surface and remains steady without flow in or out of the leaf.

3.4.1.3. Soil water content

Soil water content (*SWC*) was calculated as:

$$SWC\,(\%) = \frac{(\text{Pot weight - Minimum pot weight})}{(\text{Maximum pot weight - Minimum pot weight})} \cdot 100 \qquad [1]$$

Minimum pot weight was considered at the wilting point. To determine wilting point two pots were left without irrigation until constant weight value was achieved. Maximum pot weight was obtained weighting the pots at field capacity.

3.4.2. Chlorophyll fluorescence measurements

Chlorophyll fluorescence measurements were made with fluorescence chamber head (Li-6400–40 leaf chamber fluorometer; Li-Cor, Inc. or PAM-Fluorometer 3055-FL) integrated in an open gas exchange system (Li-6400; Li-Cor, Inc., Nebraska, USA or GFS-3000, Walz, Effeltrich, Germany). The fluorescence chambers are composed of three different light sources: modulated red and blue actinic and saturating flash lights, and infrared light to excite PSI. The light-emitting diode (LED) generates a pulsed light to induce a pulsed fluorescence in the leaf that is electronically identified and amplified.

Quantum of light is absorbed by a molecule of chlorophyll. The excitation energy of the quantum is transferred via excitons to reaction centers of photosystems II (PSII) and I (PSI), raising them to an excited state. Electrons return rapidly to their ground level, releasing the absorbed energy in one of these three ways (Fig. 3.2): (a) converted into chemical energy to drive photosynthesis (photochemistry), (b) emitted as heat (known as non-photochemical quenching) or re-emitted as light (chlorophyll fluorescence). These three processes occur in competition, such that any increased efficiency of one results in a decrease in the yield of the other two.

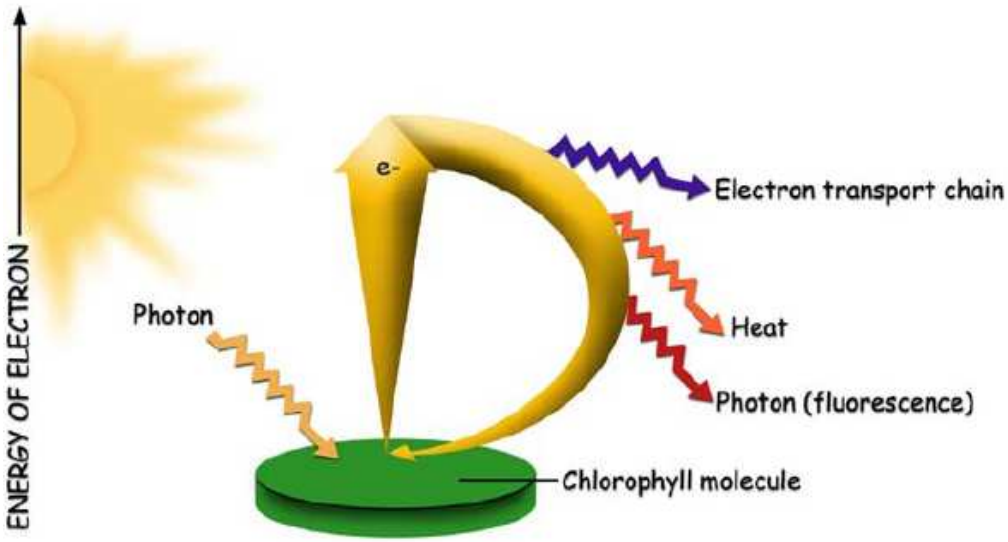

Figure 3.2. Outline representation of the three possible processes to dissipate the absorbed energy (from Li-6400XT v6.1 manual)

3.4.2.1. The Fluorescence parameters

Chlorophyll fluorescence parameters have been established as non-invasive indicators of the functionality of the photosynthetic apparatus. Most of these parameters are determined by the light-induced chlorophyll fluorescence induction kinetics of dark-adapted state, known as Kautsky effect (Kautsky *et al.* 1960).

1) Dark adapted state

$\boldsymbol{F_0}$: It is the minimal yield of fluorescence determined in darkness by a weak measuring beam (< 0.1 μmol photons m^{-2} s^{-1}) where all reaction centres are opened. In this level all molecules of quinone A (Q_A, first electron acceptor of the photosynthetic pathway) are oxidized (Fig. 3.3).

$\boldsymbol{F_m}$: It is the maximum fluorescence yield obtained after applying a saturating flash from the ground state (F_0) (Fig. 3.3). All reaction centres will close and all molecules of Q_A will be reduced.

$\boldsymbol{F_v/F_m}$: It is the intrinsic efficiency of the PSII (quantum efficiency if all PSII centres were opened). This parameter is given by the equation:

$$F_v/F_m = \frac{F_m - F_0}{F_m} \qquad [2]$$

This parameter is used as an indicator of plant photosynthetic performance, with optimal values around 0.83 (Björkman & Demmig 1987, Johnson *et al.* 1993). Lower values indicate photoinhibition, so that plants have been exposed to stressful conditions.

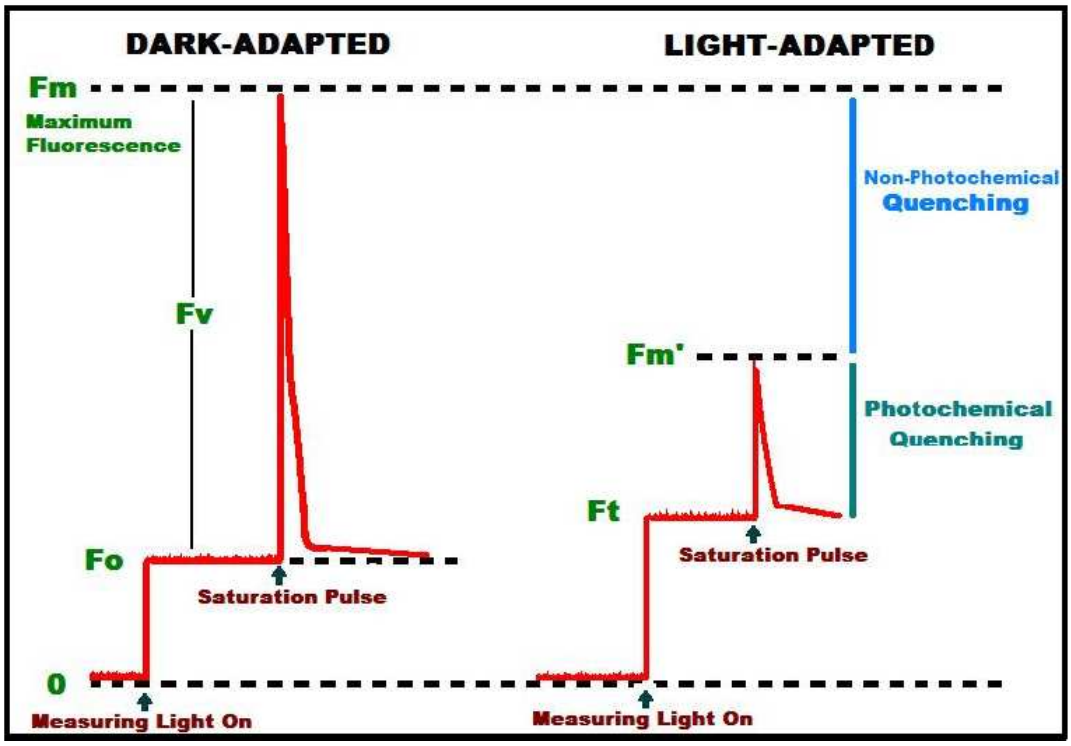

Figure 3.3. Sequence of a typical fluorescence trace. (From Gallé & Flexas 2010)

2) Illuminated state

$\boldsymbol{F_t}$: It is the steady-state yield of fluorescence in the light adapted state (Fig. 3.3).

$\boldsymbol{F_m}$**'**: It is the maximum fluorescence in the light (Fig. 3.3). A decrease of $F_{m'}$ as compared to F_m indicates the presence of non-photochemical quenching (NPQ, energy is converted to heat).

$\Delta \boldsymbol{F/F_m}$**' (**$\boldsymbol{\Phi_{PSII}}$**)**: It is the efficiency of the PSII photochemistry, calculated after Genty *et al.* (1989) as:

$$\varphi_{PSII} = \frac{F_m' - F_t}{F_m'} \qquad [3]$$

It measures the proportion of the light absorbed by chlorophyll associated with PSII that is used in photochemistry.

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:

$$J_{flu}\,(\mu mol\ m^{-2}\ s^{-1}) = \varphi_{PSII} \cdot PPFD \cdot \alpha \cdot \beta \qquad [4]$$

where PPFD is the photosynthetically active quantum flux density, α is the leaf absorbance and β reflects the partitioning of absorbed quanta between photosystems II and I. In the experiment belonging to section 5.1 leaf absorptance was calculated with spectrometer (AvaSpec-2048-2, Avantes, Apeldoorn, The Netherlands) coupled to an integrating sphere (ISP-80-8-R, Ocean Optics, Dunedin, FL, USA). β was assumed 0.5 (Laisk & Loreto 1996). In all other experiments, the product α · β was determined as the slope of the relationship between Φ_{PSII} and Φ_{CO2} obtained by varying light intensity under non-photorespiratory conditions in an atmosphere containing less than 1% of CO_2 (Valentini *et al* . 1995).

NPQ: It is an indicative of the level of non-radiative energy dissipation in the light-harvesting antenna of PSII. It is calculated as:

$$NPQ = \left(\frac{F_m}{F_m'}\right) - 1 \qquad [5]$$

The importance of this parameter results from the fact that it measures changes in heat dissipation relative to the dark adapted state. This is thought to be essential protecting the leaf from over-energizing of the thylakoid membranes (photodamage).

3.4.3. Gas exchange measurements

3.4.3.1. IRGA overview

Leaf gas exchange measurements were performed with an open circuit infrared gas analyzer (IRGA), Li-6400 or GFS-3000, depending on the experiment (see specific Material and Methods section in each chapter) (Fig. 3.4). The principles and parameters measured explained in this section are similar for both instruments used.

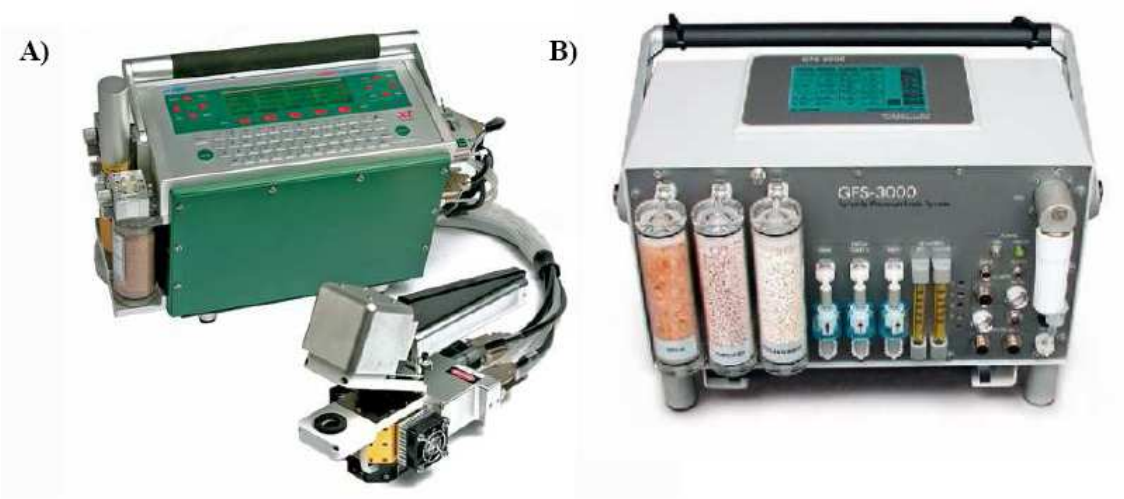

Figure 3.4. Li-6400 (A)and GFS-3000 (B) Portable Photosyntesis Systems

The system is based on the differences in CO_2 and H_2O in an air stream that is flowing through a cuvette with photosynthetic tissue. These differences are measured by two independent infra-red gas analyzers. 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}. Similarly, increases in water content of the same air stream, can be used to determine the transpiration rate (E) and express it in mmol H_2O m^{-2} s^{-1}.

$$A_N = \frac{\text{Flow} \cdot \Delta CO_2}{\text{Area}} \qquad [6]$$

$$E = \frac{\text{Flow} \cdot \Delta H_2O}{\text{Area}} \qquad [7]$$

Leaf temperature is measured with a thermocouple in contact with the bottom of the leaf. Another thermocouple within the cuvette determines the air temperature. From previous measurements, stomatal conductance for water vapour (g_s) can be deducted. Implementing leaf temperature and air pressure, leaf water vapour total conductance is calculated. The stomatal conductance is obtained from the total conductance by removing the contribution from the boundary layer conductance, which is dependent on whether the leaf has stomata on one or both sides of the leaf.

The internal or sub-stomatal conductance 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.

$$C_i = C_a - \left(\frac{A_N}{g_s} \right) \quad [8]$$

where C_a is the CO_2 concentration around the leaf.

This system can control the environmental conditions in the leaf chamber, such as the CO_2 concentration, humidity and light intensity and quality. Both instruments (Li-6400 and GFS -300) are provided with several types of leaf chamber, one of them have incorporated the LED Light Source, which permits to regulate the incident radiant flux density on the leaf, ranged from 0 to 2000 or 2500 μmol photon m^{-2} s^{-1} (GFS-3000 and Li-6400, respectively). Humidity is controlled by two mechanisms, manually by adjusting the incoming air in the leaf chamber which is routed through the drier or humidifier, and controlling the flow rate of air through the chamber. CO_2 Mixer allows control of the CO_2 concentration, which is the basis of the photosynthetic CO_2 response curves (Flexas *et al.* 2007c) (Fig. 3.5).

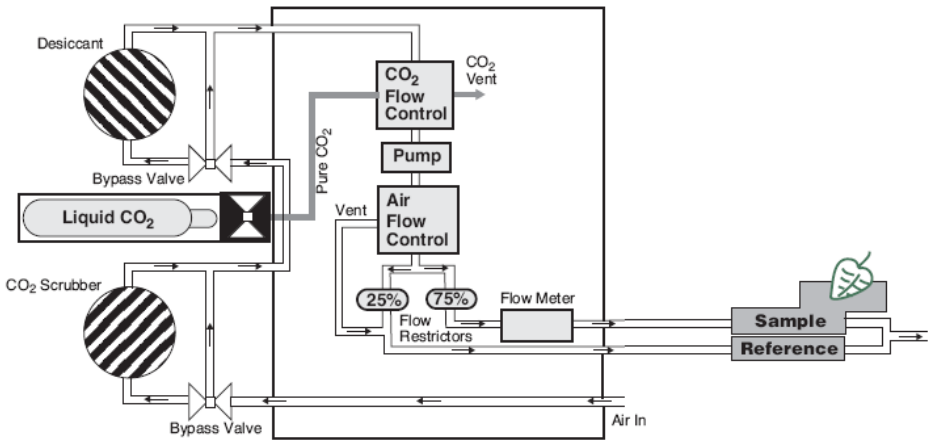

Figure 3.5. Li-6400 flow schematic (from manual Li-6400XT v6.1)

3.4.3.2. Leaf gas exchange measurements

3.4.3.2.1. Instantaneous gas exchange measurements

Instantaneous gas exchange measurements were made on fully expanded apical leaves. Measurements of A_N, g_s, E and C_i were performed at saturating red light (1500 μmol m^{-2} s^{-1}) achieved with the red LED lamp of the system, with an additional 10% blue light to maximize stomatal opening. CO_2 concentration in the leaf chamber (C_a) was set at 400 μmol CO_2 mol $^{-1}$air in the cuvette, and the relative humidity of the incoming air ranged between 40 and 60%. Block temperature was maintained at 30ºC, except the experiment of section 5.1 that was maintained at 25ºC, while water vapour pressure deficit (VPD) was not controlled.

3.4.3.2.2. A_N-C_i curves

Rapid variations of CO_2 concentrations around the leaf (A_N-C_i curves) are used to determine photosynthesis limitations (Long & Bernachi, 2003; Sharkey *et al.,* 2007). These curves can be divided in three sections. At low C_i concentrations, A_N response is linear and it refers to (maximal) Rubisco activity. The second section of the A_N-C_i curves responds to the (maximal) electron transport rate (RuBP-regeneration) while the last section A_N response is constant or decrease with an additional increase in C_i, being limited by the utilization of triose-phosphate (TPU).

CO_2 response curves were started at a CO_2 concentration (C_a) of 400 μmol CO_2 mol $^{-1}$air. After recording the steady-state A_N value under saturating light, C_a was lowered stepwise to 50 μmol mol $^{-1}$, providing at least 2 minutes at each step for stabilization. Then, C_a was raised again to 400 μmol mol $^{-1}$ until reaching steady-state value similar to that obtained at the beginning of the curve. In some cases, C_a was increased stepwise from 400 to 1500 μmol mol $^{-1}$ (Fig. 3.6).

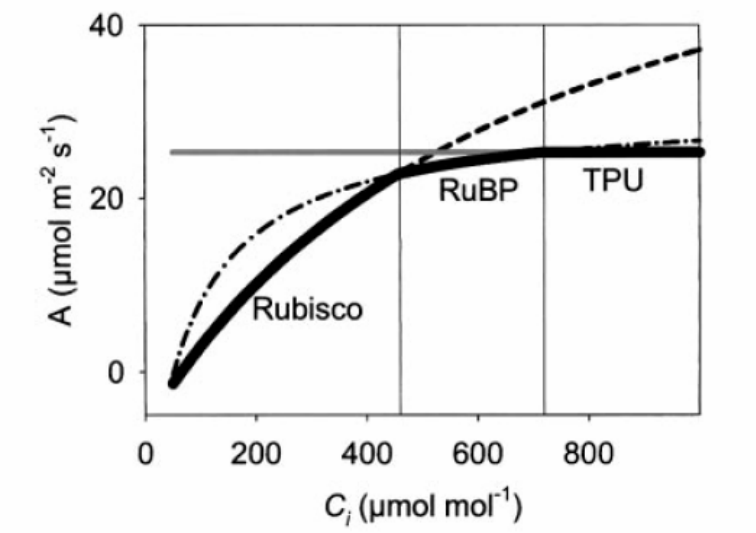

Figure 3.6. Idealized A_N/C_i response. The rates of photosynthesis that would be achieved depending on whether Rubisco, RuBP or TPU are limiting are indicated. The actual photosynthetic rate (solid line) at any given Ci is the minimum of these three potential limitations. Parameters used: $V_{c,max}$=70 µmol m^{-2} s^{-1}, J_{max}=130 µmol m^{-2} s^{-1}, V_{TPU}=9.1 µmol m^{-2} s^{-1}, , Rd=2 µmol m^{-2} s^{-1}, (From Long & Bernacchi, 2003)

3.4.3.2.3. Respiration in the light and apparent CO_2 photocompensation point

Light respiration (R_L) and apparent CO_2 photocompensation point (C_i*) were calculated according to Laisk method (1977) as described in von Caemmerer (2000) using a Li-6400 with the chamber of 6 cm^2. The Laisk method analyzes the rate of net CO_2 exchange at low internal CO_2 concentrations (C_i) at varying irradiances. This rate is mathematically expressed as:

$$A_N = v_c - 0.5v_o - R_L \qquad [9]$$

where v_c and v_o are the rate of carboxylation and oxygenation, respectively. C_i* is the CO_2 concentration at which the photorespiratory efflux of CO_2 equals the rate of photosynthetic CO_2 uptake (i.e. when $v_c = 0.5\ v_o$).

Laisk curves were obtained from A_N-C_i curves ranged from 400 to 50 µmol CO_2 mol^{-1} air, under three different light intensities (i.e. 50, 150 and 300 µmol m^{-2} s^{-1}). The intersection point of the three A_N-C_i curves were used to determine C_i* (x-axis) and R_L (y-axis). C_i* was used as a proxy for the chloroplastic CO_2 photocompensation point (Γ*) according to Warren and Dreyer (2006). The values of C_i* only were calculated for irrigated plants, because has been demonstrated that the specificity of Rubisco does not change under water stress treatment (Galmés *et al.*, 2006).

In the experiment of section 5.1 instead of measuring C_i* was used the Rubisco specificity factor (τ) obtained from the bibliography (for more details see the specific material and methods). Rubisco specificity factor is derived from Rubisco kinetics as:

$$\tau = \frac{V_{c,\max} \cdot K_o}{V_{o,\max} \cdot K_c} \quad [10]$$

where $V_{c,max}$ and $V_{o,max}$ are the maximal rates for carboxylation and oxygenation activities of Rubisco, and K_o and K_c are the Michaelis constants for these reactions (Laing *et al.*, 1974).

3.4.3.2.4. Cuticular conductance

Cuticular properties affect gas exchange in leaves. Water conductance (g_{cut}) of leaf cuticle is higher than CO_2, causing less photosynthesis per unit of water used (Boyer *et al.*, 1997). The effect of this discrimination is especially important when stomata are completely closed, that is, when g_{cut} is the main determinant of leaf CO_2 uptake and transpiration. Measurements of g_s with an IRGA system are the sum of stomatal and cuticular conductance. To determine the importance of g_{cut} in whole plant carbon and water balance, two different methods were used in the experiment of section 7.1 to measure g_{cut}. In the first method, g_{cut} was measured with the IRGA in leaves with the abaxial surface covered with silicone grease and a polyethylene filter to prevent stomatal gas exchange (Boyer *et al.*, 1997). In the second method, g_s was continuously measured on a detached leaf until the decline of g_s flattens out and its values become stable. This end point represents the remaining conductance (g_s) after stomata were completely closed (Burghardt & Riederer, 2003).

3.4.3.2.5. Leaks

In gas exchange systems, chambers usually enclose a small leaf surface (2-8 cm^2) surrounded by gaskets. The illuminated photosynthesizing leaf area is always surrounded by an area darkened by gaskets, which respires and thus affects the measurement of the photosynthetic flux, especially at the two ends of A_N-C_i curves when large CO_2 gradients between the chamber and the surrounding air are present (Fig. 3.7).

In an attempt to correct for gradient between inside and outside CO_2 concentrations and the inward diffusion of respiratory CO_2 from darkened leaf

material under the gasket, leakage corrections were performed in all the experiments of this Thesis. To avoid large changes in leaf structure, leaves were thermally killed by immersion in boiling water until no variable chlorophyll fluorescence was detectable which was taken as evidence for total photosynthesis impairment and leaf death (Schreiber *et al.*, 1998). A_N and C_i values of living leaves were corrected by simple subtraction of the relationship between C_a and the 'apparent' photosynthesis of a chamber filled with a dead leaf (Flexas *et al.*, 2007c).

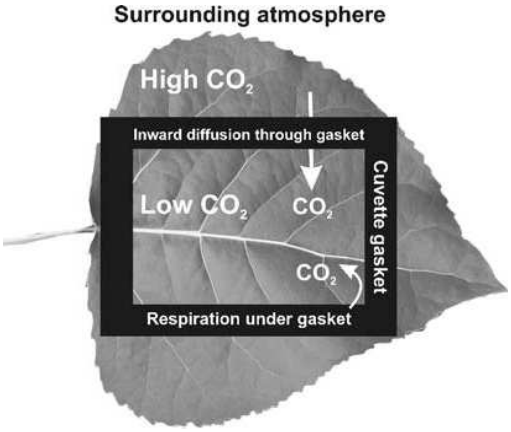

Figure 3.7. Diagram illustration of how CO_2 diffuses from the surrounding atmosphere into the leaf chamber, and how CO_2 released from respiration by leaf under the gasket can diffuse inward to the cuvette (Hurry V. *et al.*, 2005).

3.4.3.3. Respiration measurements

Respiration measurements were performed in three different ways depending on the tissue analyzed:

- Leaves: Li-6400 or GFS 3000 using the leaf chamber
- Roots: Li-6400 using the soil chamber
- Stems: oxygen electrode

3.4.3.3.1. Leaf respiration

Leaf dark respiration (R_d) was measured using Li-6400 or GFS-3000 depending on the experiment (for more details see material and methods of each section) with a chamber of 6 or 8 cm^2 (for Li-6400 and GFS-3000, respectively). Measurements were performed at 25 or 30ºC, C_a of 400 µmol CO_2 mol^{-1}air and

200-150 μmol s^{-1} flow rate after maintaining plants for at least 2 hours in darkness.

In the experiment of section 7.2, leaf respiration was measured in a controlled room temperature at 20, 25, 30 and 35ºC, after 8-10 h of dark period. Furthermore, in order to complete the plant carbon balances and to measure night transpiration (experiments 7.1 and 7.2), nocturnal night courses were made measuring at 2 hour intervals from 20:00 to 5:00h (solar time). The conditions of measurement were similar to those previously described.

3.4.3.3.2. Root respiration

Root respiration (R_{root}) was measured using the soil chamber (6400-09) attached to the Li-6400 sensor head.

The predominant processes that produce carbon dioxide in soil are respiration of roots, soil organisms and the decomposition of organic matter. The production of CO_2 in the soil is strongly correlated with soil temperature and moisture. (Norman *et al*., 1992). Therefore, soil temperature and moisture data can be useful when interpreting soil CO_2 flux measurements. Although the primary mechanism for transport of CO_2 from the soil to the atmosphere is diffusion, transport may also be influenced by fluctuations in pressure, wind, temperature, and displacement by precipitation.

The system is based on the increase of CO_2 concentration inside the chamber, which has been deployed on the soil surface for a short period of time (measured soil area is 71.6 cm^2). The air is circulated from the soil to the top of the chamber by the mixing fan placed in the sensor head. Then, the air in the chamber is withdrawn through the analyzer inlet duct and enters the optical path of the gas analyzer. Air is returned from the gas analyzer outlet ducts to the manifold near the soil surface (Fig. 3.8). Air temperature is measured with the thermocouple within the soil chamber while soil temperature is determined with a probe connected to the Li-6400 console. Soil CO_2 flux measurements were made using a soil collar inserted 3 to 4 cm into the soil as an interface between the chamber and the soil to minimize environmental fluctuations.

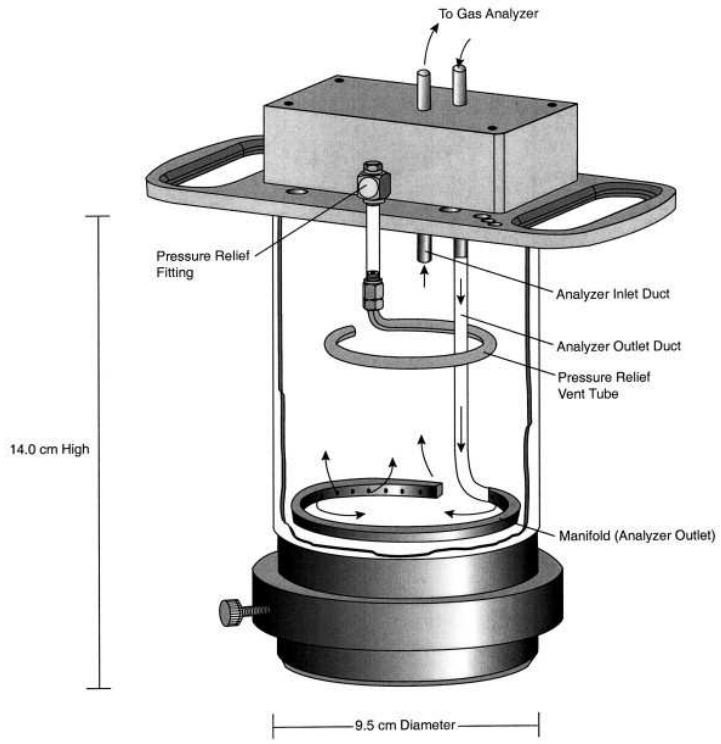

Figure 3.8. Schematic diagram of the soil chamber used in the Li-6400 (Welles *et al.*, 2001)

Measurements were made at different substrate temperatures of 20, 25, 30 and 35ºC. All measurements were performed outdoors, considering natural variations of soil temperature along the day, except at 20ºC in which case pots were left overnight in a room under controlled temperature. Irrigated and non-irrigated pots containing the same substrate than the other experimental pots but without plant were measured at each temperature to measure respiration by soil microorganisms. Finally, R_{root} was estimated as the difference between CO_2 efflux in pots containing and not-containing plants under both water conditions (Koerber *et al.*, 2010).

3.4.3.3.3. Stem respiration

Stem respiration (R_{stem}) was measured using a liquid-phase Clark-type oxygen electrode (Dual Digital Mod 20; Rank Brothers Ltd, England) at 25, 30 and 35ºC controlled with a water bath connected to the incubation chamber of the cuvette. Apical and intermediate stem sections were collected during the light period and incubated in a solution containing 30mM MES buffer and 0.2 mM CaCl2 at pH 6.2, for 30 minutes in the dark. Stem samples were placed in the closed electrode cuvette and depletion of the O_2 concentration in the rapidly stirred solution of the closed cuvette was linear with time.

The oxygen electrode allows the measurement of oxygen evolution, uptake and liberation in a closed system. The oxygen electrode works based on a polarographic measurement of the electricity that flows between an anode and a cathode. This electrode consists on a cell which contains platinum wire cathode and a silver ring wire anode, linked by an electrolyte (potassium chloride solution). On top of the electrode, a gas-permeable membrane (teflon or polyethylene) is placed, sealed with a silicone rubber 'O' ring that surrounds the electrodes (Fig. 3.9). The reaction mixture in the appropriate container is stirred constantly with a small magnetic stirring rod. When a small voltage is applied across the electrodes (-0.6V), the platinum electrode is negatively charged with respect to the silver electrode. Oxygen diffuses through the membrane and is reduced at the platinum surface. The electrical current generated is directly proportional to the amount of oxygen that is reduced. The amount of oxygen that is reduced depends on the diffusion through membrane, which is the limiting step in the reaction. Any variation in the oxygen concentration due to gas-exchange of the tissue analyzed is reflected in the electrolyte (potassium chloride solution). As a consequence of this, the electric current between cathode and anode also changes.

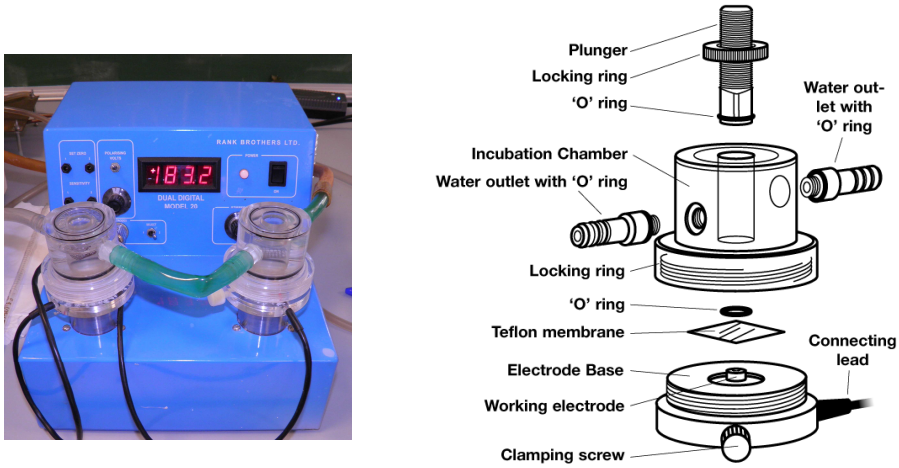

Figure 3.9. Photograph (left) and diagram (right) of the different components of the liquid-phase Clark-type oxygen electrode Dual Digital Model 20 (Rank Brothers LTD, Cambridge, England).

3.4.4. On-line measurements of stable carbon isotope discrimination

On-line measurements of stable carbon isotope discrimination were made with a Cavity Ring-Down Spectrometer (CRDS) (Isotopic CO_2 G1101-i analyzer, Picarro, Inc., Santa Clara, California, USA) connected to the leaf chamber of the GFS 3000 gas-exchange system.

The technique is based on absorption measurements using a near-infrared laser. Gas sample is circulated in an optical measurement cavity (ring down cavity) formed by two plane-concave mirrors. The mirrors are coated for an optimum reflectivity in the visible and near UV range of the spectrum reflectivity. Then, the optical absorbance of the sample is determined providing the concentration or isotopic ratio measurements of CO_2.

The instrument includes an inlet valve, pump, and pressure sensor to automatically and continuously capture the gas sample. Diode laser emits a directed beam of light energy through one partially reflecting mirror placed into the cavity. The light intensity inside the cavity then builds up over time and is monitored through a second partially reflecting mirror using a photo-detector located outside the cavity. The "ring-down" measurement is made by rapidly turning off the laser and measuring the light intensity in the cavity as it decays exponentially with a time constant that depends on the losses due to the cavity mirrors and the absorption and scattering of the sample being measured (Fig.3.10). The instrument have a precision of $\delta^{13}CO_2$ 0.3 ‰ in five minutes and a response time of 60 seconds.

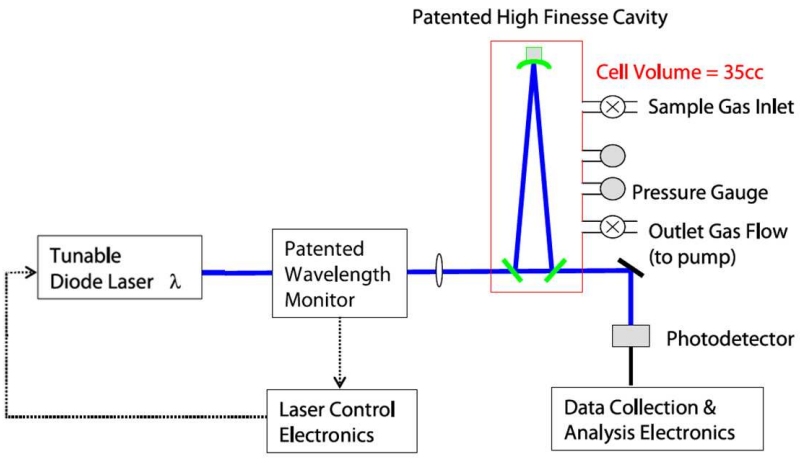

Figure 3.10. Block diagram of the Cavity Ring-Down Spectrometer (Sumner *et al.* 2011).

In our experiment (section 5.1), after recording the steady-state A_N values at each C_a, $^{12}CO_2$ and $^{13}CO_2$ concentrations entering and leaving the leaf chamber of the GFS-3000 were measured by the Spectrometer connected in a bypass loop. The values of $\delta^{13}C$ were corrected for water vapor (White paper for water vapor correction for the G2101-i Isotopic CO_2 Analyzer, C. Rella, www.piccaro.com) and methane (Isotopic carbon in CO_2 methane detection flag, K. Cunningham, www.piccaro.com) according to manufacturer's recommendations.

3.4.5. Water use efficiency

3.4.5.1. Intrinsic (A_N/g_s) and instantaneous (A_N/E) water use efficiency

At leaf level, instantaneous measurements of net assimilation of CO_2 (A_N), transpiration rate (E) and stomatal conductance (g_s) obtained by the open infrared gas-exchange system (Li-6400; Li-Cor, Inc., Lincoln, NE, USA) allowed to determine intrinsic and instantaneous water use efficiency from the ratio between A_N/g_s and A_N/E, respectively.

3.4.5.2. Carbon isotope composition in leaf dry matter

Two stable isotopes of CO_2 exist and are naturally abundant: $^{13}CO_2$ and $^{12}CO_2$. The $^{13}CO_2$ isotope has a heavier molecular mass than $^{12}CO_2$. Due to its molecular properties, $^{13}CO_2$ is discriminated against along its pathway of diffusion from the atmosphere up to the site of fixation. Moreover, carboxylating enzymes (Rubisco and PEP carboxylase) discriminate against $^{13}CO_2$ if the concentration of CO_2 is high. The concentration of CO_2 inside the leaf (C_i) depends on the rate of photosynthesis and the opening of the stomatal pores, which influences isotopic discrimination (Eq 8).

Young leaves developed after the onset of the treatments were sampled for carbon-isotope analysis. They were dried for 48h in an oven at 60ºC and ground into powder. Subsamples of 2 mg were analyzed for isotope ratio ($\delta^{13}C$). Samples were combusted in an elemental analyzer (Thermo Flash EA 1112 Series, Bremen, Germany), CO_2 was separated by chromatography and directly injected into a continuous-flow isotope ratio mass spectrometer (Thermo Finnigan Delta XP, Bremen, Germany). Peach leaf standards (NIST 1547) were run every eight

samples. $\delta^{13}C$ values were referred to a Pee Dee Belemnite standard, and was calculated according to Farquhar & Richards 1984:

$$\delta^{13}C(‰) = \left(\left(\frac{R_{sample}}{R_{standard}}\right) - 1\right) \cdot 1000 \qquad [11]$$

3.4.5.3. Whole plant water use efficiency

Plants were harvested at the beginning and at the end of the experiments to determine the whole plant biomass. Leaves, stems and roots were separated and dried in an oven at 60ºC to obtain dry weight. Total biomass increment during the experiment was estimated as the difference between whole plant dry weight at the beginning and at the end of the experiment. Plant water consumed over the experimental period was estimated from the sum of the daily water consumption calculated as described in section 3.3.

Whole plant water use efficiency was determined as follows:

$$WUE_{WP}\ (g\ L^{-1}) = \frac{(\text{dry weight final biomass - dry weight initial biomass})}{\text{total water consumed}} \qquad [12]$$

3.4.6. Leaf anatomy

Pieces of 1x1 mm were cut between the main veins of leaves. Leaf material was quickly fixed under vacuum with 4% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2). After primary fixation, the tissue was washed three times in the same buffer used in the previous step. Afterwards, samples were fixed in 1% osmium tetroxide between 1-2 hours and dehydrated in a graded ethanol series (from 30% to absolute ethanol). Then, ethanol was replaced by propylene oxide, an intermediary solvent highly miscible with the embedding media and washed three times. The solvent propylene oxide, was mixed with Spurr's resin (Monocomp Instrumentación, Madrid, Spain) and placed into vials with the dehydrated segments. Gradually, the resin-solvent ratio was increased until pure Spurr's resin was used. The pure resin specimens were transferred into molds and cured in an oven at 60 ºC during 48 h. The semi-thin

(0.8 μm) and ultra-thin (90 nm) cross sections were cut with an ultramicrotome (Reichert & Jung model Ultracut E).

3.4.6.1. Light microscopy

Semi-thin cross sections (0.8 μm) were stained with 1% toluidine blue and viewed under Olympus light microscope BX60. Pictures were taken at 200x and 500x magnifications with a digital camera (U-TVO.5XC, Olympus) to measure whole leaf thickness and the thickness of epidermal layers, palisade and spongy mesophyll. The volume fraction of intercellular air spaces was calculated as:

$$f_{\text{ias}} = 1 - \frac{\sum S_{\text{s}}}{t_{\text{mes}} w} \qquad [13]$$

where ΣS_s is the total cross sectional area of mesophyll cells, w is the width of the section and t_{mes} is the mesophyll thickness between the two epidermises.

All images were analyzed with Image analysis software (Image J; Wayne Rasband/NIH, Bethesda, MD, USA) in 4-6 different fields of view, making ten measurements for spongy tissue and ten for palisade parenchyma cells for each anatomical trait.

3.4.6.2. Electron microscopy

Ultra-thin cross sections (90 nm) for transmission electron microscopy (TEM H600, Hitachi) were contrasted with uranyl acetate and lead citrate. Photos were taken at 2000x magnification to measure the length of mesophyll cells and chloroplasts adjacent to intercellular air spaces, cytoplasm thickness, chloroplast width and thickness.

Mesophyll (S_m/S) and chloroplast (S_c/S) surface area exposed to intercellular air spaces per leaf area were calculated separately for spongy and palisade tissues as in Evans *et al*. (1994) and Syvertsen *et al.* (1995):

$$S_{\text{m}} / S = \frac{L_{\text{m}}}{w'} \cdot \frac{T_{\text{L}}}{t'} \cdot F \qquad [14]$$

where L_m represents the length of mesophyll cells exposed to intercellular airspace, T_L the leaf thickness, *w'· t'* the area of the electron micrograph and *F* the curvature correction factor. To convert lengths in cross-section to surface area, curvature correction factor was measured and calculated according to Thain (1983). This factor accounts for the circumstance that the surface is not strictly perpendicular to the section. It was obtained for palisade and spongy cells by assuming that the cells are spheroids having different width to height ratios.

Chloroplast surface area (S_c/S) exposed to intercellular air spaces per leaf area was calculated as:

$$S_c / S = \frac{L_{chl}}{L_m} \cdot S_m / S \qquad [15]$$

where L_{chl} is the length of chloroplast exposed to intercellular airspace.

Mesophyll cell wall thickness was measured at 20000 – 40000x magnifications. Micrographs were randomly selected in each section and cell wall thickness was measured for 2-3 cells per micrograph.

All parameters were analyzed at least in four different fields of view. Ten measurements for spongy and palisade cells were made for each anatomical trait. All images were analyzed with Image analysis software (ImageJ; Wayne Rasband/NIH, Bethesda, MD, USA).

3.4.7. Leaf morphological parameters

3.4.7.1. Leaf mass per area and leaf density

To determine leaf mass per area (M_A), first the leaf area (*LA*) was measured using a leaf area meter (AM-300 Area Meter, Analytical Development Co. Hoddesdon, UK), or scanning the leaves to determine *LA* from the images using Image J (ImageJ; Wayne Rasband/NIH, Bethesda, MD, USA). Then, the dry mass of these leaves was determined after drying oven for 48h at 60ºC. M_A was calculated as:

$$M_A\ (\text{g m}^{-2}) = \frac{\text{Dry mass}}{\text{Leaf area}} \qquad [16]$$

Leaf density (D_L) was determined from T_L obtained from anatomical measurements and M_A. Then, leaf density was calculated as the ratio of leaf mass per area to leaf thickness, expressed in g cm^{-3}.

3.4.8. Other measurements

3.4.8.1. Leaf area

Leaf area (*LA*) was measured using a leaf area meter (AM-300 Area Meter), or scanning the leaves as described in section 3.4.7.1. Whole plant leaf area was obtained from the leaf area of 20 to 30 leaves (subsample) and the dry weight of all the leaves of a plant. Total *LA* was then calculated as follows:

$$\text{Total } LA\,(\text{m}^2) = \frac{(\text{Total leaves dry weight } x\ LA \text{ subsample})}{\text{leaves dry weight subsample}} \qquad [17]$$

3.4.8.2. Rubisco Content

First, leaf soluble proteins were extracted following the protocol described by Pérez *et al.* (2011). Leaf discs of 3.4 cm^2 were sampled and immediately frozen in liquid N_2. Samples were ground into powder in a mortar with liquid nitrogen extraction buffer was added and grinding continued until the mixture was thawed. The extraction buffer was composed of 4 ml of 100 mM N,N-bis (2-hydroxyethyl) glycine (Bicine)-NaOH (pH 7.8), 10 mM $MgCl_2$, 0.5 mM dithiothreitol (DTT), 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM ethylene glycol tetraacetic acid (EGTA), 1% (v/v) Triton X-100, 20% (v/v) glycerol, 1 mM benzamidine, 1 mM e-aminocaproic acid, 10 μM leupeptin, and 1 mM phenylmethylsulphonyl fluoride (PMSF). Aliquots were centrifuged at 12000*g*, at 4ºC for 1.5 minutes.

Leaf soluble protein concentration was determined spectrophotometrically according to Bradford (1976). This method is based in the absorbance shift observed in an acidic solution of dye Coomassie Brilliant Blue G-250 (Bio-Read, USA). When added to a solution of protein, the dye binds to the protein resulting in a colour change from a reddish brown to blue. The peak absorbance of the acidic dye solution changes from 465 to 595 nm when binding to protein occurs. Therefore, measuring absorptance of the protein-dye complex at 595 nm allows an accurate quantification. Aliquots from different samples containing 20 μg of leaf soluble proteins were loaded in 12.5% SDS-polyacrylamide gel to determine the amount of Rubisco by quantitative electrophoresis by densitometry. Total proteins were separated by 0.75 mm thick of SDS-PAGE gel (12.5% resolving and 4% stacking). The gels were fixed in a mixture with water, methanol and acetic acid

for 1 hour, stained in EZ Blue Gel Staining (Sigma) solution for 1 hour, and finally rinsed in water to remove excess stain. Gels were scanned (HP Scanjet G3010) and images were analyzed by densitometry using an image analysis software (TotalLab v2005, Nonlinear Dynamics, Durham, NC, USA).

3.4.8.3. Specific activity of Rubisco in vivo (k_{cat})

The in vivo specific activity of Rubisco per active site k_{cat}, was calculated according to Eichelmann *et al.* (2009):

$$k_{cat} = \frac{550000\, V_{c,max}}{8[\text{Rub}]} \qquad [18]$$

where [Rub] is the Rubisco content (g m^{-2}), assuming that all the Rubisco is active and its molecular weight is 550 000 Da, and $V_{c,max}$ was obtained from A_N-C_c response curves (section 5.1.).

3.4.8.4. Extraction of leaf soluble sugars

Leaf soluble sugars were extracted as described by Brugnoli *et al.* (1988). Leaf samples were ground into powder in a mortar with liquid nitrogen where 12 ml of water were added. The mixture was mixed for 30 minutes at room temperature and boiled for 3 minutes. After centrifugation (25 minutes at 14.000*g*), the supernatants were purified using two exchange resins, Dowex-50 (H^+) and Dowex-1 (Cl^-), in sequence. The first exchange resin allows separating aminoacids from organic acids and soluble sugars, while the second resin separates organic acid from soluble sugars. The eluates obtained from the chromatography containing the soluble sugars were freeze-dried. Subsamples of 1 mg were analysed for isotope ratio ($\delta^{13}C$) using an elemental analyzer continuous-flow isotope ratio mass spectrometer (Thermo Finnigan Delta Plus, Bremen, Germany) (section 3.4.5.2).

3.4.8.5. Leaf absorptance

Leaf absorptance (α) was calculated as:

$$\alpha = 1 - (\text{reflectance} + \text{transmittance}) \quad [19]$$

Leaf transmittance and reflectance measurements were determined by spectrometry (AvaSpec-2048-2, Avantes, Apeldoorn, The Netherlands) using an integrating sphere (ISP-80-8-R, Ocean Optics, Dunedin, FL, USA). Average absorptance across 400- 700 nm region was used to characterize the fraction of incident photosynthetically active radiation absorbed by the leaf.

3.5. CALCULATIONS

3.5.1. Estimation of mesophyll conductance (g_m) to CO_2

3.5.1.1. Estimation of g_m with gas exchange and chlorophyll fluorescence

Estimation of g_m combining gas exchange and chlorophyll fluorescence, known as variable *J* method, was described by Di Marco *et al.* (1990) and further elaborated by Harley *et al.* (1992). The method relies on the basic relationship between the rate of photosynthetic electron transport rate (J), net CO_2 assimilation (A_N), and the CO_2 concentration at the site of Rubisco (C_c). This relationship can be modeled according to Farquhar *et al.* (1980):

$$J = 4\left(A_N + R_L\right) \cdot \frac{\left(C_c + 2\,C_i^*\right)}{C_c - C_i^*} \quad [20]$$

where the factor 4 denotes the minimum electron requirement for carboxylation. The variable J method has the problem that there are uncertainties in the relationship between J_f (obtained from chlorophyll fluorescence, section 3.4.2.1.) and J_a (calculated by gas exchange measurements). Equation 20 assumes that the linear electron transport calculated by gas exchange (J_a) is used in photosynthesis and photorespiration, while alternative electron sinks are negligible, and considers that chlorophyll fluorescence achieve the same cells that contribute to the gas exchange measurements. Moreover, equation 20 assumes there is no contribution of PSI to chlorophyll fluorescence at room temperature. Hence, this method requires an accurate estimate of leaf absorptance (α) (Eq. 19) or to measure the PSII optical cross-section (Eichelmann & Laisk, 1999) to

determine the distribution of light between the two photosystems. However, even taken these precautions there may not be a 1:1 relationship between J_f and J_a, therefore a partial solution to these problems is to construct a "calibration curve" under non-photorespiratory conditions varying the light intensities (section 3.4.2.1.). Under non-photorespiratory conditions linear electron transport is associated with Rubisco carboxylation and alternative electron sinks. Therefore, in this Thesis α and light curves under non-photorespiratory conditions were measured to overcome the uncertainties linked with this method.

From Fick's first law of diffusion, the relation between A_N, C_i and C_c is expressed as:

$$A_N = g_m(C_i - C_c) \quad [21]$$

Substituting C_c, equation 21 becomes:

$$J = 4(A_N + R_L)\cdot\frac{((C_i - A_N / g_m) + 2\,C_i\,*)}{(C_i - A_N / g_m) - C_i\,*} \quad [22]$$

Rearranging equation 22:

$$g_m = \frac{A_N}{C_i - \frac{\Gamma*(J_a + 8(A_N + R_L))}{J_a - 4(A_N + R_L)}} \quad [23]$$

where 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 & Dreyer (2006). C_i* and R_L were determined according to the Laisk method (section 3.4.3.2.3). Alternatively, R_d was measured according to the section 3.4.3.3.1. Mesophyll conductance (g_m) was calculated using either R_d or R_L. The results showed only estimations corresponded to R_d measurements since the differences between both respiration measurements are non-significant and measurements of R_L presented larger variability than R_d.

Determination of g_m can be used to calculate C_c (Eq. 22). Using C_c instead of C_i, it was possible to convert A_N-C_i curves into A_N-C_c curves and to obtain more reliable results of *in vivo* photosynthetic activity.

3.5.1.1.1. Parameters derived from A_N-C_c curves

The model of Farquhar *et al.* (1980) has provided a tried and tested method 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 provide a means to determine the partitioning of energy between photosynthesis and photorespiration, and therefore to convert C_i into C_c, as explained above.

In this model, the biochemical reactions of photosynthesis are considered to be in two or sometimes three phases (according to von Caemmerer *et al.* 2000) obtained from the response of A_N to C_c. At low C_c, $\partial A_N/\partial C_c$ is high and determined by the Rubisco activity. When C_c is increased there is an inflection to a lower $\partial A_N/\partial C_c$ that approaches zero, where RuBP-regeneration is limiting. In some cases, a further increase in C_c may result in another transition to plateau or a decrease in A_N with an additional increase in C_c ($\partial A_N/\partial C_c \leq 0$), if triose-phosphate utilization (*TPU*) becomes limiting (Fig. 3.6). By fitting these phases, key biochemical kinetic variables determining photosynthetic rate can be determined *in vivo*: the maximum velocity of carboxylation ($V_{c,max}$), the capacity for photosynthetic electron transport ($J_{,max}$) and the triose-phosphate utilization rate (V_{TPU}). These model parameters on the basis of C_c are calculated using the temperature dependence of kinetic parameters of Rubisco described by Bernacchi *et al.* (2002), whereby net assimilation rate is given as:

$$A_N = \min\{A_c, A_q, A_p\} - R_L \quad [24]$$

With:

$$A_c = V_{c,max}\frac{C_c - \Gamma^*}{C_c + K_c[1 + (O_i / K_o)]} \quad [25]$$

$$A_q = \frac{J(C_c - \Gamma^*)}{4(C_c + 2\Gamma^*)} \quad [26]$$

$$A_p = \frac{3V_{TPU}}{\left(1 - \frac{\Gamma^*}{C_c}\right)} \quad [27]$$

where A_c, $A_{q,}$ A_p represent photosynthesis limited by carboxylation, RuBP regeneration and TPU utilization, respectively, K_c and K_o are the Rubisco Michaelis-Menten constants for carboxylation and oxygenation, respectively, O_i is the leaf internal oxygen concentration (assumed equal to the external) and V_{TPU} is the rate of use of triose phosphates.

3.5.1.2. Estimation of g_m with gas exchange only: the curve-fitting method

The curve-fitting method is based on measurements of A_N and C_i in a wide range of CO_2 concentrations. The data are then fitted to the C_3 photosynthesis model of Farquhar *et al.* (1980), which was later modified by Ethier & Livingston (2004) and Sharkey *et al.* (2007) to include g_m. The fundamental premise of the curve-fitting methods is that a finite g_m reduces the curvature of an A_N-C_i response curve.

The Farquhar *et al.* model of C_3 photosynthesis states that photosynthesis is the minimum of rates limited by Rubisco carboxylation (A_c) or the rate of RuBP regeneration (A_j):

$$A_N = \min \{A_c, A_j\}$$

The rates of A_c and A_j are conventionally given by non-linear equations (e.g. Sharkey *et al.*, 2007), but can also be formulated as quadratic equations (Ethier & Livingston, 2004):

$$A_c = \frac{-b + \sqrt{b^2 - 4ac}}{2a}, \text{ where:}$$

$a = -1/ g_m$,

$$b = \frac{V_{c,max} - R_L}{g_m} + C_i + K_c(1 + O / K_o), \text{ and}$$

$$c = R_L[C_i + K_c(1 + O/K_o)] - V_{c,max}(C_i - \Gamma^*) \qquad [28]$$

$$A_j = \frac{-b + \sqrt{b^2 - 4ac}}{2a}$$

$a = -1/ g_m$,

$b = (J/4 - R_L)/ g_m + C_i + 2\Gamma^*$, and

$c = R_L (C_i + 2\Gamma^*) - J/4(C_i - \Gamma^*)$ [29]

where J is the electron transport rate under RuBP-limited conditions and O is oxygen concentration.

3.5.1.3. Estimation of g_m with gas exchange and ^{13}C isotope discrimination

This method is based on simultaneous measurements of leaf gas exchange and carbon isotope discrimination. Discrimination occurs during photosynthetic CO_2 fixation. $^{13}CO_2$ diffuses more slowly in the gaseous and the liquid phase, and is carboxylated much more slowly than $^{12}CO_2$ by Rubisco and PEP carboxylase (Farquhar *et al*., 1982b; Evans *et al*., 1986).

In mathematical terms, carbon isotope discrimination (Δ) is:

$$\Delta = \frac{a_b(C_a - C_s)}{C_a} + \frac{a(C_s - C_i)}{C_a} + (e_s + a_i)\frac{(C_i - C_c)}{C_a} + \frac{bC_c}{C_a} - \frac{(eR_d / k + f\Gamma^*)}{C_a} \quad [30]$$

where C_a is the concentration of CO_2 in air and C_s at the leaf surface; and a_b fractionation due to diffusion through the boundary layer (2.9‰), a diffusion through stomata (4.4‰), e_s and a_i are the fractionation occurring during the dissolution (1.1‰ Mook *et al*., 1974) and diffusion of CO_2 in the liquid phase (0.7‰ O'Leary, 1981). Value of b is the fractionation associated with the enzymatic fixation of CO_2 which accounts for the fractionation of Rubisco (95%) and PEP carboxylase (5%). Both enzymes operate in parallel affecting the isotopic composition of C fixed (Brugnoli *et al.*, 1998; Brugnoli & Farquhar, 2000). Therefore, depending on the proportion of PEP carboxylations in C_3 plants, the value of b can ranged between 27–30‰. Value of e is the fractionation due to mitochondrial respiration and f the fractionation due to photorespiration. Values of e and f are subjected to uncertainty since different measurements have provided different results (von Caemmerer & Evans 1991, Tcherkez *et al.* 2003, Gessler *et*

al. 2009). In this Thesis to estimate g_m, *b* value was considered -29‰ according to Roeske & O`Leary (1984), Guy *et al.* (1993) and Evans *et al.* (1994) and, *e* and *f* were taken as zero according to von Caemmerer and Evans (1991) and Scartazza *et al.* (1998).

To measure simultaneously carbon isotope discrimination and leaf gas exchange, the air entering (C_e) and leaving (C_o) the gas exchange chamber is transferred to the mass spectrometer to determine $\delta^{13}C$. In that way, Δ_{obs} can be calculated according to Evans *et al.* (1986):

$$\Delta_{obs} = \frac{\xi(\delta_o - \delta_e)}{1 + \delta_o - \xi(\delta_o - \delta_e)} \qquad [31]$$

$$\text{with } \xi = \frac{C_e}{C_e - C_o}$$

where δ_e and δ_o are the isotopic compositions of the CO_2 in the air entering and leaving the chamber.

According to Evans *et al.* (1986), the expected carbon isotope discrimination (Δ_i) is obtained from gas exchange assuming that g_m is infinite (i.e. when $C_i = C_c$).

$$\Delta_i = a + (b - a)\frac{C_i}{C_a} \qquad [32]$$

Substracting equation 31 from equation 32

$$\Delta_i - \Delta_{obs} = (b - e_s Ca_i)\frac{C_i - C_c}{C_a} + \frac{(eR_d / k + f\Gamma^*)}{C_a} \qquad [33]$$

and substituting equation 21 (from fisrt Fick's law) into equation 34 is derived:

$$\Delta_i - \Delta_{obs} = (b - e_s - a_i)\frac{(A_N)}{g_m C_a} + \frac{(eR_d / k + f\Gamma^*)}{C_a} \qquad [34]$$

Finally, rearranging equation 34, g_m can be calculated according to Evans *et al.* (1986) and modified by Lloyd *et al.* (1992) as:

$$g_m = \frac{(b - e_s - a_i)\, A_N / C_a}{(\Delta_i - \Delta_{obs}) - \dfrac{(eR_d / k + f\Gamma^*)}{C_a}} \quad [35]$$

In this Thesis, Δ_{obs} was obtained from two different sources to calculate g_m: (1) from the air collected during photosynthesis measurements (see section 3.4.4), or (2) from leaf soluble sugars (Δ_{obs}), proposed by Brugnoli *et al*. (1988) (see section 3.4.8.4.).

3.5.1.4. Estimation of g_m from anatomical parameters

A One-dimension within leaf gas diffusion model by Niinemets & Reichstein (2003a) and applied by Tosens *et al.* (2011) has been used in this Thesis to estimate g_m. Total diffusion conductance (g_m) is divided in three phases, gaseous (g_{ias}), liquid (g_{liq}) and lipid (g_{lip}). g_{ias} comprises from sub-stomatal cavities to outer surface of cell walls, while g_{liq} and g_{lip} liquid and lipid phases from outer surface of cell walls to chloroplasts. Then, g_m is calculated as a composite conductance for within leaf components as:

$$g_m = \frac{1}{\dfrac{1}{g_{ias}} + \dfrac{RT_k}{H \cdot g_{liq+lip}}} \quad [36]$$

where R is the gas constant (Pa m^3 K^{-1} mol^{-1}), T_k is the absolute temperature (K), and H is the Henry's law constant (Pa m^3 mol^{-1}). $H/(RT_k)$ is the dimensionless Henry's law constant form to convert g_{liq} and g_{lip} to a gas-phase equivalent conductance (Niinemets & Reichstein 2003a).

Gas-phase conductance is determined by average gas-phase thickness, $\Delta L_{ias,}$ and gas-phase porosity f_{ias} (fraction of leaf air-space):

$$g_{ias} = \frac{D_a \cdot f_{ias}}{\Delta L_{ias} \cdot \varsigma} \quad [37]$$

where ς is the diffusion path tortuosity (1.57m m^{-1}) and D_a (m^2 s^{-1}) is the diffusion coefficient for CO_2 in the gas-phase (1.51 x 10^{-5} at 25 °C). ΔL_{ias} was taken as half of the mesophyll thickness (Niinemets & Reichstein 2003b).

The inverse of total liquid and lipid-phase conductance is expressed as the sum of the inverse of serial conductances:

$$g_{\mathrm{liq+lip}} = \frac{S_{\mathrm{c}}}{\left(\frac{1}{g_{\mathrm{cw}}} + \frac{1}{g_{\mathrm{pl}}} + \frac{1}{g_{\mathrm{ct}}} + \frac{1}{g_{\mathrm{en}}} + \frac{1}{g_{\mathrm{st}}}\right) S} \qquad [38]$$

where the partial conductances are: g_{cw} for cell wall, g_{pl} for plasmalemma, g_{ct} for cytosol, g_{en} for chloroplast envelope and g_{st} for chloroplast stroma. The partial determinants of liquid and lipid phase diffusion pathway (g_i, where *i* stands either for cell wall, cytosol or stroma conductance) were determined as:

$$g_{\mathrm{i}} = \frac{r_{\mathrm{f,i}} \cdot D_{\mathrm{w}} \cdot p_{\mathrm{i}}}{\Delta L_{\mathrm{i}}} \qquad [39]$$

where ΔL_i (m) is the diffusion path length in the corresponding component of the diffusion pathway, p_i (m^3 m^{-3}) is its effective porosity, and D_w is the aqueous phase diffusion coefficient for CO_2 ($1.79 \cdot 10^{-9}$ m^2 s^{-1} at 25 °C). The dimensionless factor $r_{f,i}$ accounts for the reduction of D_w compared with free diffusion in water (Weisiger 1998), and was taken 1.0 for cell walls (Rondeau-Mouro *et al.* 2008) and 0.3 for cytosol and stroma (Niinemets & Reichstein 2003b). p_i was set 1.0 for cytosol and stroma (Nobel 1991), and from 0.02 to 0.3 for the thickest and the thinnest cell walls, respectively, in the section 5.1 (This was the linear response of porosity to cell wall thickness that gave rise to highest r^2 for this relationship). In the experiment 5.2 a constant value of p_i for cell wall (0.1) was assumed (Nobel 1991). g_{pl} and g_{en} can not be estimated from anatomical parameters, therefore a constant value described by Gutknecht *et al.* (1977) for lipid bilayers was assumed (0.0035 m s^{-1}).

3.5.2. Quantitative limitation analysis

3.5.2.1. Quantitative analysis of photosynthesis limitations

To determine the limitations imposed on photosynthesis, a quantitative limitation analysis of photosynthesis was calculated following the approach proposed by Jones (1985) and implemented by Grassi and Magnani (2005). The photosynthetic limitations were partitioned into their functional components related to stomatal conductance (l_s), mesophyll conductance (l_m) and biochemical characteristics (l_b). From previous A_N-C_c response curves, the absolute limitations for each component were calculated as:

$$l_s = \frac{g_{tot} / g_s \cdot \partial A_N / \partial C_c}{g_{tot} + \partial A_N / \partial C_c} \quad [40]$$

$$l_m = \frac{g_{tot} / g_m \cdot \partial A_N / \partial C_c}{g_{tot} + \partial A_N / \partial C_c} \quad [41]$$

$$l_b = \frac{g_{tot}}{g_{tot} + \partial A_N / \partial C_c} \quad [42]$$

where g_{tot} is the total conductance to CO_2 from ambient air to chloroplasts ($g_{tot} = 1/g_s + 1/g_m$) and $\partial A_N/\partial C_c$ the slope obtained from A_N-C_c response curves ranged between 50 and 100 μmol mol^{-1}.

3.5.2.2. Quantitative analysis of limitations on mesophyll conductance

Quantitative limitation analysis of g_m was calculated for each component of the diffusion pathway of CO_2 (Eq. 37-39). The gas phase limitation (l_{ias}) imposed on g_m was calculated as:

$$l_{ias} = \frac{1/g_{ias}}{1/g_m} \quad [43]$$

The proportion of g_m limited by liquid and lipid phase (l_i) conductance was determined as:

$$l_i = \frac{S / g_i}{S_c / g_m} \quad [44]$$

where l_i is the limitation in either cell wall, cytosol, chloroplast stroma, plasmalemma and chloroplast envelope and g_i is the component diffusion conductances of the corresponding diffusion pathways.

3.5.3. Plant carbon balance

Whole plant carbon balance (PCB) was calculated as:

$$PCB = A_{N\ leaf} - R_{leaf} - R_{stem} - R_{root} \quad [45]$$

Photosynthesis and respiration of the different organs were measured as previously described (sections 3.4.3.2.1. and 3.4.3.3., respectively).

Estimation of carbon gain by photosynthesis (A_N) was obtained from the sum of daily A_N per total leaf area (LA), which increment was considered lineal during the experiment. This parameter was considered separately, for irrigated and non-irrigated plants because they present different dependency on light intensity. In control plants, from three different daily cycles a single hyperbolic function of the relationship between A_N and the photosynthetic active radiation (PAR) was derived for each cultivar and used for the integration of daily leaf photosynthesis. The inclination of the leaf with respect to the horizontal was considered and applied to the function as a correction factor of 30º. On the other hand, in water stressed plants the major limiting factor in photosynthesis is g_s (Flexas *et al.* 2002), therefore, A_N was estimated from the daily carbon gain calculated as the mean of the three daily cycles of leaf photosynthesis measured six times along the day in three different days throughout the experiment for each cultivar.

Leaf respiration was calculated from nocturnal time courses and measurements made at different temperatures in irrigation and non-irrigation conditions (3.4.3.3.1.). Night-time leaf respiration was calculated as the integral of the nigh-time course of R_{leaf}. Then, values obtained from leaf respiration at different temperatures were used to develop calibration curves to estimate the temperature response of night leaf respiration. Leaf carbon loses during diurnal period were included in the measurements of A_N, since this equals gross photosynthesis minus the sum of photorespiration and mitochondrial respiration.

Total stem respiration (R_{stem}) was obtained from the measurements performed in apical and intermediate segments of the main stem of irrigated and water stressed plants at different temperatures. From these measurements a single exponential function was obtained and used to calculate daily R_{stem} considering hourly changes of T_{air} and a linear increase in stem biomass.

Estimation of total root respiration (R_{root}) was derived from a single exponential function obtained from measurements of R_{root} in irrigated and non-irrigated plants at different temperatures. This function was used to calculate daily R_{root} considering hourly changes of T_{soil} and daily linear increases along the experiment.

Plant Biomass (PB) was calculated considering the N content and the ash fraction of the different tissues (leaf, stem and roots) described by Vivin *et al.* (2003) for grapevines.

Chapter 4

IMPROVEMENT OF WATER USE EFFICIENCY IN GRAPEVINES

NOTE: this review paper presents many of the ideas explained in the Introduction and some of the preliminary results obtained in the present Thesis

4.1. Flexas J., Galmés J., Gallé A., Gulías J., Pou A., Ribas-Carbó M., **Tomás M.** & Medrano H. (2010) Improving water use efficiency in grapevines: potential physiological targets for biotechnological improvement. *Australian of Grape & Wine Research* 16, 106-121

Chapter 5

VARIABILITY OF MESOPHYLL CONDUCTANCE AND MECHANISTIC BASIS OF THESE VARIATIONS

5.1. Tomás M., Flexas J., Copolovici L., Galmés J., Hallik L., Medrano H., Tosens T., Vislap V. & Niinemets Ü. (2012). Importance of leaf anatomy in determining mesophyll diffusion conductance to CO_2 across species: quantitative limitations and scaling up by models. *Plant, Cell & Environment* (submitted).

5.2. Tomás M., Medrano H., Martorell S., Pou A., Escalona J.M., Ribas-Carbó M. & Flexas J. (2012) Mesophyll conductance and leaf anatomy traits in different cultivars of grapevine (in preparation).

Chapter 6

WATER USE EFFICIENCY AT LEAF AND WHOLE PLANT LEVEL

6.1. Tomás M., Medrano H., Pou A., Escalona J.M., Martorell S., Ribas-Carbó M. and Flexas J. (2012) Water use efficiency in grapevine cultivars grown under controlled conditions: effects of water stress at the leaf and whole plant level. *Australian Journal of Grape & Wine Research* 18, 164-172

Chapter 7

IMPORTANCE OF PLANT RESPIRATION, CUTICULAR AND NIGHT TRANSPIRATION IN WHOLE PLANT WATER USE EFFICIENCY

7.1. Escalona JM., Fuentes S., **Tomás M**., Martorell S., Flexas J. & Medrano H. (2012) Responses of leaf night transpiration to water stress in *Vitis vinifera* L. *Annals of Botany* (submitted).

7.2. Escalona J.M.*, **Tomás M.***, Martorell S., Medrano H., Ribas-Carbó M. & Flexas J. (2012). Carbon balance in grapevines under different soil water supply: importance of whole plant respiration. *Australian Journal of Grape & Wine Research* (in press)

Chapter 8

GENERAL DISCUSSION

The results of this Thesis have been structured in four chapters (4, 5, 6, and 7) corresponding to its publications. The relevance of the findings is profusely discussed in each of these Chapters that deal with each general objectives. Therefore, the present General Discussion chapter focuses only on broad and integrated view of all physiological processes that can affect leaf and whole plant *WUE*.

8.1. MECHANISMS THAT DETERMINE g_m AND ITS EFFECTS ON LEAF *WUE*.

The first broad objective of this Thesis was to analyze the mechanisms involved in the variability of mesophyll conductance (g_m) and whether such variability was linked to improvements of leaf *WUE* (WUE_l).

Mesophyll conductance (g_m) has been proposed as a potential physiological process to improve WUE_l, as it would increase CO_2 concentration at the carboxylation site (C_c) and consequently A_N without any effect on leaf transpiration (Parry *et al*. 2005, Flexas *et al.* 2008). From Fick's first law of diffusion, a direct dependency is expected between WUE_l (A_N/g_s) and the ratio g_m/g_s.

The results obtained in the present Thesis confirm that g_m/g_s determines WUE_l in different cultivars of grapevines. Increases in g_m/g_s corresponded to increases in A_N/g_s regardless of the water status of the plants. Recently, this positive relationship between g_m/g_s and WUE_l (A_N/g_s or $\delta^{13}C$) has been reported in several species such as in *Populus balsamifera* (Soolanayakanahally *et al*., 2009), barley (Barbour *et al*., 2010), *Picea asperata* (Duan *et al*., 2011) and tomato (Galmés *et al.*, 2011). Therefore, the positive correlation between g_m/g_s and A_N/g_s observed in this Thesis and in other studies confirms g_m as a potential target to improve WUE_l by increasing A_N without increasing plant water losses.

Leaf structural traits and biochemical components have been considered the major limitations of g_m. CO_2 diffuses through a gas phase in the intercellular air spaces, through liquid phase in cell walls, cytosol and chloroplast stroma, and through lipid phase in plasmalemma and chloroplast envelope. Moreover, CO_2

diffusion through membranes also depends on their permeability. Aquaporins and carbonic anhydrases have been proposed as main components associated to the membrane permeabilities and to rapid changes of g_m (Gillon & Yakir, 2000; Hanba *et al*., 2004; Flexas *et al*., 2006c; Heckwolf *et al*., 2011).

Recently, it has been shown that g_m is highly determined by leaf anatomical traits while the role of biochemical properties is still unclear. In particular, the surface of chloroplasts exposed to intercellular air spaces (S_c/S) and cell wall thickness (T_{cw}) have been suggested as the most important anatomical traits limiting g_m (Terashima *et al.*, 2011). Accordingly, several reports demonstrated positive relationships between g_m and S_c/S (Evans *et al.*, 1994; Evans & Loreto, 2000; Terashima *et al.*, 2006; Tholen *et al.*, 2008; Tosens *et al*., 2011). Moreover, it has been reported that T_{cw} could account for 25% or 50% of the total internal resistance (Evans *et al.*, 2009; Terashima *et al*., 2011). However, several studies have also shown negative correlations between g_m and T_{cw} in different species (Hassiotou *et al*., 2010; Scafaro *et al*., 2011; Peguero-Pina *et al*., 2012; Tosens *et al*., 2012) or between $g_m/(S_c/S)$ and T_{cw} when pooling data from multiple studies (Terashima *et al.,* 2011). Therefore, S_c/S and T_{cw} seem to be the most important components involved in internal diffusion of CO_2. In the present Thesis the importance of the anatomical traits in g_m is evaluated, in particular S_c/S and T_{cw}, in a wide range of species with contrasted leaf structural properties, from herbs to evergreen species with thick leaves (section 5.1). The inter-specific differences in g_m, and consequently in A_N, were mainly explained by both S_c/S and T_{cw}, although the importance of each in the diffusion of CO_2 varied depending on the leaf structure, being T_{cw} most important in species with high leaf dry mass per area (M_A). However, when studies are focused in a narrow range of species, as in different Australian sclerophylls species (Tosens *et al*., 2012) or when different species within the same genus are studied, *Banksia* (Hassiotou *et al*., 2010), *Oryza* (Scafaro *et al*., 2011) or *Abies* (Peguero-Pina *et al*., 2012), smaller effects of leaf anatomy are observed. In the present Thesis, where genotypic variability of g_m has been studied in grapevines (section 5.2) the role of the key anatomical traits, S_c/S and T_{cw}, is not obvious. In the present case, membrane permeability to CO_2 in the liquid and lipid phase might play an important role in the genetic variability of g_m. Differences of several orders of magnitude have been reported for CO_2 permeabilities for biological membranes (Evans *et al*., 2009). Moreover, it has

been suggested that aquaporins constitute a key factor to regulate CO_2 diffusion through membranes (Martre *et al*., 2002; Terashima *et al*., 2011). Hence, aquaporins could be involved in the genetic variability of g_m observed in grapevines, and they are potentially responsible of improvements of g_m/g_s. Therefore, it is clear that leaf anatomy plays a dominant role in setting g_m on a broad scale, while on a smaller scale other factors become compelling to explain g_m differences, as it is the case for grapevine cultivars. Hence, further studies are urged to determine the function of aquaporins in relation to variations of g_m.

8.2. DOES LEAF *WUE* DETERMINE WHOLE PLANT *WUE*?

The second goal of this Thesis faces the scale-up of WUE_l to WUE_{WP} with the specific question of how significant is WUE_l as a proxy for WUE_{WP}.

Most studies about *WUE* are focused in WUE_l, intrinsic *WUE* (A_N/g_s), instantaneous *WUE* (A_N/E) and/or carbon isotopic composition ($\delta^{13}C$) (Morison *et al*. 2008). Leaf-level measurements of *WUE* are commonly used as proxy for WUE_{WP}, however, WUE_l is not always a good parameter to predict WUE_{WP}, depending on the species studied and the experimental conditions. A negative and strong correlation between $\delta^{13}C$ and WUE_{WP} has been observed in monocotyledonous species such as wheat (Farquhar & Richards, 1984), crested wheatgrass (Read *et al*., 1991), barley (Hubick & Farquhar, 1989) and Kentuchy bluegrass (Ebdon *et al*., 1998), and in dicotyledonous species such as rice (Impa *et al*. 2005), sunflower (Virgona *et al*. 1990) and peanuts (Craufurd *et al*. 1999). However, no correlation between leaf (single-leaf or daily integrated measurements of *WUE*) and whole plant *WUE* has been observed in other plants with more complex canopies, such as in *Vitis vinifera* (Poni *et al*., 2009; Tarara *et al*., 2011; Medrano *et al*, 2012) and western redcedar populations (Fan *et al*., 2008). These studies are in agreement with the results obtained in Chapter 6, where no consistent correlation was found between WUE_l (A_N/g_s, A_N/E, $\delta^{13}C$) and WUE_{WP} in spite of the significant variability observed among the different cultivars of grapevine at both levels of *WUE*.

The possible causes of this lack of correlation could be partly associated to the patterns of biomass accumulation in the different plant organs during the growth season. Carbon isotope composition ($\delta^{13}C$) of plant tissues reflects the

variation of the photosynthesis to stomatal conductance ratio over a considerable time and under variable environmental conditions (Martinelli *et al*., 1998; Buchmann *et al*., 1997, 1998). Fast growing plants (annual herbs) with short leaf life spans, high photosynthesis and rapid carbon gain reduce the accumulation of dry matter to the growing period. On the other hand, grapevines and other evergreen/deciduous species present dry matter remaining from previous growth seasons that could interfere in the carbon composition of the newly formed tissues (Damesin *et al*., 1998; Hobbie *et al*., 2002). Therefore, these differences in the patterns of carbon accumulation and allocation during the growing season could explain why in fast growing species there is a good correspondence between $\delta^{13}C$ and whole-season *WUE* while in evergreen/deciduous woody species this correlation is unexistent

Another limitation to predict WUE_{WP} from WUE_l is related to the canopy architecture. In most studies, well-exposed leaves are used to determine WUE_l, minimizing the variability due to different locations on the canopy and interaction with sun position. However, canopies are formed by heterogeneous leaves with different irradiance directions. Open canopies with low leaf area index (LAI) or vertically inclined leaves increase light penetration to lower leaves, allowing all leaves to be well exposed and, consequently, presenting a good correspondence between WUE_l (leaf gas exchange measurements) and WUE_{WP} (Terashima & Hikosaka 1995, Xu *et al*., 2004). On the other hand, more complex canopies with high LAI or flat leaves, such *Vitis vinifera*, present many shaded leaves either temporally or permanently during the day. In this case, target leaves measured by gas exchange measurements are not fully representative of the whole canopy (Roux *et al*., 1996; Intrieri *et al*., 1997; Escalona *et al*., 2003; Petrie *et al*., 2009; Poni *et al*., 2009).

However, the main limitation to the correspondence between WUE_l and WUE_{WP} could be associated to the importance of carbon losses by plant respiration. Whole plant water use efficiency depends on the balance between assimilatory and respiratory processes. There is limited information on the spatial and temporal variation of plant respiration rates to determine the final carbon balance. Moreover, root temperature in potted plants and night temperatures during summer can induce considerable increases in carbon losses which could partially explain the lack of correlation between WUE_l and WUE_{WP}. Therefore,

other physiological mechanisms not directly related to maximum leaf photosynthesis of sun exposed leaves are involved in the regulation of WUE_{WP}; among them respiratory processes, less known than photosynthesis ones. This observation led us to formulate the last general objective of the present Thesis, which is to determine what physiological processes besides leaf photosynthesis could be potential targets to improve WUE_{WP}

8.3. PHYSIOLOGICAL MECHANISMS THAT CAN AFFECT WHOLE PLANT *WUE*

Photosynthesis, transpiration and respiration are the physiological mechanisms that directly affect carbon balance and water transpired. Therefore, water losses uncoupled to any carbon gain (cuticular and night transpiration) and, carbon losses by root, stem and leaf dark respiration have been proposed as potential causes of the lack of correlation between leaf and whole plant *WUE* (Flexas *et al*., 2010; Schultz & Stoll, 2010).

Significant water losses can occur during the night due to incomplete stomatal closure and cuticular transpiration, thus increasing water losses without any carbon gain (Caird *et al*., 2007) contributing to the discrepancy between leaf and whole plant *WUE*. Night and cuticular transpiration have been studied in the present Thesis in different cultivars of grapevine under irrigation and drought conditions (section 7.1). Important water losses were observed during the night in grapevines being reduced when progressive water stress was applied. The most significant water losses were observed through the stomata, representing 70-90% of total night transpiration, while water transpired by cuticle was of minor importance. Night transpiration, and in particular g_{night}, showed variations in response to $VPD_{ambient}$ as observed in previous reports (Oren *et al.*, 2001; Bucci *et al.*, 2004; Barbour & Buckley, 2007). Whole plant water losses were minor during the night, particularly under water stress. This fact suggests that night transpiration was compensated by dew deposition on the leaf or substrate surface as a consequence of mild temperatures and high relative humidity in the area of study, contributing to leaf hydration. These results indicate that water losses in grapevines are not significantly relevant in setting WUE_l under Mediterranean conditions with high air relative humidity.

The second physiological mechanism considered to cause the discrepancy between WUE_l and WUE_{WP} is carbon loss by respiration. This Thesis studies respiration in different organs of grapevine to determine the importance of this process in the whole plant carbon balance and consequently in WUE_{WP} (section 7.2). Most respiration studies at the whole plant level are performed in big chambers; however, these chambers imply a modified environment as compared to natural ambient (McCree, 1986; Norby *et al*., 1997; Petrie *et al*., 2003; Perez Peña & Tarara, 2004; Tarara *et al*., 2011) and do not allow measurements of respiration for each organ separately. In the experiment described in section 7.2, respiration was determined at the 'single organ level' in different cultivars of grapevine under well-watered and water stress conditions to determine the importance of each organ in the plant carbon balance. At the whole plant level, a large proportion of the carbon fixed by photosynthesis was consumed by respiration under irrigated (30-50%) and non irrigated conditions (40-60%), respectively. The most important carbon consumers by respiration were roots, which accounted for 70-80% of the total plant carbon consumption by respiration under irrigation. Under water stress root respiration was reduced, likely due to reduced photosynthesis and carbohydrate transport to roots (Bota *et al*., 2004), but it still accounted for up to 60-65% of the total plant carbon used. Leaves and stems showed lower rates of respiration than roots, with a variable response to drought, while leaf respiration decreased, as previously observed (Schultz & Stoll 2010), stem respiration slightly increased. Then, high variations among the different organs were observed (Vivin *et al*., 2003), in addition to significant intra-specific variability in root respiration. These results indicate the importance of each organ respiration in the plant carbon balance being root respiration responsible of most carbon losses. These results support the idea that reducing respiration, especially in roots, could contribute to improve WUE_{WP}. As described in the Introduction, a decrease of maintenance respiration, a process which significant part is sustained by the alternative oxidase pathway (*AOP*) described as a futile process (Flórez-Sarasa *et al.*, 2007), could be associated to an increase of carbon gained by photosynthesis. Therefore, further studies in grapevine respiration are needed to determine the possibility to use it as a potential tool to improve WUE_{WP}.

8.4. GENERAL OVERVIEW

Overall, the results of the present Thesis describe the importance of several physiological mechanisms involved in water use efficiency in grapevines that could be considered good candidates to improve WUE_{WP}. Mesophyll conductance and respiration have been described as important factors limiting *WUE*, for which improving net plant carbon uptake (mesophyll conductance) or minimizing carbon losses (respiration), are promising targets for the improvement of *WUE*. On the other hand, night transpiration, studied as a mechanism that affects water balance without any gain of carbon, showed significant water losses but largely compensated by the dew deposition on leaf and substrate surfaces that allows the leaf hydration, being this physiological process of minor importance in the regulation of *WUE* under Mediterranean conditions.

The results of my experimental Thesis combined with previous data from our research group (Escalona *et al*., 2003) are represented in a schematic diagram (Fig. 8.1) to give a general overview on factors that can directly affect WUE_{WP} and that could be targeted for its improvement (Fig. 8.1). This general overview is based on data from well-irrigated Tempranillo grafted on Richter-110 and grown in the field (Escalona *et al*., 2003), and irrigated Tempranillo un-grafted and grown in pots (experiments section 7.1 and 7.2). Potential maximum values of photosynthesis (A_N) and transpiration (E) are considered as those that would achieve a plant if all its leaves were fully exposed to sun all the day. These values are set to 100% with A/E ratio of 1. Escalona *et al*. (2003) showed that the potential carbon gain can be reduced up to more than 50% due to the complexity of the canopy structure, in which many leaves are shaded during the day especially in the inner part of the canopy, hence displaying lower rates of photosynthesis. Then, this reduces potential whole plant photosynthesis to 47% (see graph). Regarding to the transpiration, Escalona *et al*. (2003) demonstrated that 36% less water is lost when the canopy architecture is taken into account as compared the potential maximum of leaf transpiration (100%). In this case, those leaves shaded during the day present lower ratios of transpiration than leaves well exposed to the sun, inducing that potential whole plant transpiration goes down to 64% (Fig. 8.1). Therefore, from Escalona *et al*. (2003) results we can conclude that 27% of whole plant water use efficiency reduction is explained by canopy structure. On the other hand, the experimental results of this Thesis (section 7.2)

showed that 44% of carbon losses are owing to the plant respiration. This is displayed in figure 8.1 as star marks where is represented the percentage of carbon losses for each organ respiration, stems (2%), leaves (5%) and roots (37%). Then, when it is applied to potential whole plant photosynthesis, net carbon gain decreases from 47% to 26%. However, cuticular and night transpiration results of experiment of section 7.1 demonstrate that these processes only imply slight increments of total water losses (7%), incrementing potential whole plant transpiration from 64% to 71%. Hence, 36% of potential whole plant water use efficiency reduction is mainly explained by plant respiration.

Considering all above, we can conclude that canopy structure and whole plant respiration are the two factors that account for most of the discrepancies between WUE_{I} and WUE_{WP}.

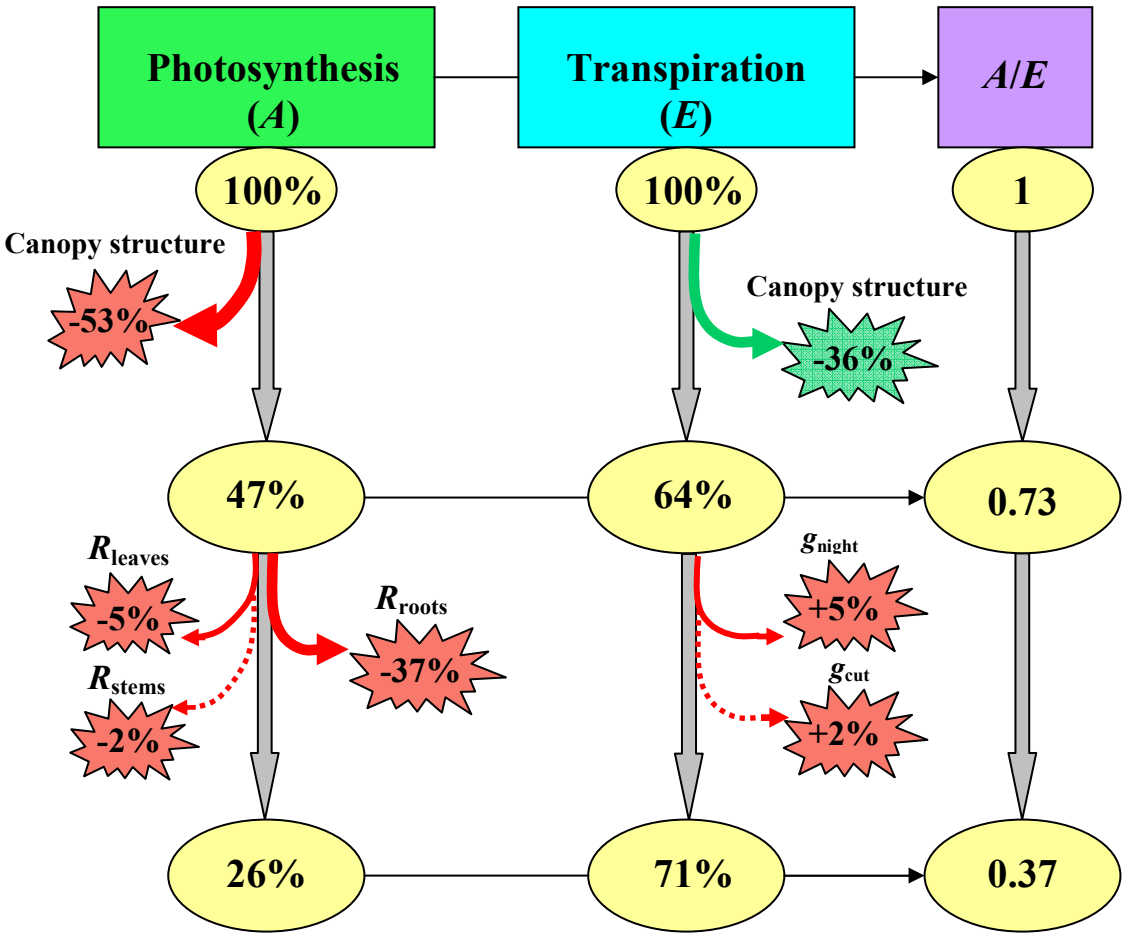

Figure 8.1. Schematic diagram of carbon and water losses in Tempranillo under irrigation conditions that affect photosynthesis, transpiration and water use efficiency. Data obtained from Escalona *et al.* (2003) and experiments of sections 7.1 and 7.2.

The results of this Thesis combined with data from Escalona *et al*. (2003) represented in the schematic diagram (Fig. 8.1) shows an illustrative overview of whole plant physiological processes that affect *WUE* under irrigation. However, it is noteworthy that plants used in the experiments of this Thesis were plants without fruits. Then, this observation led us to formulate the following questions: what would happen in plants with fruits? Is the importance of each organ respiration in WUE_{WP} maintained?

There are evidences that grapevine fruits are the most active sink of photosynthates. It has been shown that fruits import up to 70-80% of the total assimilates obtained by photosynthesis although they constitute only 20-30% of total plant dry matter (Bota *et al*. 2004). Therefore, further studies with fruiting plants are needed to determine the importance of each tissue respiration, in particular fruits and roots, to know in which organ is necessary to act to improve WUE_{WP}.

Chapter 9

CONCLUSIONS

From the experimental research presented in this Thesis established from its objectives, a series of conclusions have been reached.

1. *To analyze the mechanisms that could induce the variability of mesophyll conductance (g_m), and whether such variability can be linked to improvements of leaf WUE (WUE_l)*

1. In grapevines there was a large intra-specific variability in mesophyll conductance (g_m) under irrigation, while g_m generally declined under water stress.

2. The inter-specific variability of g_m was mainly explained by anatomical parameters. Surface of chloroplasts exposed to intercellular air spaces (S_c/S) and cell wall thickness (T_{cw}) were the most important limitations in internal CO_2 diffusion.

3. The distribution of chloroplast along the exposed mesophyll cell wall (S_c/S) was more determining in CO_2 diffusion in species with thin leaves, while T_{cw} was more limiting in thicker leaves with high leaf dry mass per area (M_A).

4. At smaller scale (among cultivars of a single species, grapevine), key anatomical traits (S_c/S and T_{cw}) were not associated to intra-specific variability of g_m. The regulation of CO_2 membrane permeability by aquaporins is proposed as a possible candidate to explain the genetic variability of g_m in this case.

5. A good correlation between intrinsic *WUE* (A_N/g_s) and g_m/g_s was observed in grapevines regardless of plant water status. Therefore, g_m could be a potential physiological target to improve leaf *WUE*, by means of improving photosynthesis without increasing water losses.

2. *To establish links between leaf and whole plant WUE*.

6. Increases in leaf *WUE* (A_N/g_s or $\delta^{13}C$) did not always correspond to increments in whole plant *WUE*. Hence, a lack of correlation between leaf and whole plant *WUE* could be observed for certain experimental conditions in *Vitis vinifera* cultivars.

3. *To determine what physiological processes could be a potential target to improve whole plant WUE (WUE_{WP}).*

7. A possible cause of the discrepancy observed in *WUE* at leaf and plant level could be the water transpired by cuticular (g_{cut}) and night conductance (g_{night}). However, the water losses during the night were a small proportion of daily ones and seemed to be of minor importance in grapevines under Mediterranean conditions due to the dew deposition on leaf and substrate surface that compensated water losses.

8. Carbon losses by plant respiration, in particular root respiration (R_{root}), explained the largest part of the lack of correlation between WUE_l and WUE_{WP} observed in grapevines. Root respiration was identified as a promising candidate target to improve *WUE*.

9. In order to improve WUE_{WP} in grapevines, acting at different levels is essential. Improving physiological processes like mesophyll conductance (anatomical and biochemical properties), respiration and parameters related to the plant structure (roots and canopy) is needed.

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