ECOPHYSIOLOGICAL TRAITS AND THEIR RESPONSES TO DROUGHT IN SPECIES FROM THE BALEARIC ISLANDS WITH DIFFERENT GROWTH FORMS

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Ecophysiological traits and their responses to drought in species from the Balearic Islands with different growth forms

PhD Thesis

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CERTIFICO:

Que Jeroni Galmés Galmés ha realitzat sota la meva direcció, en el Grup de Recerca en Biologia de les Plantes en Condicions Mediterrànies, de la Facultat de Ciències de la Universitat de les Illes Balears, el treball que, per optar al grau de Doctor en Ciències (Biologia), presenta amb el títol:

"Ecophysiological traits and their responses to drought in species from the Balearic Islands with different growth forms"

Considerant finalitzada la present memòria, autoritzo la seva presentació per a que pugui ésser jutjada pel tribunal corresponent.

Per a que així consti, signo el present certificat a Palma, el 19 de gener de 2006.

Jaume Flexas Sans

L'EXTENSIÓ DE TERRA QUE CONTEMPLES
PREN-LA COM UN REFLEX DE TU MATEIX,
VULGUES CONFONDRE'T AMB ELS SEUS EXEMPLES,
LA TERRA I TU BLEIXEU EN UN SOL BLEIX

(RAMON LLULL)

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SYMBOLS AND ABBREVIATIONS LIST

α	leaf absorptance
A	anteraxanthin
ABA	abscisic acid
A_G	gross CO_2 assimilation rate
A_N	net CO_2 assimilation rate
A_{SAT}	light- and CO_2- saturated photosynthesis
ATP	adenosine triphosphate
β	fraction of absorbed light that reaches photosystem II
B	plant dry matter
B_L	biochemical limitations to photosynthesis
C	CO_2 mole fraction
C_a	atmospheric CO_2 concentration
C_c	chloroplastic CO_2 concentration
cDNA	complementary deoxyribonucleic acid
Chl	chlorophyll
C_i	sub-stomatal CO_2 concentration
CTAB	cetyl trimethyl ammonium bromide
$\Delta F/F_m$'	operating quantum efficiency of PSII photochemistry
$\Delta G^{\ddagger}$	free energy of activation
$\Delta G_c^{\ddagger}$	carboxylation free energy of activation
$\Delta G_o^{\ddagger}$	oxygenation free energy of activation
D	seed dormancy period
DIECA	diethyldithiocarbamate
D_L	diffusive limitations to photosynthesis
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DPS	xanthophylls de-epoxidation state
DPS_{MD}	midday xanthophylls de-epoxidation state
DPS_{PD}	predawn xanthophylls de-epoxidation state
DTT	dithiothreitol
ε	leaf volumetric elastic modulus
E	leaf transpiration rate
EDTA	ethylene diamine tetraacetic acid
EGTA	ethylene glycol tetraacetic acid
ES	evergreen shrubs
ESS	evergreen semi-shrubs
ETR	electron transport rate
ϕ_{CO2}	apparent quantum efficiency of CO_2 fixation
F_m	maximum fluorescence in the dark-adapted state
F_m'	maximum fluorescence in the light-adapted state
F_o	basal fluorescence of a dark adapted leaf
F_s	steady-state fluorescence emission
F_v/F_m	maximum quantum efficiency of PSII photochemistry
Γ	CO_2 compensation point

Γ^*	CO_2 compensation point in the absence of mitochondrial respiration
G	cumulative seed germination
g_i	internal (mesophyll) conductance
GRC	growth response coefficient
g_s	stomatal conductance
g_{sc}	stomatal conductance to CO_2
g_{sw}	stomatal conductance to H_2O
HEPES	hydroxyethyl piperazinyl ethasulfonic acid
J_{max}	electron transport driving regeneration of RuBP
K_c	Michaelis constant for the carboxylase activity of Rubisco
k_c	rate constant for carboxylation activity of Rubisco
k_c^{cat}	reaction turnover rate for carboxylation activity of Rubisco
K_L	leaf-specific hydraulic conductance
K_o	Michaelis constant for the oxygenation activity of Rubisco
k_o	rate constant for the oxygenase activity of Rubisco
L	Rubisco large subunits
LA	leaf area
LAR	leaf area ratio
LDM	leaf dry matter
LMR	leaf mass ratio
MC_L	mesophyll limitations to photosynthesis
NAR	net assimilation rate
NARp	partial net assimilation rate
NARt	total net assimilation rate
NPQ	Stern-Volmer non-photochemical quenching of chlorophyll fluorescence
NS_L	non-stomatal limitations to photosynthesis
O	O_2 mole fraction
PAR	photosynthetically active radiation
PCR	polymerase chain reaction
PEG	polyethylene glycol
PGA	phosphoglycerate
PH	perennial herbs
PMSF	phenylmethylsulphonylfluoride
PPFD	photosynthetic photon flux density
P_r	photorespiration rate
PSI	photosystem I
PSII	photosystem II
PVP	polyvinylpyrrolidone
*rbc*L	Rubisco large subunits encoding genes
*rbc*S	Rubisco small subunits encoding genes
R_D	rate of mitochondrial respiration in the dark
RDM	root dry matter
RGR	relative growth rate
RGRp	partial relative growth rate
RGRt	total relative growth rate
R_L	rate of mitochondrial respiration in the light
RMR	root mass ratio
RNA	ribonucleic acid
RT	reverse transcription reaction
Rubisco	ribulose-1,5-biphosphate carboxylase/oxygenase

RuBP	ribulose-1,5-biphosphate
RWC	relative water content
RWC_{MD}	midday relative water content
RWC_{PD}	pre-dawn relative water content
RWC_0	relative water content at zero turgor
RWC_{100}	relative water content at full turgor
S	Rubisco small subunits
SAI	stomatal area index
SD	stomatal density
SDM	stem dry matter
SDS	semi-deciduous shrubs
S_L	stomatal limitations to photosynthesis
SLA	specific leaf habitat
SLW	specific leaf weight
SMR	stem mass ratio
SR	stomatal responsiveness to pre-dawn leaf water potential
SWC	soil water content
τ	*in vitro* Rubisco specificity factor
τ^*	*in vivo* apparent Rubisco specificity factor
T_l	leaf temperature
TEA	triethylamine
TPU	triose-phosphate utilization
T_{50}	seed average time response
V	violaxanthin
V_c	carboxylation activity of Rubisco
v_c	velocities of carboxylation of Rubisco
$V_{c,max}$	maximum rates for the carboxylation activity of Rubisco
V_o	oxygenation activity of Rubisco
v_o	velocities of oxygenation of Rubisco
$V_{o,max}$	maximal rates for the oxygenation activity of Rubisco
V_{TPU}	triose-phosphate utilization rate
w_c	potential rates of CO_2 assimilation supported by Rubisco
w_j	potential rates of CO_2 assimilation supported by RuBP regeneration
w_p	potential rates of CO_2 assimilation supported by triose-phosphate utilization
Ψ	leaf water potential
Ψ_{MD}	midday leaf water potential
$\Psi_{\pi 0}$	osmotic potential at zero turgor
$\Psi_{\pi 100}$	osmotic potential at full turgor
Ψ_{PD}	pre-dawn leaf water potential
Ψ_{p100}	leaf water potential at full turgot
Z	zeaxanthin

CONTENTS

Chapter 1
INTRODUCTION

1.1. CLIMATE AND VEGETATION OF THE MEDITERRANEAN BASIN

1.1.1. The Mediterranean climate and the Mediterranean basin

In one of the first efforts to divide the Earth into a limited number of geoclimatical regions, Köppen, in 1900, defined Mediterranean regions as regions subject to climates receiving rain primarily during winter season from the mid-latitude cyclone. These areas experience climatic conditions characterized by a hot drought period in summer and a cool wet period in winter, and can be considered as the transition between dry tropical and temperate climates. This basic definition constitutes the core of what it is called Mediterranean climate (Aschmann, 1973). It is important to note that the Mediterranean climate is very recent in geological terms and first appeared approximately 3.2 million years ago during the Pliocene, evolving from a nearly Tropical climate (Raven, 1973; Suc, 1984). Mediterranean climates have attained their greatest extent at present. A Mediterranean climate occurs on about two percent of the world's total land area (Thrower & Bradbury, 1973; World Conservation Monitoring Centre, 1992). This area includes specific lands of four continents, all of them located on the western or southwestern coasts of these continents. Specifically, Mediterranean climate is found in regions of California, central Chile, South Africa, Southwestern and Southern Australia and the Mediterranean basin.

The present Thesis was developed in the Balearic Islands, located in the Mediterranean basin, which is the largest region of land with Mediterranean climate, representing about 60% of the world's total Mediterranean climate. The Mediterranean basin has a far larger area and more complex geography than any other Mediterranean climate region, reflected by a series of distinctive features that will be discussed in this section. The Mediterranean basin is the only Mediterranean climate region that includes parts of three continents, comprising regions of Southern Europe, Northern Africa and the Western edge of Asia, resulting in a rich flora particularly where the continents meet (Dallman, 1998). However, only a part of the Mediterranean basin can be considered to be Mediterranean from a climatic point of view. This area extends approximately about 1.68 MKm^2 (Le Hourérou, 1981). The other areas of the Mediterranean basin have very different climates, from the deserts of Libya to the cold temperate areas of North Italy and the steppes of Turkey. The proximity of these climates affects the Mediterranean areas and their vegetation.

In the Mediterranean region, rainfall is generally greater in the north than in the south, and increases with elevation in the numerous mountain ranges throughout the region. The dry season lasts from 1 to 8 months, and the average duration of the summer drought varies considerably among regions within the Mediterranean basin. During this dry period, plants undergo water deficit conditions. The most important consequence of this type of climate is that there is no period in which high temperatures and high soil water availability co-occur. This has important implications for vegetation as it limits the active growing season to the humid period between fall and spring. Similarly, organic matter decomposition is constrained during most of the year (Serrasoles *et al*., 1999).

Another important feature of the Mediterranean climate is that precipitation is not uniformly distributed along the year and it is highly variable from year to year. Most of the precipitation falls between September and May, but usually concentrated in few torrential events, which implies the possible occurrence of short drought periods even during the humid season. Year to year variations of precipitation are greater in the drier areas, as occurs in desert climates (Ehleringer & Mooney, 1983). The inter-annual variations often lead to alternation drought and humid cycles of several years.

In addition to water limiting conditions during the dry season, plant growth is also constrained by cold temperatures during winter. Minimum temperatures can be below freezing during winter months in many areas of the northern part of the basin as well as in the mountains. Moreover, the growth of many Mediterranean species is limited when daily mean temperatures are below 10ºC (Rambal, 2001), a common event in the Mediterranean basin. Mediterranean areas with severe summer drought have less severe cold winters, and vice-versa (Mitrakos, 1980). This climatic trade-off within the Mediterranean region greatly affects plant species distribution. In the Balearic Islands, although cold winter stress can affect plant performance in the mountain areas of Mallorca (Flexas *et al*., 2003; Gulías, 2004), the predominant xeric areas imply that summer drought is the most important constraint for plant performance in this region. Therefore, in the present Thesis drought stress will be considered as the most determinant factor to determine both short term and adaptive plant ecophysiological responses.

Despite the general features that characterise the Mediterranean climate, great climatic differences exist along the basin, even when considering small territories. Within the biggest island of the Balearic Archipelago, Mallorca, this high climatic

variability is reflected by a steep gradient of both precipitation and temperature, which imposes different degrees of environmental stresses. For instance, mean annual precipitation ranges from less than 300 mm in the southern coastal areas to more than 1400 mm in the highest mountains in the north (Guijarro, 1986). Such climatically-variable areas appear as an excellent target to search for species-specific differences in plant adaptation and acclimation processes to environmental constraints.

1.1.2. Main traits of the Mediterranean vegetation types

Although it is difficult to make generalisations on Mediterranean vegetation types, especially when considering the climatic diversity of the Mediterranean climate, some general traits can be defined. There is a great diversity of annual and perennial herbs in the Mediterranean vegetation, while the number of shrubs and tree species is rather low. Drought conditions and disturbances, like human activities, enhance the presence of herb species, while trees and shrubs are more abundant in the less dry and less disturbed areas. However, evergreen sclerophyll shrubs are very characteristic of the Mediterranean flora and they dominate an important part of the Mediterranean landscapes (Di Castri, 1981). In these regions evergreen species are more abundant than deciduous ones. As a consequence, ecological studies have largely focused on evergreen sclerophyll species and less attention has been devoted to deciduous broadleaved species.

Sclerophylly literally describes leaves which are hard, tough and stiffened. This strengthening is primarily a result of increased thickness and/or density of the leaf tissues, and is commonly described in terms of specific leaf weight (Salleo & Lo Gullo, 1990; Witkowski & Lamont, 1991; Groom & Lamont, 1997). Historically, the functional role of sclerophylly has been interpreted as an adaptation to the unique environmental conditions associated with the Mediterranean climate, in different ways: adaptation to drought, adaptation to soil nutrient deficiency and/or adaptation to herbivory (Small, 1973; Poole & Miller, 1975; Levitt, 1980; Mooney, 1982; Turner *et al*., 1993; Turner, 1994; Salleo *et al*., 1997). However, the most common view is that, although sclerophylly is a successful feature in dry environments, it is a consequence of long leaf life span more than an adaptation to drought (Grubb, 1986), since long-lived leaves need more protection against biotic and abiotic damage. Long leaf live span is an adaptation to limited resources availability, nutrients and water (Reich *et al*., 1991), as

occurs in the Mediterranean basin. Moreover, whether sclerophylly is under strict climatic control is questioned by the presence of sclerophyllous vegetation in non-Mediterranean environments, e.g. chaparral in Arizona, heathland communities in eastern Australia (Specht, 1979), and fynbos-like vegetation in the Afromontane region of Africa (Killick, 1979).

Winter deciduousness is a typical feature of cold climates; many Mediterranean species that hold this character have been originated in the Temperate or Irano-Tourarian regions. This feature is clearly disadvantageous in a Mediterranean climate, since the combination of winter deciduousness and dry summers leads to a very short carbon assimilation period, not enough, in many cases, to pay off the costs of the leaf construction (Ne'eman & Goubitz, 2000; Flexas *et al*., 2003).

Among the perennial species, summer semi-deciduous shrubs seem to be the best adapted species to the driest Mediterranean conditions, where they replace the evergreen shrub communities, dominating low maquis (Margaris, 1981; Ne'eman & Goubitz, 2000). Compared to evergreen sclerophylls, the seasonal dimorphism is the most common feature among these plants (Kyparissis & Manetas, 1993). Drought semi-deciduous species avoid excessive water loss with a reduction of their transpiring surface through partial fall of leaves during the dry period (Orshan, 1963). In accord to this decreased water requirement during the months of drought, semi-deciduous shrubs have shallower root systems than evergreen sclerophyll shrubs (Dallman, 1998). Deciduousness has been well correlated with drought-avoiding strategies for Mediterranean plant growth forms (Mooney & Dunn, 1970; Schulze, 1982). However, these growth form-type species are also typically classified as drought-tolerant species, and associated to the possession of a number of physiological and biophysical adaptations that enhance tolerance mechanisms to water stress (Grammatikopoulos, 1999; Werner *et al*., 1999), with intermediate characteristics between evergreen shrubs and herbaceous species (Grant & Incoll, 2005). For instance, regarding to their photosynthetic capacity, semi-deciduous species present somewhat lower photosynthetic capacity compared to annual herbs, but maintain it during a longer period (Flexas *et al*., 2003). By contrast, they have higher stomatal conductance and assimilation rates than evergreen sclerophylls (Ehleringer & Mooney, 1983), but with shorter periods of activity (Mooney & Dunn, 1970).

The Mediterranean region presents a high percentage of herbaceous species (Heywood, 1989). According to Schulze (1982), herbaceous species can be divided in

two main groups: annual and perennial. The former group have a short life cycle which may be completed within a year or less, and the plant usually dies after seed formation. A rapid development after germination is very important for the success of the herbaceous plants, especially those annuals (Schulze, 1982). This group of species is the most effective in colonizing bare lands. On the contrary, perennial herbs may complete their life cycle after two years (biennial) while others require many years (perennial *sensu strictu*). The first implication is that they are exposed to the Mediterranean summer stressing conditions. The perennial herbs differs from annual species in that production is directed to the prolonged survival of the plant individual, whereas annuals rely solely on successive generations of new individuals for the continuation of dry matter production.

1.1.3. The Mediterranean region as a major centre of plant diversity

The importance of the Mediterranean region derives from a number of considerations (based partly on Heywood, 1977). First, the Mediterranean region has been described as one of the world's major centres of plant diversity, containing 11 of the 231 centres selected for their global importance (Davis *et al*., 1994). Moreover, since many crop relatives occur in the Mediterranean basin (Zohary & Hopf, 1993; Heywood & Zohary, 1995), this description can be extended to include one of the centres of diversity for crop plants (Harlan, 1995).

The Mediterranean region, which covers some 2.3 million km^2 that represents some 1.6% of the land surface, yet contains about 10% of the world's flowering plants (Quézel, 1985; Greuter, 1991; Heywood, 1995; Quézel & Medail, 1995), which explains the floristic richness of the Mediterranean region in comparison with adjacent temperate and desert regions. As a whole, the Mediterranean region comprises approximately 24 000-25 000 species (or approximately 29 000-30 500 taxa including subspecies) (Greuter, 1991; Heywood, 1995; Quézel, 1985, 1995). Greuter (1991) found, plotting taxon numbers against area sizes, that the flora of the Mediterranean region as a whole is about twice as large as would be expected, even though deserts make up half of its total surface area adding significantly to its floristic diversity.

In addition to this floristic richness, it is noticeable the high degree of local endemicity of the region. It has been estimated by Quézel that about 50% of the flora is endemic to the region, while Greuter (1991), in his analysis of the flora based on the

published volumes of Med-Checklist, gives an extrapolated figure where nearly 37.5% is considered to be locally endemic (i.e. confined to a single area) and 63.5% endemic to the region covered by the Med-Checklist. Certain areas in the region are especially rich in endemics, among them the islands (Cardona & Contandriopoulos, 1979).

Another characteristic trait of the Mediterranean region is its high degree of human interference and disturbance of the vegetation. This dates back over ten thousand years, and has been responsible for the transformation of much of the native vegetation, leading to the formation of many secondary or subserial communities such as the characteristic shrubland communities (maquis, phrygana, matorral, garrigue, etc.) that form such a conspicuous part of Mediterranean landscapes. Naveh & Dan (1973) described the region as "composed of innumerable variants of different degradation and regeneration phases". The various components of this human disturbance are largely responsible for the high degree of floristic and ecological diversity shown by the region, particularly fire, desertification, grazing and thinning (Le Houérou, 1981; Trabaud, 1981).

Finally, the region presents both a high percentage of annual species in the flora, especially in families such as the *Caryophyllaceae, Cruciferae, Compositae, Umbelliferae*, and a large number of exotic or invasive species that have become established in the region. In a reverse direction, many Mediterranean weeds are noxious weeds in other parts of the world (Heywood, 1989).

1.2. ECOPHYSIOLOGICAL PROCESSES INFLUENCING PLANT DISTRIBUTION AND SUCCESS

According to Lambers *et al*. (1998) the floristic composition at any given site is determined by a series of historical, physiological, and biotic filters, which restrict the actual vegetation to a relatively small number of species. First, many species are absent from a given plant community for historical reasons, which means that they may have evolved in a different region and never dispersed to the study site. Secondly, of those species that arrived to a site, some of them may lack the appropriate physiological traits to survive that particular physical environment. Finally, biotic interactions exert an additional filter that eliminates many species that may have arrived and are capable of surviving the physical environment. Because of biotic interactions, the actual distribution of a species (realized niche as determined by ecological amplitude) is more

restricted than the range of conditions where it can grow and reproduce (its fundamental niche as determined by physiological amplitude).

Historical, physiological, and biotic filters are constantly changing and interacting. Because of these interacting filters, the species present at a site are simply those that arrived and survived. This Thesis undertake studies on physiological filters at three different plant developmental stages, in Mediterranean species: seed, seedling and adult stages, with the aim of understanding critical factors in species success in a Mediterranean context. As has been pointed out in previous section, drought is the main stress limiting plant productivity worldwide (Boyer, 1982), and even in a major extent under the Mediterranean climate (Flexas *et al*., 2003). Moreover, there is a high variability in the extent and duration of drought. Therefore, it might be expected that, within species found over a wide range of Mediterranean environments, differentiation would have occurred as a result of changing selective pressures (Grant & Incoll, 2005). In fact, there is now some evidence that wide physiological or morphological variation exists between populations of some Mediterranean species, both along large-scale climatic gradients (Villar-Salvador at el., 1997) and within smaller geographical areas (e.g. Díaz-Barradas *et al*., 1999). For this reason, the present work will focus on understanding the physiological responses to water stress in several Mediterranean species with different growth forms, and inhabiting different environments, endemic to the Balearic Islands or widespread distributed.

1.2.1. Germination

In seed physiology, germination is usually defined as the total sum of processes preceding and including protrusion of the radicle through the surrounding structures until the radicle becomes visible. After radicle emergence, germination is considered complete and growth commences (Hilhorst & Toorop, 1997). This seed phase is considered as the most important stage in the higher plant life cycle with respect to survival. More specifically, after seed production and dispersal, the significance of seed stage capacity has long been recognized as one of the critical steps in species spatial and temporal establishment success (Harper, 1977; Silvertown & Lovett-Doust, 1993; Hilhorst & Toorop, 1997). The response pattern of seed germination is also regarded as a key characteristic in plant life history strategy (Angevine & Chabot, 1979; Mayer & Poljakoff-Mayber, 1989).

The term ‘germinability’, or ‘germination capacity’, refers to the potential to germinate under specific conditions. The germination capacity may be affected by the level of dormancy, but also by the availability of the environmental cues required for germination. Another crucial aspect of germination concerns its timing in relation to the onset of favourable conditions for seedling development. This timing of germination is controlled by the interplay between the physiological state of the seed (i.e. dormancy) and seed’s responsiveness to environmental factors, such as temperature, moisture, aeration, fire and light (Beardsell & Richards, 1987).

The term ‘dormancy’ refers to a physiological condition in seeds that prevents them form germination, and plays an important part in preventing seed from germinating at times that would be unfavourable for growth and establishment (Fenner & Kitajima, 1999). A dormant seed may be induced to germinate, but only under particular conditions. This is linked with the duration of germination, which is the time elapsing between hydration of the seed and the appearance of the radicle.

Both physiological parameters, i.e. germination capacity and germination duration, that define and characterise seed germination for a given species, are known to be regulated not only through genotypic characteristics (Gutterman, 1993), but also by environmental conditions. After seed hydration occurs, soil temperature is the most important environmental factors controlling seed germination (Beardsell & Richards, 1987). Temperature can affect germination capacity through its effects on seed deterioration, loss of dormancy and the germination process itself (Roberts, 1988). For seeds to be able to germinate, their ‘cardinal temperatures’ must correspond to external conditions that ensure sufficiently rapid and successful development of the young plants. The temperature range for the onset of germination is broad in species that are widely distributed and in those adapted to large temperature fluctuations in their habitat (Larcher, 1995).

The peculiarity of the Mediterranean climate has important implications on plant germination physiology. Dry summer conditions limit water availability and thus germination and growth, while cool winter temperatures also limit germination during the season with high water availability (Rundel, 1996). The germination season, and for extension the temperature requirements for germination, are crucial for plant survival. For instance, it is obvious that a species with high, massive germination relatively independent of temperature might not be favoured, as any short precipitation in late spring would cause its seeds to germinate, but seedlings will not be capable to overcome

the summer stresses (Lloret *et al*., 1999). Moreover, the presence of dormancy that delays germination is often advantageous in a competitive or seasonal environment (Harper, 1977; Vleeshouwers *et al*., 1995).

1.2.2. Seedling establishment and growth

After seed production, dispersal and germination have been successfully achieved, the next step is the establishment of the seedling. Seedling establishment is another critical developmental stage in a plant life cycle, and seedlings within this stage are especially susceptible to those biotic and abiotic factors that limit and constraint plant development and survival. Seedlings are susceptible to many hazards, such as desiccation (Moles & Westoby, 2004), pathogens (Augspurger, 1984), 'winter death' and grazing, as well as competition with the existing vegetation (Kolb & Robberecht, 1996).

One way to study the seedling establishment and development is analysing seedling growth. Growth analysis is a conceptual framework for resolving the nature of genotype × environment interactions on plant growth. It allows to get insights in the growth parameters and to factorise them into physiological (net assimilation rate, NAR) and morphological (specific leaf area, SLA, and leaf mass ratio, LMR) components that determine the plant's carbon economy (Lambers *et al*., 1989). Moreover, it also permits the study of the allocation patterns of the assimilated carbon. In this sense, it is well-known that the allocation of biomass to different plant organs depends on species, ontogeny and on the environment experienced by the seedling (Poorter & Nagel, 2000).

Variation among species in growth rate reflects inter-specific variety in an array of plant physiological traits (Lambers & Poorter, 1992), and arises from evolutionary selection that results in species having certain combinations of those traits (Chapin, 1980). Hence, there is a link between the potential growth rate of a species and its characteristic habitat, functional type, phylogeny, phenology, plant strategy, stress tolerance, successional habit, plant architecture, leaf life span and growth form. Regarding to the habitat, plant species characteristic of favourable environments often have inherently higher maximum relative growth rate (RGR) than species from less favourable environments (Lambers & Poorter, 1992). This fact already points to the relatively low growth rates, and therefore production, of the Mediterranean-type ecosystems compared to other biome-type ecosystems, such as tropical, boreal or

temperate forests (Ehleringer & Mooney, 1983; Schulze, 1982). Regarding to plant growth form, herbaceous species show higher rates of growth than shrubs and trees (Ehleringer & Mooney, 1983; Lambers & Poorter, 1992). With respect to successional habit, early-successional species, as well as annual species, tend to be characterised by high RGRs, while late-successional species and perennials have, in general, a lower RGR (Poorter & Garnier, 1999). Even within a single functional type, differences in RGR, due to, e.g., a different evolutionary history, may lead to differences in competitiveness (Pattison *et al*., 1998; Baruch & Goldstein, 1999; Durand & Goldstein, 2001).

These general patterns describe species adaptation and strategies under close-to-optimal conditions. However, these are generally not the conditions that prevail in the natural environment. In natural environments, growth and development cycles have to be completed within a time frame dictated by environmental conditions where light, temperature, moisture and nutrients often limit the expression of genetic potential (Atwell *et al*., 1999). Among these factors, water stress is again a major limiting factor for RGR in semi-arid climates, such as the Mediterranean, strongly constraining both growth and seedling survival during late spring and summer (Volaire *et al.*, 1998; Moles & Westoby, 2004). In addition to a decreased water uptake, drought may also have an indirect effect on growth, reducing nutrient uptake, as the delivery of nutrients by mass flow is hampered in dry soil (Marschner, 1995). Growth is primarily reduced by low water availability due to inhibition of very sensitive processes, such as leaf cell elongation and protein synthesis, which in global affect plant expansion (Bradford & Hsiao, 1982). Water stress induces changes in seedling development and allocation patterns, by increasing the allocation of biomass below than above ground, decreasing the evaporative surface, and increasing the leaf mass per unit leaf area (Ludlow, 1989). It is also commonly observed that roots of unwatered plants grow deeper into the soil than those of well-watered plants (Sharp & Davis, 1985), which is related to promote, together with an increased allocation biomass to underground tissues, substantial improvement in plant yield under water stressed conditions (Jordan *et al*., 1983).

It has been suggested that it is particularly important to study differences in the adaptability to water stress at the seedling stage because morphological and physiological attributes during this period are key factors for the recruitment and survival of species (Donovan & Ehleringer, 1992). Furthermore, the analysis of growth in terms of RGR and its components in Mediterranean species comprising different

growth forms may explain some of the still existing uncertains over the underlying causes of drought-induced changes in seedling development.

1.2.3. Plant water relations and stomatal regulation

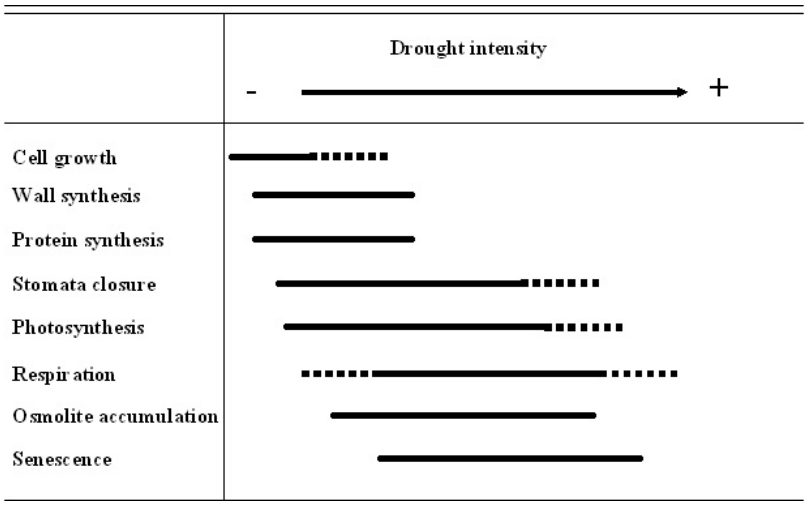
Life evolved in water, and water remains the essential medium in which biochemical processes take place.

Plant water relations are a large and diverse subject, and plant responses to water scarcity are complex. Plants are composed mainly of water, and on average the protoplasm contains 85-90% water. Terrestrial plants growing in any natural environment are rarely free from water stress during more than a few days. Even in the humid tropics, the plants undergo brief stress due to dynamic changes in the climatic conditions (Grace *et al*., 1995). However, it is in the arid and semi-arid regions, such as the Mediterranean basin, where water becomes the paramount factor in determining the distribution of species, and where the responses and adaptations of a species to water stress are critical for its success.

Plant water status is determined by the balance between water losses in transpiration to the atmosphere and water absorption from the soil. When transpiration exceeds absorption, cell turgor falls, which negatively affects physiological processes and therefore their productivity, distribution and competitive relationships (Tretiach, 1993; Damesin *et al*., 1998). Numerous studies have reported a myriad of changes in physiological processes by water stress (Fig. 1.1).

Figure 1.1

Theoretical sequence of metabolic processes as affected at different levels of water stress. Modified from Hsiao (1973).



However, species inhabiting in drought-prone environments, among them the Mediterranean plants, present different strategies which enable them to overcome soil water deficits (Schulze, 1988; Joffre *et al*., 1999). Classically, plant resistance to drought has been divided into escape, avoidance and tolerance strategies (Levitt, 1980; Turner, 1986). Escape strategies rely on successful reproduction before the onset of severe stress. Plants can also endure drought conditions by avoiding tissue dehydration, while maintaining tissue water potential as high as possible, or by tolerating low tissue water potential. Dehydration avoidance is common to both annuals and perennials and is associated with a variety of adaptive traits, which involve minimising water loss and maximising water uptake (Chaves *et al*., 2003). On the other hand, drought tolerance is determined by a number of mechanisms that increase tolerance of plant tissues to dehydration (Kramer, 1988). Dehydration tolerant species function under low plant water potentials to facilitate water uptake from drying soils by maintaining a soil-to-leaf water potential gradient, which also facilitates a rapid recovery after drought (Tschaplinski *et al*., 1998). Both high osmotic potential and low elasticity help in rapid decreases of water potential with a given change in water content (Abrams, 1990).

Minimising water loss is strongly related to tight stomatal regulation. Stomatal apertures are the major pathway for the movement of CO_2 from the atmosphere into the photosynthetic mesophyll of leaves. Stomatal aperture appears to be controlled by complex mechanisms which operate to maintain a variable balance between allowing CO_2 uptake to proceed, while restricting the loss of water vapour, and preventing leaf desiccation (Schulze & Hall, 1982). Hence, stomatal morphological and physiological aspects are of key interest in the study of drought-adaptation to semi-arid conditions. Stomatal morphological features, including density and size, they have been shown to vary widely among species, showing evidence of adaptive acclimation and heritable variation (Hetherington & Woodward, 2003; Masle *et al*., 2005; Pearce *et al*., 2006). For instance, stomatal size is smaller and stomatal density greater in species typical of xeric environments (Dunlap & Stettler, 2001; Pearce *et al*., 2006). Finally, stomatal closure is a common response to drought stress, but there is also a considerable interspecific variability in the sensitivity of the response, as evidenced, for example, by the water potential where the stomata closes (Poole & Miller, 1975; Duhme & Hinckley, 1992; White *et al*., 2000; Mediavilla & Escudero, 2003, 2004). These differences in the ability to regulate stomatal conductance are of main interest in

Mediterranean focussed studies, since they may contribute to determine individual species persistence on a changing environment (Abril & Hanano, 1998).

1.2.4. Photosynthetic processes

Life on earth is carbon based and is sustained by photosynthetic use of energy from sunlight to fix atmospheric. Understanding the biophysical, biochemical, and physiological basis for the impairment of photosynthesis in plants which experience internal water deficits becomes of major interest in order to improve plant responses to environmental stresses.

There has been some controversy regarding the main physiological targets responsible for photosynthetic impairment under drought (Boyer, 1976; Sharkey, 1990; Chaves, 1991; Cornic & Massacci, 1996; Massacci & Loreto, 2001; Flexas & Medrano, 2002a; Lawlor & Cornic, 2002; Loreto *et al*., 2003). However, there is now substantial consensus that reduced CO_2 diffusion from the atmosphere to the site of carboxylation is the main cause for decreased photosynthesis under most water stress conditions (Chaves & Oliveira, 2004; Flexas *et al*., 2004a). Such reduced leaf diffusive capacity is due to at least two components that are regulated almost simultaneously: stomatal closure and reduced mesophyll conductance. Although restricted CO_2 diffusion across leaves is likely to be the most usual cause for decreased photosynthesis rates under water stress, metabolic impairment may also occur, particularly under severe stress (Flexas & Medrano, 2002a; Lawlor & Cornic, 2002; Flexas *et al*., 2006). Furthermore, photosynthetic capacity also depends on the efficiency of the process of CO_2 fixation into organic compounds, which is related to the affinity of Rubisco for CO_2 with respect to O_2, i.e. the Rubisco specificity factor (Roy & Andrews, 2000; Lawlor, 2001).

In the following sections, the importance of diffusive, both stomatal and mesophyll, and metabolic limitations on photosynthetic impairment by water stress will be discussed, as well as the importance of an improved specificity of Rubisco under drought.

1.2.4.1. CO_2 diffusion: stomatal and mesophyll limitations

For photosynthesis to occur, carbon dioxide must diffuse from the atmosphere to the sites of carboxylation, in the chloroplast stroma. Carbon dioxide in the chloroplast stroma is removed by the RuBP carboxylase reaction and a gradient of CO_2 develops

across the chloroplast envelope, cytosol, cell membranes and walls to the intercellular spaces and, via the stomata, to the ambient air. This gradient is the driving force for CO_2 diffusion, but the rate at which diffusion to the reaction site occurs depends on the conductances to CO_2 diffusion in the gas and liquid phases in the leaf and atmosphere and on external CO_2 concentration (Lawlor, 2001). In the gaseous phase, CO_2 must diffuse across a boundary layer in the air above the foliage surface, through stomatal opening and across intercellular air spaces in the sub-stomatal cavity (Warren *et al*., 2004). In the liquid phase there are resistances as CO_2 enters the liquid phase at the surface of mesophyll cells, as CO_2 diffuses within the cell to the chloroplast membrane and from there to the sites of Rubisco (Aalto & Juurola, 2002). The stomatal (including the boundary layer) and the mesophyll (or internal) conductances account then for the gaseous and liquid phases of CO_2 diffusion and, therefore, determine CO_2 concentrations at the sub-stomatal (C_i) and chloroplastic (C_c) levels, respectively.

An assumption of much gas exchange work, including the original photosynthesis model of Farquhar *et al*. (1980), has been that C_i approximates CO_2 concentration at Rubisco (C_c), i.e. that $C_i = C_c$. However, there is now mounting evidence that this assumption is invalid and C_c may be significantly less than C_i (Evans *et al*., 1986; Harley *et al*., 1992; Loreto *et al*., 1992; Epron *et al*., 1995; von Caemmerer, 2000; Warren *et al*., 2004; Flexas *et al*., 2006).

Stomatal closure is widely documented to be one of the most sensitive and earliest leaf physiological responses to water stress (Hsiao, 1973; Boyer, 1976; Sharkey, 1990; Chaves, 1991; Lawlor, 1995; Cornic & Massacci, 1996; Flexas *et al*., 2002). Stomata closes as drought progresses to limit water loss from the leaf. However, this water-saving strategy is irremediably followed by parallel decreases on net photosynthesis. Furthermore, as stated above, not only stomatal barriers affect the CO_2 availability for carboxylation, but also the presence of leaf mesophyll barriers limits the rate of photosynthesis. There is increasing evidence that plants regulate mesophyll conductance to CO_2 in response to varying environmental conditions, such as water stress (Renou *et al*., 1990; Roupsard *et al*., 1996; Flexas *et al*., 2002, 2004a; Warren *et al*., 2004), which perhaps could be linked to a fine and rapid regulation of expression and/or activity of plasma membrane aquaporins (Uehlein *et al*., 2003; Hanba *et al*., 2004).

In addition to this rapid regulation of mesophyll conductance in response to water availability, mesophyll conductance appears related to leaf morphology and

anatomy (Vitousek *et al*., 1990; Parkhurst & Mott, 1990; Evans *et al*., 1994; Syvertsen *et al*., 1995; Kogami *et al*., 2001), although this correlation may be weak (e.g., Hanba *et al*., 2002). The potential for CO_2 diffusion in the liquid phase is a function of cell wall thickness (Nobel, 1991) and the surface area of mesophyll cells or chloroplasts exposed to the intercellular air spaces (Laisk *et al*., 1970; Nobel *et al*., 1975; Nobel, 1991; Evans *et al*., 1994).

As previously discussed, the natural vegetation of Mediterranean areas results in a high diversity of forms, which differ, among other characters, in leaf morphology (Ehleringer & Mooney, 1983). Evergreen sclerophyll trees and shrubs, typically of Mediterranean evergreen shrubs, are characterized by thick cuticles, numerous sclereids and fibres, lignified epidermal cells and increased cell wall thickness (Lo Gullo & Salleo, 1988), which improve the resistance of foliage to drought (e.g., Niinemets, 2001) at expenses of decreases in leaf internal conductance (Evans & Loreto, 2000). On the other hand, the thin and porous malacophyll leaves of drought tolerant semi-deciduous shrubs are likely to present relatively lower mesophyll limitations to CO_2 assimilation.

1.2.4.2. Photosynthetic metabolism

While a reduced CO_2 diffusion from the atmosphere to the site of carboxylation is the main cause for decreased photosynthesis under most water stress conditions (Chaves & Oliveira, 2004; Flexas *et al*., 2004a), metabolic impairment appears when water stress becomes severe (Boyer, 1976; Lawlor, 1995; Tezara *et al*., 1999; Flexas & Medrano, 2002a; Lawlor & Cornic, 2002).

Evidences for metabolic impairment under drought stress have been assessed both by *in vitro* and *in vivo* measurements. Regarding to *in vitro* measurements, they have been mostly focussed on the activity of some enzymes and the size of some enzymatic pools of intact leaves submitted to water stress. This approach has led to different conclusions. Hence, while some studies have demonstrated strong reductions of leaf RuBP content (Giménez *et al*., 1992; Gunasekera & Berkowitz, 1993), others have shown constant contents over a drought period (Lal *et al*., 1996). Similarly, some reports have shown strong drought-induced reductions of Rubisco activity (Maroco *et al*., 2002; Parry *et al*., 2002), but others have observed no effect of drought (Lal *et al*., 1996; Pankovic *et al*., 1999). Recently, Flexas *et al*. (2004a), compiling data from literature, stated that metabolic impairment eventually appears when the stomatal

condictance (g_s) falls below a certain threshold (generally lower than 0.15 mol H_2O m^{-2} s^{-1}).

Regarding *in vivo* metabolic impairment measurements, they have been mostly based on the interpretation of A_N-C_i curves over a drought experiment. Hence, it has been suggested that both the capacity for ribulose-1,5-bisphosphate (RuBP) regeneration and the maximum velocity of carboxylation by Rubisco ($V_{c,max}$) are substantially reduced under drought, and each of these processes has been proposed to be the main limitation to photosynthesis imposed by drought under saturating light and current atmospheric CO_2 concentrations (Wilson *et al*., 2000; Centrito *et al*., 2003; Xu & Baldocchi, 2003; Peña-Rojas *et al*., 2004; Aranda *et al*., 2005). However, it is controversial whether A_N-C_i analysis is reliable under drought. Among other considerations, as discussed in the previous section, drought-induced changes in the mesophyll conductance, which may invalidate conclusions derived from A_N-C_i analysis, for instance, underestimation of $V_{c,max}$ (Flexas *et al*., 2002; Grassi & Magnani, 2005; Flexas *et al*., 2006). Effectively, the few studies that have converted A_N-C_i into A_N-C_c curves show that Mediterranean plants presents a fairly conservative carboxylation capacity until drought stress becomes severe (Grassi & Magnani, 2005).

In summary, there are still some uncertain in drought-induced stomatal and non-stomatal limitations to photosynthesis. The Mediterranean vegetation attains resistance by different traits which may vary considerably among species.

1.2.4.3. The efficiency of the carbon fixation: the specificity of Rubisco

The biochemistry supporting life on earth depends, in terms of gain of energy, on oxidative reactions. The final product of metabolic pathways based on carbon is carbon dioxide, which is released into the atmosphere. To close the carbon cycle it is necessary to feed the carbon dioxide back to the food chain. The only enzyme capable of this task is a protein located at the chloroplast stroma, Rubisco (Ribulose-1,5-bisphosphate carboxylase/oxygenase, EC 4.1.1.39) (Andrews & Lorimer, 1987). Rubisco then comprises the starting point of any food chain. Rubisco plays its role in a cyclic, autocalatytic process, called the Calvin cycle, reductive pentose phosphate pathway or photosynthetic carbon reduction cycle (PCR cycle), and its importance resides in catalysing the first step, the reaction of CO_2 with an acceptor molecule, ribulose bisphosphate (RuBP), producing 3-phosphoglycerate (3PGA).

Besides its unique role in incorporating carbon from atmospheric CO_2 into the organic substances of the biosphere, Rubisco has a peculiar catalyst inefficiency: its carboxylase activity is reduced by a major competing reaction with another atmospheric gas, O_2, involving the same active site as the natural substrate, CO_2. The opposing oxygenase activity of Rubisco catalyses the first reaction in the photorespiratory pathway (Ogren & Bowes, 1971; Laing *et al*., 1974), causing in C_3 plants the loss of up to 50% of the carbon fixed by Rubisco and greatly decreasing the efficiency with which light energy is used (Zelitch, 1973). The balance between the two competitive reactions is determined by the kinetic properties of Rubisco and the CO_2 and O_2 concentrations at the site of the enzyme. Therefore, the specificity (τ) expresses the intrinsic carboxylation capacity of Rubisco relative to oxygenation and is used as a measure of the efficiency of Rubisco from different organisms. The comparison of τ values, which vary over a 20-fold range, from divergent photosynthetic organisms shows that Rubisco specificity increases from the lower photosynthetic forms to higher plant forms (Jordan & Ogren, 1981; Tortell, 2000). Despite the strong phylogeny-dependence of Rubisco specificity, it has been hypothesised that the enzyme could evolve according to the organisms' specific needs for CO_2 assimilation depending on environmental conditions in which the organism evolved (Horken & Tabita, 1999). More specifically, Delgado *et al*. (1995) and Kent & Tomany (1995) hypothesised that hot environments associated with water stress may impose increased selection pressure on Rubisco for improved specificity. Such postulation may be based on the following facts:

- Temperature dependence of τ. Rubisco τ decreases with increasing temperature (Chen & Spreitzer, 1991).
- Under water stress conditions, leaf conductances to gas diffusion decrease and, as a consequence, the CO_2 concentration at the site of Rubisco (C_c) decreases (Flexas *et al*., 2004a). Rubisco is estimated to work at a C_c just one-quarter of its effective K_c under optimal conditions (Sharkey, 1998). Therefore, under water limitation, C_c may be far lower than its effective K_c, increasing the ratio of photorespiration to photosynthesis (Flexas & Medrano, 2002a).

Although it is well known that Mediterranean species are well adapted to drought, the Mediterranean basin is characterised by a long dry, hot summer, which severely stresses plants and influences their distribution. Within the Balearic Islands there is a steep gradient of both precipitation and temperature, which may impose

different degrees of such environmental stresses. Therefore, such a climatically-variable area appears ideal to search for species with specific differences in τ.

In addition to these hypothetically adaptation processes on Rubisco kinetics, another way of variation in the specificity of the enzyme could exist: a short-term acclimation to environmental stresses. The Rubisco large subunit is encoded by multiple identical copies of *rbc*L in the chloroplast genome (Eilenberg *et al.*, 1998), whereas a *rbc*S gene family having 2 to 12 nuclear genes encodes small subunit peptides (Dean *et al.*, 1989; Spreitzer, 2003). Thus, while the copies of the large subunit would likely be the same, the differential expression of *rbc*S genes may depend on the environment. For instance, transcription of specific *rbc*S genes appears to be dependent on light quality in the fern *Pteris vittata* (Eilenberg *et al.*, 1998). Although large subunits have the main influence on catalytic properties, the Rubisco small subunits have also been hypothesized to affect key kinetic characteristics, like τ (Roy & Andrews, 2000; Parry *et al.*, 2003). Hence, in principle, any environmental condition capable of modulating *rbc*S gene expression could induce changes in Rubisco specificity.

1.2.5. Leaf respiration and carbon balance

A large portion of the carbohydrates that plants assimilate is used in respiration, which is needed to produce the energy and carbon skeletons to sustain plant growth (Lambers *et al.*, 2005) Hence, the carbon economy of plants, which will finally determine growth, can be viewed in terms of balance between the carbon fixation processes (i.e., photosynthesis) and the carbon consuming processes (i.e., respiration). Much is know about the metabolic pathways of the respiratory system, however, and opposing to the abundant studies on the effects of environmental stresses on photosynthesis, much less is known on how respiration is affected by external conditions. For instance, the regulation of respiration by drought has been only scarcely studied and debated (Flexas *et al.*, 2005, and references therein). However, since photosynthesis is limited temporally (i.e., to daytime hours) and spatially (i.e., to green biomass) and respiration occurs continuously in every plant organ, the latter may be the most important factor controlling productivity, particularly when photosynthesis is largely depressed, such as under drought conditions. Nevertheless, and in spite of its well-recognized importance, the regulation of respiration by drought at the plant physiological level is largely unknown, partly because only a limited number of studies

are available and partly because of the apparent contradictions among these studies. Certainly, the available experimental evidences do not support a clear pattern of respiration response to drought, different studies showing either increased, unaffected or decreased rates of respiration (Hsiao, 1973; Amthor, 1989; Flexas *et al*., 2005).

The lack of enough studies on plant respiration responses to drought is especially dramatic in Mediterranean species. Mediterranean vegetation, which suffers extreme variations of water availability, has developed an array of adaptations to such stressing conditions, which may include essential clues for a better understanding of the effects of drought on respiratory rates and, for extend, on carbon balance.

1.2.6. Photoinhibition and photoprotection

Photochemistry seems a likely target for water stress inhibition of photosynthesis under Mediterranean summer conditions, where plants are subjected to high light and temperature in addition to water deficit. However, a high stability of dark-adapted PSII efficiency (F_v/F_m) has been demonstrated under mild to moderate drought in well-adapted species, such as Mediterranean evergreen sclerophylls (Epron & Dreyer, 1990; Epron & Dreyer, 1992, 1993; Epron *et al*., 1992; Méthy *et al*., 1996; García-Plazaola *et al*., 1997; Epron, 1997; Faria *et al*., 1998; Werner *et al*., 1999; Martínez-Ferri *et al*., 2000; Flexas *et al*., 2001; Gulías *et al*., 2002), deciduous sclerophylls (Epron & Dreyer, 1990; Dreyer *et al*., 1992; Damesin & Rambal, 1995; Gulías *et al*., 2002), and annual species (Stuhlfauth *et al*., 1988, 1990). In these species, only when drought is severe enough, dark-adapted PSII efficiency slightly declines. Mediterranean summer semi-deciduous shrubs seem to be an exception to this rule, and some of them rapidly lose dark-adapted PSII efficiency in response to drought (Karavatas & Manetas, 1999; Werner *et al*., 1999; Zunzunegui *et al*., 1999; Munné-Bosch & Alegre, 2000a,b).

Under conditions of illumination under drought, as photosynthesis decreases, a larger portion of the incoming light becomes in excess of that used for photosynthetic assimilation. It is generally observed that when drought is severe, the plants present a somewhat decreased electron transport rate (ETR) (Demmig-Adams *et al*., 1989; Epron *et al*., 1993; Epron, 1997; Faria *et al*., 1998; Peñuelas *et al*., 1998; Gulías *et al*., 2002). However, when gas exchange is measured simultaneously with chlorophyll fluorescence, it is shown that electron transport is reduced by drought to a lesser extent

than CO_2 assimilation (Valentini *et al*., 1995; Méthy *et al*., 1996; Faria *et al*., 1998; Munné-Bosch *et al*., 1999a; Gulías *et al*., 2002). This may be due to an increased electron transport to alternative energy-dissipation pathways, mostly photorespiration and Mehler reaction, which could provide photoprotection against excess light, through drainage of excess electrons (Kozaki & Takeba, 1996; Osmond *et al*., 1997; Flexas & Medrano, 2002c).

The drought-induced excess light decreases the rate of photosynthetic electron transport increases in parallel the rate of thermal dissipation, as indicated by increased NPQ (Stuhlfauth *et al*., 1988, 1990; Epron & Dreyer, 1992; Faria *et al*., 1998; Flexas *et al*., 2002c; Gulías *et al*., 2002). Increased thermal energy dissipation and reduced electron transport under excess light can be occasioned mainly by two different phenomena: increased photoprotection ('dynamic photoinhibition', *sensu* Osmond, 1994) and/or photodamage ('chronic photoinhibition', *sensu* Osmond, 1994). The former describes changes that are reversed within an hour upon returning leaves to low light conditions, while the second term refers to changes whose reversion takes place in several hours (Osmond, 1994; Osmond & Grace, 1995). The term 'permanent photoinhibition' indicates changes in the photosynthetic efficiency, which did not reverse overnight (Long *et al*., 1994). In Mediterranean plants, drought enhances 'dynamic photoinhibition' and, to a lesser extent – at least in well acclimated sunny leaves –, 'chronic photoinhibition' and 'permanent photoinhibition', except in some semi-deciduous shrubs (Werner *et al*., 2001). Therefore, Mediterranean plants may have developed effective ways to avoid or delay permanent photoinhibition.

The most obvious way to avoid excess irradiance during drought periods is to avoid its capture. This can be achieved by the particular orientation of the leaves, which has been termed structural photoprotection, and is typical of many Mediterranean plants (Puigdefábregas & Pugnaire, 1999; Valladares & Pugnaire, 1999). Another way to reduce excess light capture is to reduce chlorophyll content, which has been shown an adaptive mechanism typical of some Mediterranean species, mainly found in semi-deciduous malacophylls (Kyparissis *et al*., 1995; Munné-Bosch & Alegre, 2000a, b), but also in *Pinus halepensis* (Elvira *et al*., 1998). Finally, non-glandular leaf hairs are known to modify the internal radiation environment of a leaf (Karabourniotis & Bornman, 1999). Leaf pubescence has been reported to be an adaptation to the Mediterranean environment by reducing transpiration, increasing the probability of

water uptake by leaves, maintaining favourable leaf temperature, and protecting against UV-B radiation responsible for photosynthetic inhibition (Savé *et al*., 2000).

In addition to avoiding light capture, plants have evolved other mechanisms to cope with the excess light absorbed. The most important physiological mechanism associated with photoprotection consists on a thermal dissipation of the energy absorbed in the antenna of PSII, which involves the so-called xanthophyll cycle and the increased trans-tylakoid ΔpH (Demmig *et al*., 1987; Demmig-Adams & Adams, 1992). Although the precise mechanism of photoprotection involving the xanthophyll cycle is not yet clarified (Gilmore, 1997; Niyogi, 1999; Li *et al*., 2000), a close correlation is often observed between NPQ and the concentration of zeaxanthin or the de-epoxidation state of the cycle (DPS), the correlation being similar for different species (García-Plazaola *et al*., 1997; Demmig-Adams, 1998; Fleck *et al*., 1998, 2000; Gulías *et al*., 2002). However, no correlation was observed between DPS and NPQ in some Mediterranean species (Faria *et al*., 1998; Munné-Bosch & Alegre, 2000c; Gulías *et al*., 2002).

Other photoprotective mechanisms have been related to increased antioxidant activity (Kyparissis *et al*., 1995; Faria *et al*., 1996; Elvira *et al*., 1998; Schwanz & Polle, 1998) and volatile compound synthesis (Tyson *et al*., 1974; Ross & Sombrero, 1991; Loreto & Sharkey, 1993; Bertin & Staudt, 1996; Llusià & Peñuelas, 1998).

1.3. THE IMPORTANCE OF BIODIVERSITY AND THE ROLE OF ENDEMIC SPECIES IN THE MEDITERRANEAN INSULAR ECOSYSTEMS

Global biodiversity is changing at an unprecedented rate as a complex response to several human-induced changes in the global environment (Pimm *et al*., 1995). Five *drivers of change* have been identified for terrestrial ecosystems as the most important determinants of such changes: changes in land use, atmospheric CO_2 concentration, nitrogen deposition and acid rain, climate, and biotic exchanges (Sala *et al*., 2000). Gene pool erosion can occur as a direct result of these changes or indirectly due to fragmentation and isolation of previously intact areas, which reduce habitat requirements below the minimum needed for population and species viability. Among terrestrial biomes, it is predicted that Mediterranean ecosystems, as well as grasslands, will experience the largest biodiversity loss, because of their sensitivity to all drivers of biodiversity change, particularly land-use change and biotic exchanges (Sala *et al*.,

2000). This biodiversity loss, yet now, affects the stability, productivity and other aspects of the functioning of natural ecosystems, such as the intespecific relationships. Diversity is also functionally important, because it is linked to an increase of the endemic taxa, with the concomitant enrichment of the genetic resources (Vitousek *et al*., 1997; Mooney & Hobbs, 2000). The Balearic Islands are not an exception from this general rule, and as occurred in other Mediterranean islands, the human establishment, approximately 5000 years ago (Alcover *et al*., 2001), resulted in profuse changes in the structure and composition of the insular ecosystems, with the introduction of new animal and plant species, and causing the extinction of many others, like the well-documented case of *Myotragus balearicus* (Alcover *et al*., 1981; Bover & Alcover, 2000).

The term biological diversity (often shortened to 'biodiversity') is a measure of the variation in genes, species and ecosystems, and is most commonly taken as a synonymous of the species richness of an ecosystem, although it may also be applied at higher or lower points in the taxonomic hierarchy (Whittaker, 1998). As introduced in a previous section, the Mediterranean region is one of the world's major centres of plant diversity. In addition to this biome diversity richness, both in species and growth forms, it is well established that islands contribute disproportionately for their area to the biodiversity of the Mediterranean region. Islands can be considered as species poor for their size but rich in forms found nowhere else, i.e. endemic to that island or archipelago. It is in this sense that islands warrant the description as 'biodiversity hot-spots', constituting authentic centres of speciation and genetic source conservation (Eliasson, 1995). In this context, the Balearic Islands, with approximately a 7% of its flora being endemic (Cardona & Contandriopoulos, 1979), contribute to the maintenance of a high biodiversity in the Mediterranean basin. Moreover, the Balearic Islands, with approximately 5000 km^2 are considered a rich area in endemic species (Bykov, 1979), as well as the Iberian Penninsula (Bykov, 1979, 1983), Corsica (Gamisans *et al*., 1985), Crete (Valentine, 1972), and in general the whole southern Europe. Each of about 150 Balearic endemic taxons show different and unique cases, from species already gone extinct in the wild, as *Lysimachia minoricensis*, through species in strong danger of extinction, as *Ligusticum luteri*, *Apium bermejoi* and *Cymbalaria fragilis*, to many others that apparently do not suffer any severe threat, as *Astragalus balearicus* (Alomar *et al*., 1997). Despite differences between species, what can be generalized is that this high proportion of endemic species confined to a few

geographically very restricted populations leads to a high fragility of island ecosystems (Carlquist, 1974). This involves a higher risk of extinction for island species compared to those growing in continental areas (Alcover *et al*., 1999; Hylton-Taylor, 2000). For instance, of the 4251 threatened taxons of the whole Mediterranean basin, up to 48 of them inhabit only in the Balearic Islands (Mus, 1995).

Communities and their component species are inevitably exposed to changes in the environment. Drastic impacts may affect their species composition. More commonly, changes result in adaptive responses moderated by the available genetic diversity of the organisms represented in a community. The integrated genetic diversity within an ecosystem constitutes its evolutionary potential (Frankel *et al*., 1995). By a human point of view, the maintenance of biodiversity is valuable in terms of social benefits:

- First, the species presently inhabiting Earth provide ecosystem goods and services. For instance they act as catalysts of energy capture and production of materials, but also recycle wastes, drive global biogeochemical cycles, regulate global and local climate, generate soil fertility, and may serve for recreation purposes.
- Second, biodiversity, viewed at its elemental level, consists of differences between individuals and species in the presence of particular DNA sequences, or their location in the genome. This means that genes contained in natural populations may be viewed as a focus of conservation interest and so considered a ‘resource’. Two reasons are pre-eminent: this genetic variation is a resource for the species’ own survival and its future evolution and, second, a fraction of the genes are a potential source for improving the productivity of other populations or species (Fehr, 1984; Roelfs, 1988). Nowadays, modern biotechnology and genetic engineering provide new tools to extract useful genes from wild species and, unconstrained by mating barriers, to transfer them to related or unrelated species. Mediterranean plants, in particular, may have developed an array of different adaptation to drought, and therefore constitute a potential source of interesting genes to improve agriculture in arid regions (Schulze, 1988).
- Third, a high biodiversity is linked to large inter-specific differences in the response of environmental conditions. In this sense, understanding adaptation and acclimation responses of natural plant population to constrained environments, such as low water availability, can provide essential rules for crops improvement, in terms of, first, detecting key factors determining plant success and productivity, and second,

helping plant breeders to identify critical traits and clues among natural vegetation that may serve as a guide in the research of more efficient crop wild relatives. For instance, a strategy to improve crop water-use efficiency would be to generate crop plants with high specificity factor and catalytic rate (Spreitzer & Salvucci, 2002; Parry *et al.*, 2003). In this sense, exploiting existing variability in Rubisco specificity among C_3 plants appears a promising way to obtain genetically modified crops with improved yield under drought (Parry *et al.*, 2005).

- Fourth, taking into account the present situation and the future perspectives, with increasing demographic pressures in the Mediterranean basin, efforts will be required in order to maintain the characteristic landscape of the region, which supposes the major reclaim for the maintenance of the main economic activity of the region, tourism. In this sense, an increased knowledge of the biology of the endemic species becomes an essential step towards an efficient and successful landscape management that may ensure the maintenance of the genetic heritage of the region.

Chapter 2
OBJECTIVES AND OUTLINE

2.1. GENERAL OBJECTIVES

As stated in the Introduction, plants inhabiting the Mediterranean region are subjected to fluctuating environmental conditions. Summer drought, in particular, imposes strong limitations to plant productivity and survival. Such stressing conditions may have acted as driving forces for evolution, enforcing plants to develop an array of adaptation and acclimation strategies to ensure species competitiveness.

In the present Thesis, the ecophysiology of a number Mediterranean plants, representative of the most important growth forms and inhabiting the Balearic Islands, has been analysed in terms of germination, seedling growth and establishment, water status and stomatal regulation capacity, diffusive and metabolic limitations to photosynthesis, photoinhibition and photoprotection capacity, leaf dark respiration and carbon balance and the efficiency of carbon fixation into organic molecules. All these physiological traits except germination have been analysed under different degrees of water availability.

The general objectives of the present work were: (1) to analyze how biodiversity and adaptation to Mediterranean climate is reflected in a diversity of ecophysiological traits and their responses to drought; and (2) to study whether such diversity was related to growth forms and / or endemicity.

2.2. SPECIFIC OBJECTIVES

1. To compare plant establishment capacity – from germination to early growth under different water availabilities – in a range of Mediterranean species, belonging to different taxonomic, evolutionary and growth form groups.

2. To analyse the diversity in water relations and stomatal regulation among Mediterranean species representative of several functional groups.

3. To study the detailed photosynthetic responses to water stress and re-watering in these species, as well as to make a preliminary survey of how respiration – the other component of plant carbon balance – responds to water stress.

4. To elucidate the diversity among Mediterranean species in the responses of photoinhibition and photoprotection to water stress.

5. To survey the variability in the Rubisco specificity factor among Mediterranean species and to discern whether such variability corresponds to evolutionary or adaptative trends.

6. To determine whether ecophysiological responses to drought could have contributed to the limited success of *Lysimachia minoricensis*, the only endemic species from the Balearic Islands that is currently extinct in the wild but preserved in nurseries.

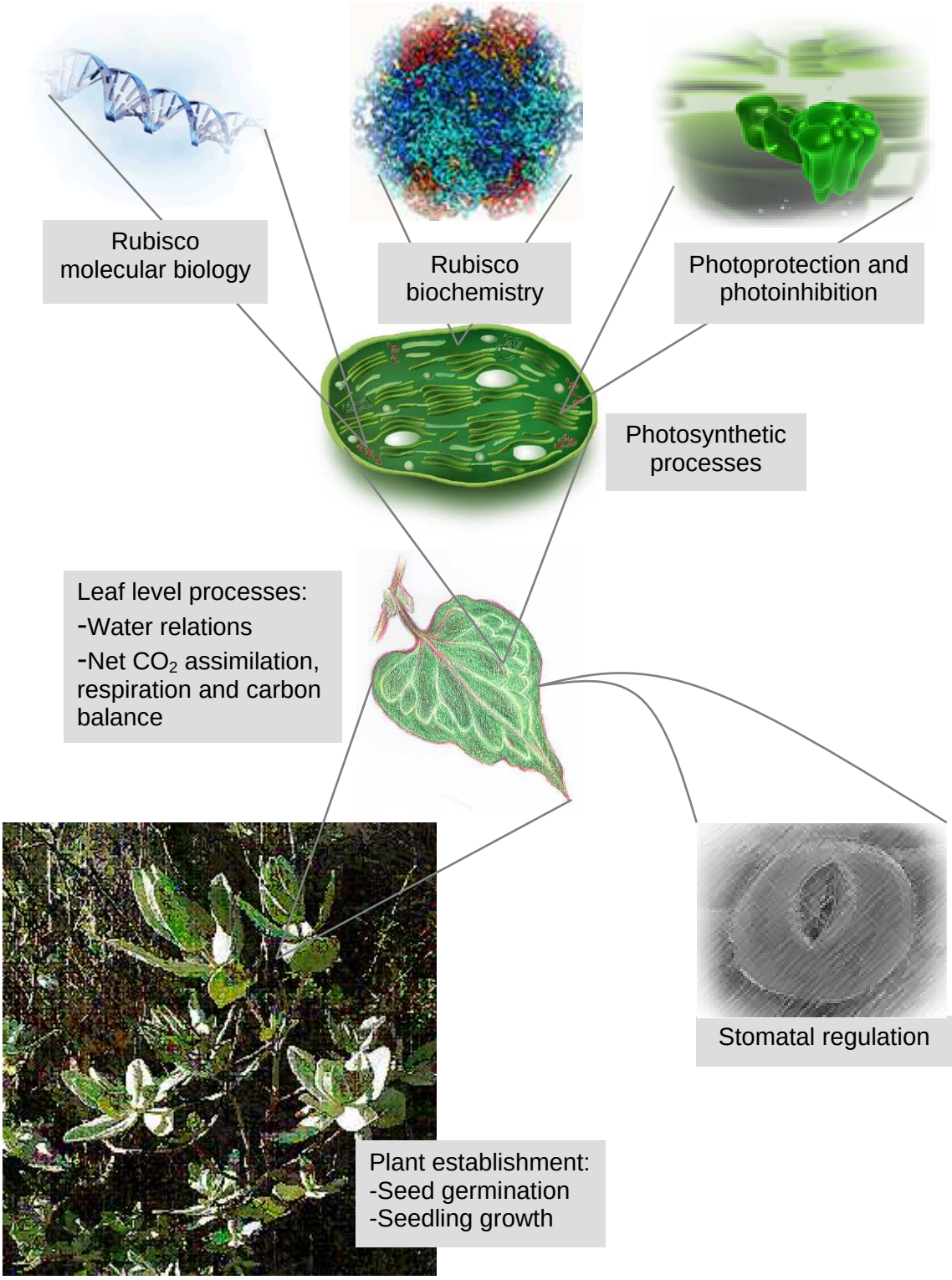
7. To check whether differences between varieties of *Digitalis minor* in a specific morphological trait, such as the presence of leaf trichomes, provides some ecophysiological advantage under the Mediterranean climate.

2.3. OUTLINE OF THIS THESIS

The contents of this thesis are organised in 14 chapters that cover the study of adaptation and responses of Mediterranean species to the typical stressing environments of this region. The existence of such adaptations were analysed at different levels (Fig. 2.1), from the organism level (e.g. seedling growth) to genome studies (i.e. Rubisco molecular biology approach), through the organ (e.g. leaf respiration and carbon balance) and organelle (e.g. photoprotection and photoinhibition) levels.

Figure 2.1

Different concretion levels of the studies included in the present Thesis.



Chapter 1: INTRODUCTION

This chapter introduces the background and sets the context for this Thesis. It includes a general overview of the Mediterranean climate and vegetation main traits, as well as the ecophysiological factors determining plant distribution and success, and the importance of the biodiversity in the Mediterranean basin.

Chapter 2: OBJECTIVES AND OUTLINE

In this chapter the general and specific objectives are presented, as well as a brief outline of the Thesis. Finally, the publications derived from this Thesis are listed.

Chapter 3: MATERIAL AND METHODS

This chapter presents a brief description of the methodology used, with an emphasis in general aspects, such as the plant material description, the environmental conditions, and the principles of the methods used in the experiments, as well as a basic assessment of the parameters calculated.

Chapter 4: SEED GERMINATION

This chapter focuses on the germination capacity and germination temperature dependence of several Mediterranean species. In particular, seeds were exposed to three different temperature regimes that simulate those typically occurring in the different seasons, to discern different strategies adopted by different species as a consequence of the heterogeneity of habitats and climatic seasonality intrinsic to Mediterranean ecosystems.

Chapter 5: SEEDLING GROWTH

This chapter presents the effects of water availability on seedling growth of eight Mediterranean species naturally occurring in the Balearic Islands. The seedling growth and establishment capacity is finally analysed in terms of the relative contribution of each of the underlying growth parameters to the decrease in RGR caused by water deficit across species comprising different growth forms.

Chapter 6: WATER RELATIONS AND STOMATAL REGULATION

This chapter covers the effects of water stress on leaf water relations and the capacity to regulate water loss through stomatal control. Specifically, stomatal

morphological attributes, stomatal conductance and its responsiveness to water stress, and leaf water relations were determined in ten Mediterranean species, with different growth forms, during an experiment of increasing drought stress, followed by re-watering.

Chapter 7: PHOTOSYNTHETIC LIMITATIONS

This chapter describes the effects of water stress on photosynthetic capacity of ten Mediterranean species, using the same experimental design as in chapter 6. Photosynthetic limitations during drought and re-watering were partitioned into their functional components, i.e. stomatal diffusion, mesophyll diffusion and biochemical components.

Chapter 8: PHOTOINHIBITION AND PHOTOPROTECTION

In this chapter, the pigment composition, photoinhibition and photoprotection events, and the relationship between them, in the same experiment as in chapters 6 and 7.

Chapter 9: LEAF RESPIRATION AND CARBON BALANCE

This chapter focuses in the effects of water stress on leaf dark respiration rates and how they may affect the leaf carbon balance of ten Mediterranean species (the same as in the experiment set out in chapters 6, 7 and 8).

Chapter 10: BIOCHEMISTRY OF RUBISCO

This chapter covers the intrinsic carboxylation capacity of Rubisco relative to oxygenation, i.e. the Rubisco specificity factor, and especially the potential acclimation and adaptation processes of the specificity of Rubisco that may have occurred due to evolution of Mediterranean species under different stresses. This is then discussed in terms of possibilities to construct genetically modified organisms with higher productivities under water limited environments.

Chapter 11: ENDEMICITY CASE 1. *LYSIMACHIA MINORICENSIS*

This chapter represents an attempt to test whether photosynthesis and photoprotection responses to water stress of a wild-extinct Mediterranean endemic

species, *Lysimachia minoricensis*, could help explaining the lack of success of this species in its natural habitat.

Chapter 12: ENDEMICITY CASE 2. *DIGITALIS MINOR*

This chapter focuses on ecophysiological differences on morphological and photosynthetic traits in two varieties of a Balearic endemic species, *Digitalis minor*, which are recognized according to its differences in pubescence: var. *minor* is pubescent while var. *palaui* is glabrous. Water status, leaf mass area, gas-exchange and chlorophyll fluorescence measurements were performed under two different water availability treatments: field capacity and 25% field capacity.

Chapter 13: GENERAL DISCUSSION

In this chapter, a general discussion is presented with the aim to link the different discussions of each specific experiment. It also includes a critical point of view on the advances achieved in respect to the background stated in the Introduction.

Chapter 14: CONCLUSIONS

The last chapter presents a list of the main conclusions drawn from this Thesis covering the objectives set out in Chapter 2.

2.4. PUBLICATIONS

The results presented in this Thesis have been published, submitted or have been prepared for publication in the following articles and gene bank notes:

Chapter 4

Galmés J., Medrano H. & Flexas J. (2006) Germination capacity and temperature dependence in Mediterranean species of the Balearic Islands. *Investigación Agraria: Sistema y Recursos Forestales* (in press).

Chapter 5

Galmés J., Cifre J., Medrano H. & Flexas J. (2005) Modulation of relative growth rate and its components by water stress in Mediterranean species with different growth forms. *Oecologia* 145, 21-31.

Chapter 6

Galmés J., Medrano H., Savé R. & Flexas J. (2006) Ecophysiological responses to water stress and recovery in Mediterranean plants with different growth forms. I. Water relations and stomatal conductance. *Plant, Cell and Environment* (submitted).

Chapter 7

Galmés J., Medrano H. & Flexas J. (2006) Ecophysiological responses to water stress and recovery in Mediterranean plants with different growth forms. II. Photosynthetic limitations. *Plant, Cell and Environment* (submitted).

Chapter 8

Galmés J., Medrano H., Abadía M. & Flexas J. (2006) Ecophysiological responses to water stress and recovery in Mediterranean plants with different growth forms. III. Photoprotection, pigment composition and energy dissipation. *Plant, Cell and Environment* (submitted).

Chapter 9

Galmés J., Ribas-Carbó M., Medrano H. & Flexas J. (2006) Ecophysiological responses to water stress and recovery in Mediterranean plants with different growth forms. IV. Leaf respiration and carbon balance (in preparation).

Chapter 10

Galmés J., Haslam R.P., Madgwick P.J., Keys A.J., Medrano H., Flexas J. & Parry M.A.J. (2004) Ribulose 1,5 bisphosphate carboxylase/oxygenase, large subunit [*Limonium gibertii*]. NCBI Gen Sequence Bank (www.ncbi.nlm.nih.gov). Accession number: CAH10354.

Galmés J., Haslam R.P., Madgwick P.J., Keys A.J., Medrano H., Flexas J. & Parry M.A.J. (2004) Ribulose 1,5 bisphosphate carboxylase/oxygenase, small subunit [*Limonium gibertii*]. NCBI Gen Sequence Bank (www.ncbi.nlm.nih.gov). Accession numbers: CAH10355 and CAH10356.

Galmés J., Flexas J., Keys A.J., Cifre J., Mitchell R.A.C., Madgwick P.J., Haslam R.P., Medrano H. & Parry M.A.J. (2005) Rubisco specificity factor tends to be larger in

plant species from drier habitats and in species with persistent leaves. *Plant, Cell and Environment* 28, 571-579.

Galmés J., Medrano H. & Flexas J. (2006) Rubisco specificity factor does not acclimate to drought in tobacco (in preparation).

Chapter 11

Galmés J., Abadía A., Medrano H. & Flexas J. (2006) Photosynthesis and photoprotection responses to water stress in the wild-extinct plant *Lysimachia minoricensis*. *Environmental and Experimental Botany* (submitted).

Chapter 12

Galmés J., Medrano H. & Flexas J. (2006) Photosynthesis and photoinhibition in response to drought in a pubescent (var. *minor*) and a glabrous (var. *palaui*) variety of *Digitalis minor*. *Environmental and Experimental Botany* (submitted).

Chapter 3
MATERIAL AND METHODS

This section describes the plant material, the main characteristics of the selected species, an overview of the methods and techniques used, and finally how diverse parameters shown in the results section were calculated. Because of the diversity of methods used in this Thesis, for a more comprehensive lecture, each chapter presents a detailed description of the materials used and methods specifically performed in each experiment.

3.1. PLANT MATERIAL

Twenty-four dicotyledonous species (Fig. 3.1) from different habitats in the Balearic Islands were selected for study. The number of the species used in each work depended on the experiment (Table 3.1).

The criteria used to select these species were based on their evolutionary history, ecological characters and phylogenetic relationships.

Regarding evolutionary history, species were classified into: endemic species, those only occurring in the Balearic Islands, and non-endemic species, species that are not restricted to the Balearic Islands.

Two different criteria were used to classify the species with respect to their ecology. Firstly, species were classified depending on their growth form into: annual herbs, evergreen/perennial herbs, semi-deciduous shrubs and evergreen shrubs. Annual herbs comprised all non-woody species that complete their life cycle in one year. Perennial herbs comprised all non-woody species that complete their life cycle in at least two years. Among them, evergreen herbs maintain functional leaves during the whole year. Semi-deciduous, malacophyll shrubs comprised all woody species that lose a certain amount of their leaves during the unfavourable season, depending on its length and severity. Evergreen shrubs comprised all woody sclerophyll hard-leaved species that maintain their leaves during the whole year. Depending on the specific experiment this species classification was slightly modified, according to the need for comparative relationships and depending to the amount of species included in the comparison. In particular, the annual herbs and the evergreen/perennial herbs were sometimes grouped in a single group of herbaceous plants. Another possible modification resulted in a more specific classification of evergreen shrubs: those with a normal size below 30 cm (i.e. *Limonium* sp.) were further classified in evergreen semi-shrubs, while for the remaining

evergreen shrubs (i.e. for those species with plant adult height higher than 1 m, that is *P. lentiscus* and *H. balearicum*) the same category of evergreen shrub was maintained.

The second ecological classification was made on the basis of habitat xericity. Group 1 comprised the species inhabiting the coastal, driest and hottest areas with annual precipitation typically below 400 L m^{-2}. Species typical of Mediterranean macchia with annual precipitation typically between 400 and 800 L m^{-2} were classified in group 2 together with some ruderal species. Group 3 comprised species inhabiting the wettest and coolest mountain areas with annual precipitation above 800 L m^{-2}, species growing only near open water sources, and species maintaining their leaves only during the wet season.

Table 3.1

List of species selected for study in each group of experiments.

Species	Seed germination	Seedling growth	Leaf ecophysiological responses to drought	Rubisco specificity factor
Diplotaxis ibicensis Pau	X		X	X
Urtica atrovirens subsp. *bianorii* (Knoche) Paira	X			X
Urtica membranacea Poiret	X			X
Beta maritima L. subsp. *marcosii* A. Juan & M. B. Crespo	X	X	X	X
Beta maritima L. subsp. *maritima*	X	X	X	X
Lysimachia minoricensis J. J. Rodr.	X		X	X
Mentha aquatica L.	X			X
Pimpinella bicknelli Briq.	X			X
Kundmannia sicula (L.) D. C.	X			X
Paeonia cambessedesii Willk.				X
Helleborus foetidus L.				X
Crepis triasii (Camb.) Nyman				X
Digitalis minor L. *var. minor*			X	X
Digitalis minor L. *var. palaui* (G. Font) Hinz & Rosselló			X	X
Lavatera maritima Gouan	X		X	X
Phlomis italica L.	X	X	X	X
Cistus albidus L.	X	X	X	X
Rhamnus ludovici-salvatoris R. Chodat				X
Rhamnus alaternus L.				X
Hypericum balearicum L.	X	X	X	X
Pistacia lentiscus L.	X	X	X	X
Limonium magallufianum L. Llorens	X	X	X	X
Limonium gibertii (Sennen) Sennen	X	X	X	X
Limonium virgatum (Willd.) Fourr.				X

Figure 3.1

List of the species used in this thesis with their family and a brief description.

Some of these pictures have been kindly given by the *Herbari virtual* of the Botany group of the University of the Balearic Islands (http://www.uib.es/depart/dba/botanica/herbari/index.html).

Diplotaxis ibicensis Pau

Brassicaceae

Upright annual herb that can reach up to half a meter in height. It is endemic of the Balearic Islands and inhabits a few coastal locations

Urtica atrovirens subsp. *bianorii* (Knoche) Paira

Urticaceae

Stinging nettle (annual herb) endemic to the mountains of Mallorca

Urtica membranacea Poiret

Urticaceae

Widespread stinging nettle (annual herb), found everywhere, typically in human disturbed areas

Beta maritima L. subsp. *marcosii* A. Juan & M. B. Crespo

Chenopodiaceae

Perennial herb. Endemic of the Balearic Islands, inhabiting a few small islets subjected to strong saline spray

Beta maritima L. subsp. *maritima*

Chenopodiaceae

Perennial herb inhabiting coastal ecosystems. Widespread in Mediterranean and temperate climates

Lysimachia minoricensis J. J. Rodr.

Primulaceae

Biannual herb endemic to the island of Menorca, considered to be now extinct in the wild, although some specimens are still conserved in botanical and private gardens. Its natural habitat was close to water streams

Mentha aquatica L.

Labiatae

Widespread perennial herb found in streams and permanent ponds

Pimpinella bicknelli Briq.

Umbelliferae

Perennial herb endemic of few populations in the mountains of Mallorca

Kundmannia sicula (L.) D. C.

Umbelliferae

Biennial herbaceous plant found in ruderal fields at the end of spring

Paeonia cambessedesii Willk.

Paeoniceae

Perennial herb with an underground trunk, it produces new leaves every year during winter and afterwards they dry up in summer. It lives in shady, humid places, usually at the foot of rocky walls

Helleborus foetidus L.

Ranunculaceae

Perennial herb which can reach half a meter in height. It grows in the Serra de Tramuntana in Mallorca. Some authors consider it a particular race of the island. It has characteristic leaves with coriaceous consistency. It is a plant typical of roadsides and clearings in mountain holm-oak woods

Crepis triasii (Camb.) Nyman

Compositae

Perennial herb endemic of Mallorca, Menorca and Cabrera. It always lives in rocky crevices, where it is quite frequent and easy to find. It has a rosette of basal leaves

Digitalis minor L. var. *minor*

Scrophulariaceae

Perennial herb which lives in rocky crags, often colonizing the soil at the foot of mountain cliff faces. This endemic variety has pubescent leaves and white flowers

Digitalis minor L. var. *palaui* (G. Font) Hinz & Rosselló

Scrophulariaceae

Perennial herb which lives in rocky crags, often colonizing the soil at the foot of mountain cliff faces. This endemic variety has glabrous leaves and white flowers

Lavatera maritima Gouan

Malvaceae

Semi-deciduous shrub up to 2 m, densely covered by hairs. It is found in the littoral areas or those of marine influence, always in places with high irradiance

Phlomis italica L.

Labiatae

Semi-deciduous shrub up to 1 m, densely covered by hairs. Endemic of the Balearic Islands. It lives mostly in the mountains of Mallorca, but also in the clearings of the garrigues of Menorca and Mallorca

Cistus albidus L.

Cistaceae

Semi-deciduous shrub up to 1 m. Commonly found in the Mediterranean garrigue. Its leaves are densely covered by hairs

Rhamnus ludovici-salvatoris R. Chodat

Rhamnaceae

Endemic woody evergreen shrub. Inhabits Mediterranean garrigues and understores of holm oak forests, mostly in Serra de Tramuntana and Cabrera, having almost disappeared from Serra de Llevant and Menorca.

Rhamnus alaternus L.

Rhamnaceae

Mediterranean woody evergreen shrub, eventually grows into a small tree. Commonly found in the garigue

Hypericum balearicum L.

Guttiferae

Woody evergreen shrub up to 2 m, endemic of the Balearic Islands. The biggest populations are found in the garigue 500 m above the sea level, where it competes with *P. lentiscus*

Pistacia lentiscus L.

Anacardiaceae

Woody evergreen shrub, the mastic tree or the lentisk can grow in to a small tree, and it is very abundant in the Mediterranean garrigues and under stories of the pine and holm oak forests

Limonium magallufianum L. Llorens

Plumbaginaceae

Woody evergreen semi-shrub, in cushion-like rosettes. Endemic of the Balearic Islands, inhabiting just in a single coastal marsh located in Magalluf, Mallorca

Limonium gibertii (Sennen) Sennen

Plumbaginaceae

Woody evergreen semi-shrub, in cushion-like rosettes. Occurring in West Mediterranean rocky and sandy coastal areas

Limonium virgatum (Willd.) Fourr.

Plumbaginaceae

Woody evergreen semi-shrub, in cushion-like rosettes, can be found either in coastal rocky substrates or wetland clayey soils

Natural populations of each species were selected for sampling (Table 3.2). In these locations, to obtain a representative sample of populations in the nature, at least 10 adult, healthy specimens were selected and enough mature seeds collected from each. The smallest and the biggest seeds of those species showing high variability in the seed weight were discarded to homogenize initial seedling weight. From these seeds, 10 to 20 plants per species were obtained, and grown outdoors at the University of the Balearic Islands (Mallorca, Spain). Completely developed seeds from these plants were collected in 2002 and 2003 and germinated to the plants used for the experiments as described specifically in each chapter. Seasonal seed collection depended of the phenology of each species (Table 3.2).

Table 3.2

List of species with their natural population (location and coordinates) and season for seed collection.

Species	Location	Coordinates	Season
Diplotaxis ibicensis Pau	Colònia de Sant Jordi	N 39º19'31"	May
	Ses Salines	E 02º59'22"	
Urtica atrovirens subsp. *bianorii* (Knoche)	Cosconar	N 39º51'08"	June
Paira	Escorca	E 02º50'40"	
Urtica membranacea Poiret	Mortitx	N 39º52'25"	May
	Escorca	E 02º55'10"	
Beta maritima L. subsp. *marcosii* A. Juan	Illot de ses Bledes	N 39º08'19"	July
& M. B. Crespo	Cabrera	E 02º57'41"	
Beta maritima L. subsp. *maritima*	Cap Salines	N 39º15'58"	July
	Ses Salines	E 03º03'02"	
Lysimachia minoricensis J. J. Rodr.	Botanic Gardens		August
	Jardí Botànic de Sóller		
Mentha aquatica L.	Calicant	N 39º39'01"	October
	Sant Llorenç	E 03º17'11"	
Pimpinella bicknelli Briq.	Mortitx	N 39º52'44"	June
	Escorca	E 02º55'12"	
Kundmannia sicula (L.) D. C.	Camp Gran	N 39º36'40"	June
	Sant Llorenç	E 03º15'30"	
Paeonia cambessedesii Willk.	Font des Quer	N 39º37'49"	September
	Estellencs	E 02º26'53"	
Helleborus foetidus L.	Serra d'Alfàbia	N 39º44'11"	June
	Sóller	E 02º42'46"	
Crepis triasii (Camb.) Nyman	Mortitx	N 39º52'25"	June
	Escorca	E 02º54'57"	
Digitalis minor L. *var. minor*	Mortitx	N 39º52'25"	June
	Escorca	E 02º54'57"	
Digitalis minor L. *var. palaui* (G. Font)	Son Fortuny	N 39º38'27"	June
Hinz & Rosselló	Estellencs	E 02º27'32"	
Lavatera maritima Gouan	Cap Blanc	N 39º27'02"	June
	Llucmajor	E 02º44'35"	
Phlomis italica L.	Alqueria Vella	N 39º44'44"	August
	Artà	E 03º20'55"	
Cistus albidus L.	Alqueria Vella	N 39º43'58"	July
	Artà	E 03º21'57"	
Rhamnus ludovici-salvatoris R. Chodat	Font des Quer	N 39º38'16"	July
	Estellencs	E 02º26'51"	

Species	Location	Coordinates	Season
Hypericum balearicum L.	Mortitx	N 39º52'25"	August
	Escorca	E 02º55'10"	
Pistacia lentiscus L.	Mortitx	N 39º52'25"	October
	Escorca	E 02º55'10"	
Rhamnus alaternus L.	Font des Quer	N 39º38'09"	June
	Estellencs	E 02º26'16"	
Limonium magallufianum L. Llorens	Magalluf	N 39º30'22"	September
	Calvià	E 02º32'46"	
Limonium gibertii (Sennen) Sennen	Es Carnatge	N 39º32'39"	September
	Palma	E 02º41'50"	
Limonium virgatum (Willd.) Fourr.	Ses Fontanelles	N 39º32'45"	September
	Palma	E 02º32'15"	

3.2. METHODS

3.2.1. Germination parameters

Seeds were placed in 9 cm diameter Petri-dishes on two layers of filter paper (Whatman no. 1) moistened to saturation with distilled water. Germination tests were conducted in controlled environment chambers (Koxka, Spain) and the experiment lasted for 91 days. Seeds were incubated in continuous darkness at three alternating temperatures (12h-12h): 5-15, 10-20 and 15-25°C.

The percentage of cumulative seed germination (G) for each replicate was calculated at the end of the experiment as:

$$\%G = \frac{SG}{IS - ES} \cdot 100$$

where: SG = number of germinated seeds.

ES = number of empty seeds.

IS = number of seeds initiated in each replicate.

The dormancy period (D) of a homogeneous group of seeds was determined as the number of days needed to observe the first seed germinated.

The average time response (T_{50}) of germination was determined as the number of days elapsed from the initial until germination of 50% of total germinated seeds.

3.2.2. Leaf morphological parameters

3.2.2.1. Specific leaf weight (SLW) and specific leaf area (SLA)

To characterise leaf morphology, SLW or its inverse SLA were measured. First, the leaf area was determined with an AM-100 Area Meter (Analytical Development Company, Herts, UK). Then, the dry mass of these leaves was determined after oven drying for 48 h at 60ºC. SLW was calculated as the ratio of dry mass to leaf area, and

expressed in g m^{-2}. SLA was calculated as the ratio of leaf area to dry mass, and expressed in m^2 kg^{-1}.

3.2.2.2. Stomatal characterisation

Stomatal density (SD) was determined using the silicon leaf impression method (Weyers & Johansen, 1985) on the abaxial lamina (since all the species analysed were hypostomatic). Samples were taken immediately from the right of the mid-vein of fully exposed mature leaves detached plants. The number of stomata was counted with a microscope at 400 × magnification on four different vision fields of separate impressions of the lamina obtained from different leaves.

Guard cell length was measured on randomly selected stomata from the same impressions used for SD determinations.

Stomatal area index (SAI) was calculated by taking the product of the stomatal length and the SD, according to Ashton & Berlyn (1994).

3.2.3. Plant growth analysis

3.2.3.1. Growth parameters

The basis for the analysis of plant growth was laid in the 1910s, when it was first realized that the increase in biomass of a seedling is more or less proportional to the amount of biomass already present (Brenchley, 1916). In this approach, the rate of plant growth is given as ‘relative growth rate’:

$$B_2 = B_1 \cdot e^{RGR(t_1 - t_2)}$$

where B_1 and B_2 are the dry mass of the plants at time t_1 and t_2, respectively, and RGR is defined as the rate of increase in biomass per unit plant mass already present. If RGR is constant over time, then plants increase in mass in an exponential way (Fig. 2.2). Strictly speaking, plant growth is never truly exponential, as the increase of mass depends on the rate of photosynthesis, which in turns depends on fluctuating environmental resources (light, for instance).

At any time, RGR can be expressed as:

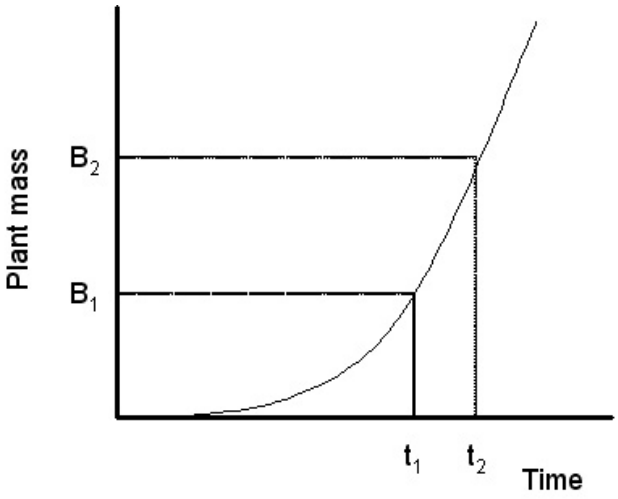
$$RGR = \frac{1}{B} \times \frac{dB}{dt}$$

And, averaged over a time interval t_1 to t_2 during which biomass increases from B_1 to B_2, RGR can be calculated as:

$$RGR = \frac{\log_e B_2 \cdot \log_e B_1}{t_2 \cdot t_1}$$

Figure 3.2

Time course of plant mass following growth in an exponential increase.



The main driving factor for growth is the process of CO_2 assimilation. Part of the photosynthetically fixed carbon will be respired, providing the energy and reducing equivalents to power the processes of nutrient uptake, transport, growth and maintenance of biomass. Therefore, the net gain in biomass results from the difference between CO_2 assimilation by leaves and respiratory loss by the entire plant. Then, leaf area can be viewed as a driving variable, and biomass increment (d*B*) per unit time (d*t*) can be then divided by leaf area (LA) to yield the net assimilation rate, NAR, where:

$$NAR = \frac{1}{LA} \times \frac{dB}{dt}$$

Averaged over a short time interval (t_1 to t_2 days) and provided whole-plant biomass and leaf area are linearly related (Radford, 1967):

$$NAR = \frac{(B_2 - B_1)\cdot(\log_e LA_2 - \log_e LA_1)}{(t_2 - t_1)\cdot(LA_2 - LA_1)}$$

NAR thus represents a plant's net photosynthetic effectiveness in capturing light, assimilating CO_2 and storing photoassimilate.

Since leaf area is a driving variable for the whole-plant growth, the proportion of a plant biomass invested in leaf area will have an important effect on RGR, and can be defined as leaf area ratio, LAR, where:

$$LAR = \frac{LA}{B}$$

At any instant (in practice, at any harvest), LAR can be taken as LA/B and can be factorised into two components, namely specific leaf area (SLA) and leaf weight ratio (LMR). As stated in the previous section, SLA is simply a ratio of the leaf area (LA) to leaf dry mass (LDM), and LMR is a true ratio of the leaf dry mass (LDM) to total plant dry mass (B). Thus,

$$LAR = \frac{LA}{LDM} \times \frac{LDM}{B} = SLA \times LMR$$

If both LA and B are increasing exponentially so that B is proportional to LA, it follows that:

$$\frac{1}{B} \times \frac{dB}{dt} = \frac{1}{A} \times \frac{dB}{dt} \times \frac{A}{B}$$

or, summarized:

RGR = NAR × LAR

and therefore:

$$RGR = NAR \times LMR \times SLA$$

Table 3.3

Summary of growth indices.

Growth index	Symbol	Mathematical definition	Units	Functional definition
Relative growth rate	RGR	$1/B \cdot dB/dt$	$mg\ g^{-1}\ d^{-1}$	Rate of mass increase per unit mass present
Net assimilation rate	NAR	$1/LA \cdot dB/dt$	$g\ m^{-2}\ d^{-1}$	Rate of mass increase per unit leaf area (physiological component of growth)
Leaf area ratio	LAR	LA/B	$m^2\ kg^{-1}$	Ratio of leaf area to total plant mass (morphological component of growth)
Specific leaf area	SLA	LA/LDM	$m^2\ kg^{-1}$	Ratio of leaf area to leaf mass (a measure of relative thickness, density or spread of leaves)
Leaf mass ratio	LMR	LDM/B	$g^{-1}\ g^{-1}$	Ratio of leaf mass to total plant mass (a measure of biomass allocation to leaves)

Similarly to LMR, stem mass ratio (SMR) and root mass ratio (RMR) can be calculated as the ratio of stem and root mass, respectively, to total plant mass.

3.2.3.2. Growth response coefficients

Growth response coefficients were calculated according to Poorter & Nagel (2000). Suppose a species that is grown at two water resource levels: high (H) and low (L), and that we have obtained data on the RGR (RGR_H and RGR_L), NAR (NAR_H and NAR_L), SLA (SLA_H and SLA_L) and LMR (LMR_H and LMR_L) of plants of both treatments. Given that RGR = NAR × SLA × LMR, the ratio between RGR_H and RGR_L equals:

$$\frac{RGR_H}{RGR_L} = \frac{NAR_H \cdot SLA_H \cdot LMR_H}{NAR_L \cdot SLA_L \cdot LMR_L}$$

Ln-transformation of both sides of the equation gives:

$$(\ln RGR_H - \ln RGR_L) = (\ln NAR_H - \ln NAR_L) + (\ln SLA_H - \ln SLA_L) + (\ln LMR_H - \ln LMR_L)$$

Thus, the difference in ln-transformed RGR values is the sum of the differences in ln-transformed values of NAR, SLA and LMR. This can be converted to:

$$l = \frac{(\ln NAR_H - \ln NAR_L)}{(\ln RGR_H - \ln RGR_L)} + \frac{(\ln SLA_H - \ln SLA_L)}{(\ln RGR_H - \ln RGR_L)} + \frac{(\ln LMR_H - \ln LMR_L)}{(\ln RGR_H - \ln RGR_L)}$$

Consequently, the first part of the right-hand term gives the fraction of the RGR difference that is associated with the difference in NAR, the second part the fraction associated with the difference in SLA, and the third part the fraction associated with the difference in LMR. If the components in the first equation have been calculated correctly and RGR is indeed exactly the product of NAR, SLA and LMR, then these values, which we call Growth Response Coefficients (GRCs), should add up to 1:

$$GRC_{NAR} + GRC_{SLA} + GRC_{LMR} = 1$$

A GRC value of 1 indicates that the proportional change in the growth parameter of interest equals the proportional change in RGR. A GRC value of 0 indicates that there is no change in that growth parameter at all. GRC values can be higher than 1 (as in the case of NAR at various irradiances) if the increase in the growth parameter is stronger than the increase in RGR, and can be lower than 0 if an increase in a growth parameter parallels a decrease in RGR.

3.2.4. Plant and soil water status

3.2.4.1. Soil water content

To determine the soil water capacity, soil samples were watered at saturation and, after over-night drainage under high atmospheric humidity, their weight was determined and considered as the pot weight at field capacity. Then, the same sample was desiccated in an oven at 70ºC for at least five days. After reaching constant weight (five days) this value was taken as the pot weight at zero soil water content. Finally, the soil water content capacity was determined as the difference between the soil weight at field capacity and the soil weight at zero water content.

3.2.4.2. Leaf water potential

Leaf water potential was determined with a Scholander chamber (Soilmoisture Equipment Corp., USA). This chamber consists on a pressure vessel, a compressed gas source and a pressure gauge (Fig. 3.3).

Figure 3.3.

Scholander chamber.

Water potential represents a reliable and simple method for evaluating the physiological condition of a plant, since cell growth, photosynthesis and crop productivity are strongly influenced by water potential and its components (Repellin *et al.*, 1997; Pardossi *et al.*, 1998; Taiz & Ziegler, 1998). The pressure method was pioneered by H. Dixon at the beginning of the twentieth century, but it did not come into widespread use until P. Scholander and coworkers at the Scripps Institution of Oceanography improved the instrument design and showed its practical use (Scholander *et al.*, 1965). Since the pressure chamber method does not require delicate instrumentation or temperature control, it has been used extensively under field conditions (Tyree & Hammel, 1972).

In this technique, a leaf or shoot to be measured is excised from the plant and is partly sealed in a pressure chamber. Before excision, the water column in the xylem is under tension. When the water column is broken by excision of the organ, water is pulled rapidly from the xylem into the surrounding living cells by osmosis. The cut surface consequently appears dull and dry. To make a measurement, the chamber is pressurised with compressed gas until the distribution of water between the living cells and the xylem conduits is returned to its initial, pre-excision state. This can be detected visually by observing when the water returns to the open ends of the xylem conduits that can be seen in the cut surface. The accurate measure of this end point is facilitated by the use of a magnifying glass. The pressure needed to bring the water back to its

initial distribution is called the balance pressure, indicator of the xylem water potential, and is readily detected by the change in the appearance of the cut surface, which becomes wet and shiny when this pressure is attained.

3.2.4.3. Leaf relative water content

The RWC, stated by Slatyer in 1967, express in percentage the water content at a given time and tissue as related to the water content at full turgor, and was determined as follows:

RWC = (Fresh weight − Dry weight) / (Turgid weight – Dry weight) × 100

To determine the turgid weight of the samples, these were kept in distilled water in darkness at 4°C to minimise respiration losses, until they reached a constant weight (full turgor, typically after 12 h). Their dry weight was obtained after 48 h at 70ºC in an oven.

3.2.4.4. Leaf-specific hydraulic conductance

Leaf-specific hydraulic conductance (K_L) was estimated from the slope of the relationship between leaf transpiration rate (E) and the leaf water potential (Ψ), and was calculated as $E/(\Psi_{MD}-\Psi_{PD})$ (Sperry & Pockman, 1993).

3.2.5. Chlorophyll fluorescence measurements

Chlorophyll fluorescence was measured with the PAM-2000 fluorimeter (Walz, Effeltrich, Germany) or with the integrated fluorescence chamber head (Li-6400-40 leaf chamber fluorometer; Li-Cor, Inc.) of the open gas exchange system (Li-6400; Li-Cor, Inc., Nebraska, USA), depending on the experiment (see specific Material and Methods' section in each chapter). Irrespective of the instrument used, the principles and parameters, explained in this section, remain the same.

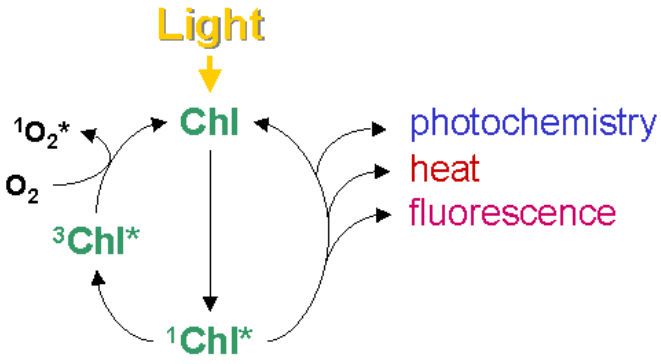
3.2.5.1. Principles

Light energy that is absorbed by chlorophyll in a leaf can undergo three fates: a) it can be used to drive photosynthesis (photochemistry), b) it can be dissipated as heat or c) it can be re-emitted as red fluorescence (Fig. 3.4). These three processes occur in competition. Since the sum of rate constants is constant, any increase in the efficiency of one process will result in a decrease in the efficiency of the other two. Therefore,

determining the efficiency of chlorophyll fluorescence will give information about changes in the efficiency of photochemistry and heat dissipation.

Figure 3.4

Possible fates of excited chlorophyll.



The most useful and widely used chlorophyll fluorescence technique is the so-called quenching analysis of modulated fluorescence by the saturation pulse method. In this method, several different light sources are required: modulated actinic light (red, at 650 nm, or white <710 nm), saturating flash (>5000 µmol m^{-2} s^{-1} in the PAR spectrum, for 0.6 - 1 s) and infrared light to excite PSI. The pulse-modulated fluorescence method use a pulsed light to induce a pulsed fluorescence in the leaf; that signal is electronically amplified and identified, allowing a 'relative' measurement of chlorophyll fluorescence even in the presence of a strong background ambient light.

A typical measurement is shown in Fig. 3.5. A leaf is dark adapted prior to the measurement. Upon the application of a saturating flash (<730 nm, about 8000 µmol m^{-2} s^{-1} for 0.6 - 1 s), fluorescence raises from the ground state value (F_o), which is the fluorescence determined in darkness by a weak measuring beam, to its maximum value, F_m. This measurement allows the determination of the maximum quantum efficiency of photosystem II (PSII) primary photochemistry, given as F_v/F_m.

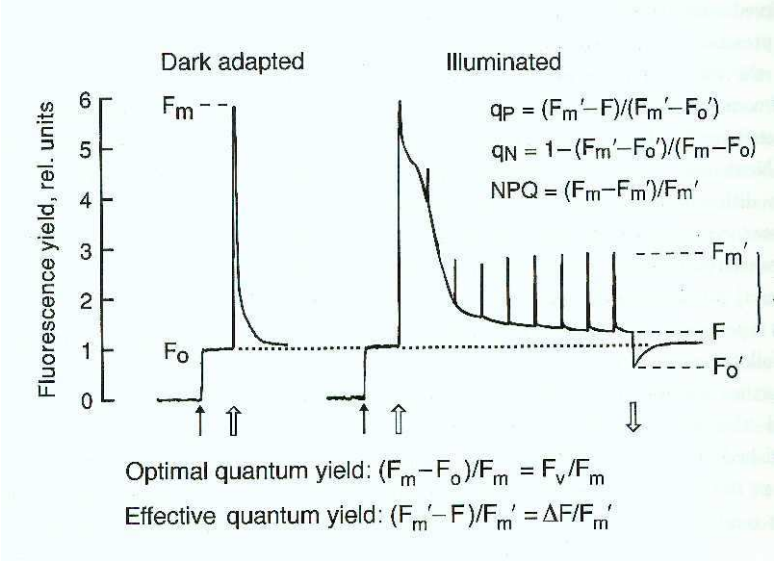
Upon subsequent application of constant illumination, a transient rise in fluorescence yield is observed. This is due to a lag phase before carbon fixation starts. Whilst electron transport starts within milliseconds upon illumination, carbon fixation needs first to be light-activated. Thereafter, upon the onset of photochemical and heat

dissipation processes, the fluorescence efficiency is quenched and reaches a steady state value (F_s). This effect was first observed by Kautsky in 1931.

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

Figure 3.5

Measurement of chlorophyll fluorescence by the saturation pulse method (from Schereiber *et al*., 1998). The thin arrows indicate the switching on of modulated light. The thick arrows indicate switching on and switching off actinic light.



3.2.5.2. The fluorescence parameters

$\Delta F/F_m$'

Perhaps the single most useful fluorescence parameter is the so-called *Genty* parameter, which is calculated after Genty *et al*. (1989) as:

$$\Delta F/F_m' = (F_m'-F_s)/F_m'$$

Since $\Delta F/F_m$' is theoretically proportional to the operating quantum efficiency of PSII photochemistry, it is a measure of the proportion of the light absorbed by PSII that is used in photochemistry. The PSII quantum efficiency is affected by the level of electron acceptors, usually $NADP^+$, available at the acceptor side of PSI. Consequently,

$\Delta F/F_m'$ decreases in situations with limiting consumption of NADPH, for example, at low internal CO_2 concentration.

ETR

Multiplied by the amount of absorbed light by PSII, $\Delta F/F_m'$, is a measure of the rate of linear electron transport through PSII. The electron transport rate can be calculated as:

$$ETR = \Delta F/F_m' \cdot PPFD \cdot \alpha \cdot \beta$$

where PPFD is the photosynthetic photon flux density (in µmol quanta m^{-2} s^{-1}), α is the leaf absorptance, and β equates to the ratio of light absorption between PSII and I. When stated, leaf absorptances were calculated with spectroradiometer coupled to an integration sphere (UniSpec, PP-Systems, USA). β was assumed 0.5 (Laisk & Loreto, 1996). In *N. tabaccum* experiments, the product $\alpha \cdot \beta$ was previously determined for each treatment as the slope of the relationship between $\Delta F/F_m'$ and ϕ_{CO2} obtained by varying light intensity under non-photorespiratory conditions in an atmosphere containing less than 1% O_2 (Valentini *et al*., 1995).

NPQ

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

The most straightforward way to quantify non-photochemical quenching is by measuring the fluorescence parameter NPQ, which is calculated as:

$$NPQ = (F_m/F_m') - 1$$

The parameter NPQ is derived from the Stern-Volmer equation and can be used to follow changes in apparent quencher concentration.

Non-photochemical quenching is induced under conditions where the photosynthetic apparatus cannot use the total absorbed light energy for photochemistry. Stress conditions such as high light intensity and low internal CO_2 concentration

markedly promote non-photochemical quenching. Therefore, the amount of non-photochemical quenching is an indicator of the stress severity.

F_v/F_m

The maximum quantum efficiency of PSII photochemistry is calculated as:

$F_v/F_m = (F_m-F_o)/F_m$

A decrease in F_v/F_m may be originated by a decrease in F_m in combination with an increase in F_o. An increase of F_o is provoked by dissociation of light harvesting pigment system of PSII from the PSII core, which is related to photoinactivation of PSII. By contrast, if decreased F_v/F_m is accompanied by a progressively decline in F_o, it may be considered indicative of sustained non-photochemical quenching or photoprotection in the dark (Osmond, 1994). The parameter F_v/F_m is then very useful to estimate the extent of photoinhibition of photosynthesis, and a decrease in F_v/F_m might be a mix of photoprotection and photodamage. A reduction in F_v/F_m that remains overnight is usually called 'permanent photoinhibition' (Long *et al*., 1994).

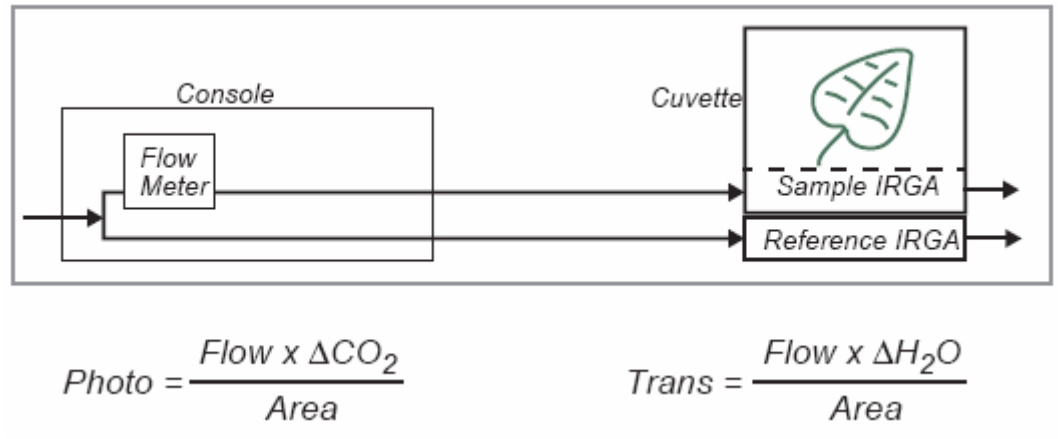
3.2.6. Gas-exchange measurements using an infrared gas analyser

Gas-exchange measurements were made with an open circuit infrared gas analyser (IRGA), Li-6400, which allows the measurement of the instantaneous net CO_2 assimilation rate in intact attached leaves (Fig. 3.6).

The system is based in the measure of the CO_2 concentration decrease in the air stream which flows through a cuvette with photosynthetic tissue. For any given decrease, air flow rate and leaf surface, the CO_2 assimilation rate (A_N) can be calculated and expressed in μmol CO_2 m^{-2} s^{-1}. In an analogous way, measuring the increase in water content of the same air stream, the transpiration rate (E) can be determined, and expressed in mmol H_2O m^{-2} s^{-1}. The system consists in two gas analysers in the sensor head, which allow obtaining absolute CO_2 and H_2O values as well from the reference as from the sample. The basic principle is based in the fact that both CO_2 and vapour H_2O are strong absorbents of infrared radiation, at different wavelengths.

Figure 3.6

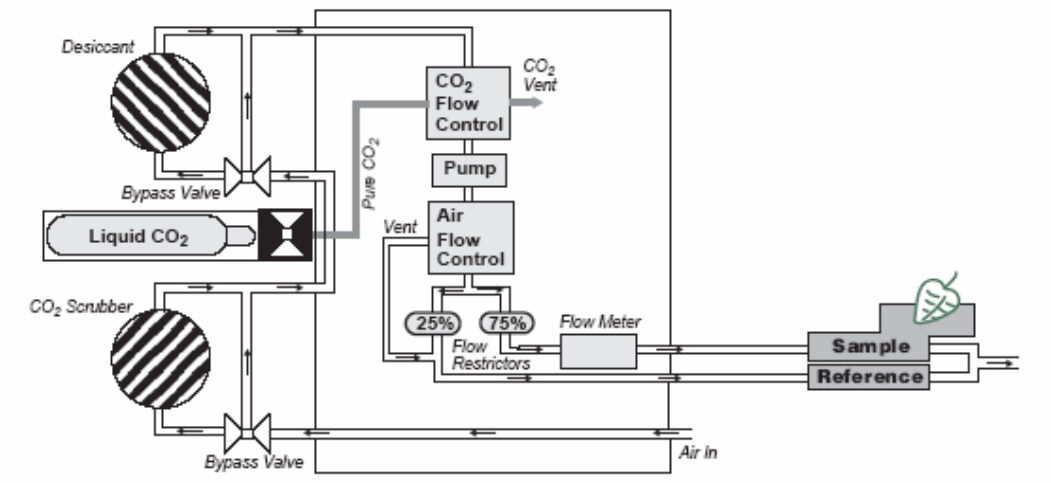
In an open system, photosynthesis and transpiration are computed from the differences in CO_2 and H_2O between in-chamber conditions and pre-chamber conditions. From Li-6400 manual 5.2v.



The following figure shows the flow schematic of the Li-6400:

Figure 3.7

LI-6400 flow schematic, with a CO_2 mixer incorporated. From Li-6400 manual 5.2v.



A thermocouple mounted inside the leaf chamber allows the measurement of the temperature of the attached leaf. Another thermocouple within the cuvette determines the air temperature. Following Farquhar *et al*. (1980) and Farquhar & Sharkey (1982), from these measurements, other photosynthetic parameters are derived, i.e. the stomatal conductance (g_s), expressed in mol H_2O per unit area and time, and the internal CO_2 concentration (C_i), expressed in μmol CO_2 mol^{-1} air.

Figure 3.8

The LI-6400 Portable Photosynthesis System.

Likewise, this system has the possibility to fix all desired parameters, and so the user can control the environmental conditions in the leaf chamber, such as light, temperature, humidity and CO_2 concentration.

LI-6400 is provided with several leaf chamber types, one of them has an incorporated a silicon diode that monitors and controls the optional 6400-02 LED Light Source, which permits to regulate the incident radiant flux density on the leaf, in a range from 0 to 2500 μmol photon m^{-2} s^{-1}, approximately. Measurements were done at saturating light of 1000-1500 μmol photon m^{-2} s^{-1} 10% blue light, and at 25ºC.

The humidity control in the LI-6400 is done by a combination of two mechanisms, manually by adjusting the incoming air in the leaf chamber which is routed through desiccant, and controlling the flow rate of air through the chamber. Measurements were done at a vapour water deficit between 1.0-1.5 kPa.

Finally, the 6400-01 CO_2 Mixer allows the control of the CO_2 concentration inside the leaf chamber, which is the basis of the photosynthetic CO_2 response curves (Escalona *et al*., 1999; Gulías *et al*., 2002). The CO_2 response curves of photosynthesis (A_N-C_i) were started at a cuvette CO_2 concentration (C_a) of 400 μmol CO_2 mol air^{-1}. After inducing photosynthesis under these conditions and once steady-state was reached, photosynthesis response curves to varying CO_2 concentration were performed. First, the C_a was lowered stepwise from 400 to 50 μmol mol^{-1} and then raised again to 400 μmol mol^{-1} until reaching a steady-state value similar to that obtained at the beginning of the curve. Then, C_a was increased stepwise from 400 to 1500 μmol mol^{-1}.

Gas exchange measurements were determined at each step after maintaining the leaf until steady-state was obtained, i.e. A_N showed no systematic decrease or increase (± 2%) for at least over 2 min. Measurements consisted in 10-13 measurements for each curve.

3.2.7. Leaf respiration measurements

Leaf respiration in the dark (R_D) was determined in two ways:

- Using the LI-6400 for *N. tabaccum* experiments.
- Using an oxygen electrode for the remaining species.

3.2.7.1. Dark respiration measurements using the LI-6400

Leaf respiration was measured as the net CO_2-exchange rate at 25ºC and at a C_a of 400 μmol mol^{-1} in the same leaves used for photosynthetic measurements after keeping them for 30 min in darkness.

3.2.7.2. Dark respiration measurements using the oxygen electrode

For dark respiration measurements, leaf samples were collected during the light period and stored 20 min in the dark in 0.2 mM $CaCl_2$ for membrane stabilization. O_2 uptake rates were measured in the dark, using a liquid-phase oxygen electrode (Hansatech Instruments Ltd., England) in ambient air-equilibrated 10 mM Mes buffer (pH 5.7), as previously described (Delieu & Walker, 1981; Azcón-Bieto *et al*., 1994). Leaf 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, except at low O_2 concentrations. To avoid oxygen-limiting conditions inside the cuvette, all measurements were determined with O_2 concentration above 60% of saturation. Respiration measurements were performed with the oxygen electrode technique to avoid the gasket-related leak with the CO_2 gas exchange measurements (Long & Bernacchi, 2003; Hurry *et al*., 2005). It is well known that the precision of the oxygen electrode techniques for respiration measurements are much higher than techniques based on CO_2 gas-exchange measurements (Hurry *et al*., 2005).

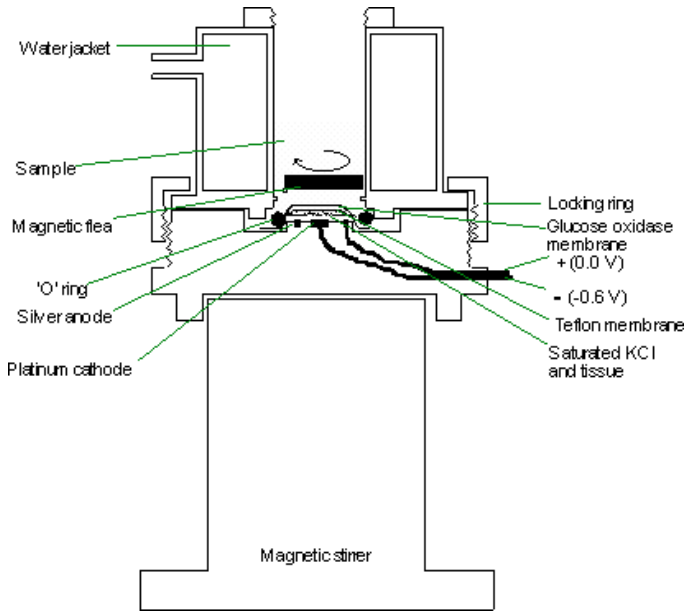
The oxygen electrode allows the measurement of oxygen evolution, uptake and liberation, during a reaction in a closed system. The oxygen electrode works based on

the principle of Clark; that is, using a polarographic measurement of the electricity that flows between an anode and a cathode.

This electrode consists on a cell which contains a circular silver wire anode and a platinum wire cathode, linked by an electrolyte (potassium chloride solution). A thin teflon or polyethylene membrane which is permeable to oxygen and a piece of paper (beneath the membrane) are usually placed over the electrodes surface (Fig. 3.9) in order to protect them and to provide a uniform layer of electrolyte between both anode and cathode. The reaction mixture in the appropriate container is stirred constantly with a small magnetic stirring rod. A voltage applied across the two electrodes undergoes oxygen electrolyte reduction in the solution.

Figure 3.9

Oxygen electrode components (after Walker, 1987).



Any variation in the oxygen concentration due to leaf gas-exchange is reflected in the electrolyte. As a consequence of this, the electric current between cathode and anode also changes. Variation in the oxygen concentration inside the chamber is measured by connecting the electrode to a recorder throughout a control box. Taking into account that oxygen solubility is temperature dependent, the whole electrode and reaction chamber was kept to a constant temperature of 25ºC by means of a water jacket attached to a temperature controlled water flow.

When a small voltage is applied across these electrodes (0.6-0.7 V), so that the platinum is made negative with respect to the silver, the current which flows is at first negligible and the platinum becomes polarised. Oxygen diffuses through the membrane and is reduced at the platinum surface, initially to hydrogen peroxide (H_2O_2). A thin layer of KCl solution closes the current circuit. The silver is oxidised and silver chloride deposited on the anode. The electrical current generated, by the reduction of oxygen at the cathode, is stochiometrically related to the oxygen consumed and converted to a voltage output signal by a control box.

3.2.8. Photosynthetic parameters calculated from chlorophyll fluorescence and gas-exchange measurements

3.2.8.1. Estimation of chloroplastic CO_2 concentration and mesophyll conductance

The CO_2 chloroplastic concentration (C_c) can be estimated combining gas-exchange and chlorophyll fluorescence measurements (Di Marco *et al*., 1990; Harley *et al*., 1992; Epron *et al*., 1995). In this work, C_c was calculated following to the method described by Epron *et al*. (1995). According to this method, the linear electron transport rate (ETR), obtained from chlorophyll fluorescence measurements, is assumed to be uniquely devoted to the carboxylation and oxygenation cycles driven by Rubisco. Therefore:

$$ETR=ETR_A+ETR_P \quad (1)$$

where ETR_A and ETR_P are the electron flow costs attributable to carboxylative and oxygenative reactions of Rubisco, respectively.

This calculation assumes that all other processes consuming light-driven electrons distinct than carboxylation and oxygenation of Rubisco are negligible or at least constant. This assumption has been proved to be mostly acceptable (Valentini *et al*., 1995).

As four electrons are required for one carboxylation and oxygenation cycle and one CO_2 molecule is realised every two oxygenation cycles by glycine decarboxylation in the photorespiratory pathways (Laing *et al*., 1974), ETR_A can be expressed as:

$$ETR_A= 4\ (A_N + R_L + P_r) \quad (2)$$

where R_L is the rate of mitochondrial respiration in the light and P_r is the rate of CO_2 production by photorespiration.

The oxygenation of two molecules of RuBP leads, via the photorespiratory pathway, to the metabolism of two glycolate-P molecules into one phosphoglyceric acid molecule and the release of one CO_2 (from the decarbobylation of one of the two glycines during the glycine-serine conversion in the mitochondrion) (Ögren, 1984; Sharkey, 1988; Peterson, 1989; Harley & Sharkey, 1991; Zelitch, 1992). In consequence, ETR_P can be expressed as:

$$ETR_P = 4\ (2\ P_r) \tag{3}$$

Finally, a system with three equations (1, 2 and 3) and three unknows (P_r, ETR_A and ETR_P) can be solved as follows, assuming that the respiration rate (R_L) measured in the dark is equal to that measured in the light:

$$ETR_A = \frac{1}{3}\left[ETR + 8\left(A_N + R_L\right)\right] \tag{4}$$

$$ETR_P = \frac{2}{3}\left[ETR + 4\left(A_N + R_L\right)\right] \tag{5}$$

$$P_r = \frac{1}{12}\left[ETR - 4\left(A_N + R_L\right)\right] \tag{6}$$

The Rubisco specificity factor (τ) is defined as the ratio $V_{c,max}\ K_o/V_{o,max}\ K_c$, where $V_{c,max}$ and $V_{o,max}$ are maximal rates for the carboxylation and oxygenation activities of Rubisco, and K_o and K_c are the Michaelis constants for these reactions (Laing *et al*., 1974). The ratio of carboxylation versus oxygenation rates (V_c/V_o) is the product of τ and the ratio of CO_2 and O_2 mole fractions (C and O, respectively):

$$\frac{V_c}{V_o} = \tau\frac{C}{O} \tag{7}$$

From ETR_A and ETR_P, the apparent Rubisco specificity factor operating *in vivo* (τ^*) can be calculated according to Laing *et al*. (1974) as follows:

$$\tau^* = \frac{\mathrm{ETR}_A}{\mathrm{ETR}_P} \cdot \frac{C_c}{O} \tag{8}$$

The CO_2 concentration at the carboxylation site (C_c) is approximated by C_i, and the oxygen molar fraction at the oxygenation site (O), is assumed to be equal to the molar fraction in the air. Alternatively, if τ as determined *in vitro* is assumed to be valid for the Rubisco *in vivo*, then C_c and g_i can be calculated. In both cases, the corresponding concentrations in the liquid phase were calculated from the published solubility of the gases in water (Harned & Davis, 1943; Harned & Bonner, 1945) at the measuring temperature and partial pressure. The mesophyll conductance to CO_2 was then calculated as:

$$g_i = A_N \cdot \frac{C_i}{C_c} \tag{9}$$

3.2.8.2. Parameters derived from A_N –C_c curves

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

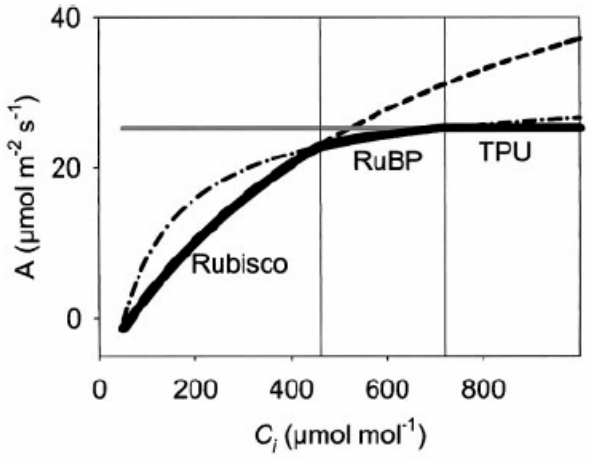
The model of Farquhar *et al*. (1980) has provided a tried and tested means to partition quantitatively biochemical and stomatal limitations on photosynthesis, from the response of CO_2 uptake to intercellular mole fraction of CO_2. Simultaneous measurement of chlorophyll fluorescence now extends this analysis, providing a means to determine the partitioning of energy between photosynthesis and photorespiration, and therefore to convert C_i into C_c, as explained above.

The response of A_N to C_c describes two, sometimes three, phases (Fig. 3.10). As C_c is increased from its minimum concentration, dA_N/dC_c is high and determined by Rubisco activity. With a further increase, there is an inflection to a lower dA_N/dC_c that approaches zero, where RuBP-regeneration is limiting. In some instances, a further increase in C_c may result in another transition to a plateau or a decrease in A_N with an

additional increase in C_c ($dA_N/dC_c \leq 0$) if triose-phosphate utilization (TPU) becomes limiting. These phases are mathematically predicted by the model of Farquhar *et al*. (1980), as modified to account for TPU-limitation (von Caemmerer, 2000). By fitting these phases, as mathematically defined (von Caemmerer, 2000) key biochemical kinetic variables determining photosynthetic rate can be determined *in vivo*, specifically: the maximum rates for the carboxylation activity of Rubisco ($V_{c,max}$), the electron transport driving regeneration of RuBP (J_{max}) and the triose-phosphate utilization rate (V_{TPU}).

Figure 3.10

Idealized A_N/C_i response (after Long & Bernacchi, 2003), equally valid for the analogue A_N-C_c 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 C_i 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}, R_D=2 μmol m^{-2} s^{-1}.



Both CO_2 and O_2 compete for the Rubisco binding site in the processes known as carboxylation and oxygenation, respectively (Farquhar *et al*., 1980). To account for the competitive inhibition between CO_2 and O_2, A_N is mathematically expressed as:

$$A_N = v_c - 0.5v_o - R_L = v_c\left(1 - \frac{\Gamma^*}{C_c}\right) - R_L \qquad (1)$$

The term $(1-\Gamma^*/C_c)$ is used to take into account the proportion of recently assimilated carbon that is released in photorespiration. The photosynthetic

compensation point (Γ^*) is the CO_2 concentration at which the photorespiratory efflux of CO_2 equals the rate of photosynthetic CO_2 uptake (i.e. when $v_c = 2v_o$). It is distinct from Γ which is the CO_2 compensation point, i.e. the CO_2 concentration at which $v_c = 2v_o + R_L$. Γ^* is determined by the properties of Rubisco (Brooks & Farquhar, 1985):

$$\Gamma^* = \frac{0.5O}{\tau} \tag{2}$$

where τ, the Rubisco specificity factor, is derived from Rubisco kinetics as:

$$\tau = \frac{V_{c,\max} \cdot K_o}{V_{o,\max} \cdot K_c} \tag{3}$$

The actual rate of carboxylation at Rubisco is determined by the minimum of the three potential rates (Harley & Sharkey, 1991; von Caemmerer, 2000):

$$v_c = \min\{w_c, w_j, w_p\} \tag{4}$$

Substituting for v_c into Eq. 1:

$$A_N = \min\{w_c, w_j, w_p\} \cdot \left(1 - \frac{\Gamma^*}{C_c}\right) - R_L \tag{5}$$

where:

$$w_c = \frac{V_{c,\max} \cdot C_c}{C_c + K_c\left(1 + O/K_o\right)} \tag{6}$$

$$w_j = \frac{ETR \cdot C_c}{4.5C_c + 10.5\Gamma^*} \tag{7}$$

$$w_p = \frac{3V_{TPU}}{\left(1-\frac{\Gamma*}{C_c}\right)} \quad (8)$$

where w_c, w_j and w_p are the potential rates of CO_2 assimilation that can be supported by Rubisco, RuBP regeneration and triose-phosphate utilization, respectively.

Since at ambient CO_2 concentration (i.e. close to 400 ppm), photosynthesis is limited by Rubisco capacity, among the three parameters that can be derived from the Farquhar model of analysis of A_N-C_c curves, we only estimated $V_{c,max}$. The quantities of $V_{c,max}$ can be then deduced by substituting equation (6) into (5). Hence, the A_N may be fitted to C_c with R_L as the y-axis intercept, and with K_c and K_o assumed temperature-dependent constants (Bernacchi *et al*., 2001):

$$K_c = e^{38.05-79.43/(R(T_l+273.15))} \quad (9)$$

$$K_o = e^{20.30-76.38/(R(T_l+273.15))} \quad (10)$$

where T_l is the leaf temperature (ºC). Fitting may then solve for the two unknowns $V_{c,max}$ and R_L. This may be achieved by a non-linear maximum likelihood best-fit (e.g. Microsoft Excel, Microsoft Corporation, USA). It is importanct to notice that estimations of $V_{c,max}$ and R_L were derived according to this model, but with the following modifications. First, we applied the 'one point method' proposed by Wilson *et al*. (2000), i.e. from the measure of assimilation and C_c at ambient CO_2 and the treatment average of Γ* for the species. This method requires that photosynthesis is substrate-limited (i.e. that C_c is on the Rubisco-limiting portion of the curve), which was the case in all our data-set according to the full A_N-C_c curves. Second, the model assumes constant τ values among C_3 species to estimate Γ*; however, we calculated the treatment average of Γ* using specific τ values for each one of the species (Galmés *et al*., 2005).

3.2.8.3. Quantitative photosynthetic limitation analysis

Photosynthetic limitations were partitioned into their functional components following the approach proposed by Jones (1985) and recently modified by Grassi &

Magnani (2005). This approach makes it possible to compare absolute or relative limitations to assimilation over any period of time, assuming that a reference maximum assimilation rate can be defined as a standard. The analysis was extended so as to partition photosynthesis limitations into components related to stomatal conductance, mesophyll conductance and leaf biochemical characteristics.

As shown in the previous section, light-saturated photosynthesis is generally limited by substrate availability and can be expressed as (Farquhar *et al*., 1980):

$$A_N = \frac{V_{c,\max} \cdot C_c}{C_c + K_c \cdot (1 + O/K_o)} \cdot \left(1 - \frac{\Gamma^*}{C_c}\right) - R_L \quad (1)$$

where $V_{c,max}$ is the maximum rate of carboxylation of Rubisco, K_c and K_o are the Michaelis–Menten constants for CO_2 and O_2, respectively, Γ* is the CO_2 compensation point in the absence of mitochondrial respiration, and R_L is the rate of non-photorespiratory CO_2 evolution in the light. Neglecting any changes in leaf respiration, a small shift in leaf assimilation can be then expressed in terms of the concurrent changes in $V_{c,max}$ and C_c as:

$$dA_N = \frac{\partial A_N}{\partial C_c} \cdot dC_c + \frac{\partial A_N}{\partial V_{c,\max}} \cdot dV_{c,\max} \quad (2)$$

The draw-down of CO_2 between the leaf surface and carboxylation sites is in turn a function of stomatal and mesophyll conductances and assimilation itself:

$$C_c = C_a - \frac{A_N}{g_{sc}} - \frac{A_N}{g_i} \quad (3)$$

where C_a is the atmospheric CO_2 concentration, g_{sc} is the stomatal conductance to CO_2 ($=g_{sw}/1.6$) and g_i is the mesophyll conductance to CO_2. The effects of boundary-layer conductance have been neglected, since all measurements were taken in well-ventilated chambers. Changes in C_c can then be expressed as:

$$dC_c = \frac{A_N}{g_{sc}} \cdot \frac{dg_{sc}}{g_{sc}} + \frac{A_N}{g_m} \cdot \frac{dg_m}{g_m} - \left(\frac{1}{g_{sc}} + \frac{1}{g_m} \right) \cdot dA_N \tag{4}$$

Assuming that only negligible changes in mitochondrial respiration take place over the experiments, from Eqn. 1 the sensitivity of assimilation to carboxylation potential can be finally expressed as:

$$\frac{\partial A_N}{\partial V_{c,\max}} = \frac{A_N}{V_{c,\max}} \tag{5}$$

When Eqns. 4 and 5 are substituted into Eqn. 2, relative changes in light-saturated assimilation can be expressed in terms of parallel relative changes in stomatal and mesophyll conductance and in biochemical capacity (i.e. in maximum carboxylation rate):

$$\frac{dA_N}{A_N} = S_L + MC_L + B_L = l_s \cdot \frac{dg_{sc}}{g_{sc}} + l_{mc} \cdot \frac{dg_m}{g_m} + l_b \cdot \frac{dV_{c,\max}}{V_{c,\max}} \tag{6}$$

$$\begin{aligned} l_s &= \frac{g_{tot} / g_{sc} \cdot \partial A_N / \partial C_c}{g_{tot} + \partial A_N / \partial C_c} \\ l_{mc} &= \frac{g_{tot} / g_m \cdot \partial A_N / \partial C_c}{g_{tot} + \partial A_N / \partial C_c} \\ l_b &= \frac{g_{tot}}{g_{tot} + \partial A_N / \partial C_c} \end{aligned} \tag{7}$$

where g_{tot} is total conductance to CO_2 between the leaf surface and carboxylation sites ($1/g_{tot} = 1/g_{sc} + 1/g_i$), S_L, MC_L and B_L are the contributions of stomatal and mesophyll conductance and of maximum carboxylation rate to dA/A, respectively, and l_s, l_{mc} and l_b are the corresponding relative limitations, with value between zero and one. Although exactly valid only for very small changes in gas exchange parameters, following Jones (1985) Eqn 6. was then applied to partition contributions to a change in photosynthesis, relative to a reference value. We then defined the drought-induced

relative change in light-saturated assimilation as the ratio of the actual value of A_N over the maximum value obtained under well-watered conditions, both measured at 25ºC:

$$\frac{dA_N}{A_N} \approx \frac{A_{N\max}^{ref} - A_{N\max}}{A_{N\max}^{ref}} \quad (8)$$

Relative changes in stomatal and mesophyll conductance and carboxylation potential were obtained in a similar way and used for the computation of individual components of photosynthesis limitations from Eqns. 6 and 7. Relative limitations in Eqn. 6 were approximated by the mean of the values corresponding to the two measurement times (Jones 1985; Wilson *et al*. 2000).

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

3.2.9. Leaf pigment determinations

Leaf disks were cut with a cork borer, wrapped in aluminium foil, dropped in liquid nitrogen, and stored (still wrapped in foil) at -20ºC. A few ml bulk acetone, neutralized with calcium carbonate, were put in a mortar and a pinch of sodium ascorbate was added. A small known amount of leaf tissue was dropped in the mortar and ground. The mixture was then poured into a volumetric flask and acetone was added until the desired volume was attained. The mixture was filtered through a 5 μm Millipore filter (Millipore Co., Billerica, USA). The whole extraction process took approximately 2 min per sample. The filtered extract was stored in foil-wrapped stoppered plastic tubes at -20ºC until the analysis was carried out. Analysis by HPLC of pigment extracts obtained with this procedure indicated that decomposition of pigments did not occur (Abadía & Abadía, 1993).

Pigment extracts were analyzed by an isocratic HPLC method based on that developed by De las Rivas *et al*. (1989). Determination of the concentrations of the individual pigments requires previous separation of the pigments. The reverse-phase HPLC method used resolves the major higher plant photosynthetic pigments in leaves, including neoxanthin, violaxanthin, taraxanthin, antheraxanthin, lutein, zeaxanthin,

lutein, chlorophyll b, chlorophyll a, and β-carotene. The separation was carried out on a C_{18} column using a single high-pressure pump and two different mobile phases in two isocratic steps. The two steps used were: mobile phase A (acetonitrile:methanol, 7:1 v:v) was pumped for 3.5 min, and then mobile phase B (acetonitrile:methanol:water:ethyl acetate, 7:0.96:0.04:8 by volume) was pumped for 4.5 min. To both solvents 0.7% (v:v) of the modifier triethylamine (TEA) was added to improve pigment stability during separation. All chemicals used were HPLC quality. The column was equilibrated before injecting each sample by flushing with mobile phase A for 5 min. The analysis time for each sample was 13 min, including equilibration time.

3.2.10. Rubisco biochemical procedures

3.2.10.1. Rubisco extraction and purification

Leaf material from each species was detached and immediately frozen in liquid nitrogen. The leaf material was ground to a powder in a mortar, buffer was added and grinding continued from time to time as the mixture thawed.

The most appropriate protein extraction media for each species was tested after extensive preliminary tests. Depending on the species, these were found to be:

(A) 0.1 M bicine, 50 mM β-mercaptoethanol, 11 mM Na-DIECA, 6% (w/v) PEG 4000, 1 mM benzamidine, 1 mM ε-amino-n-caproic acid and 1 mM PMSF, at pH 8.

(B) 0.1 M HEPES, 3% (w/v) PVP 25000, 6% (w/v) PEG 4000, 50 mM β-mercaptoethanol, 2 mM DTT, 10% glycerol, 5 mM $MgCl_2$, 5 mM EGTA and 2 mM PMSF, at pH 8.0.

(C) 0.1 M bicine, 10 mM Na-DIECA, 6% PEG 4000, 3% (w/v) PVP 25000, 1 mM DTT, 1 mM benzamidine, 1 mM ε-amino-n-caproic acid and 1 mM PMSF, at pH 8.

Buffer A was used to extract Rubisco from most of the species. Buffer B was used with *M. aquatica*, *P. lentiscus* and *C. albidus*. For *H. balearicum*, buffer A was used with the following modifications: the concentration of bicine was increased to 0.2 M and the volume used was doubled. For *P. lentiscus*, the following modifications were made to buffer B: the concentration of HEPES was increased to 0.25 M and the volume used was doubled. Buffer C was used for *N. tabaccum* extractions.

All the purification steps were carried out at 0 to 4 ºC. Fully thawed but still cold homogenates were filtered through butter muslin and then centrifuged at 22000 x g for 20 min. The supernatant liquid was decanted through 50 μm mesh nylon and PEG 4000 was added as a 60% (w/v) aqueous solution to the supernatant liquid to produce a final concentration of 20% (w/v). Also, 1 M $MgCl_2$ was added to a final concentration of 20 mM followed by gentle mixing. After standing for 10 min the mixture was centrifuged again at 22000 x g for 20 min. Then, the following steps in purification procedure depended on the experiment:

- For all the species except for *N. tabaccum*, the pellet was re-suspended in 40 ml of column buffer (10 mM Tris pH 8.0 with 10 mM $MgCl_2$, 10 mM $NaHCO_3$, 1 mM EDTA and 1 mM KH_2PO_4) containing 1mM each of PMSF, benzamidine and ε-amino-n-caproic acid. The suspension was centrifuged to remove insoluble material. The supernatant liquid was applied to an 88 x 1.6 cm column of Q Sepharose Fast Flow anion exchanger (Pharmacia Biotech, Uppsala, Sweden) previously equilibrated with Column buffer and operated at 1 ml min^{-1}. The effluent was monitored for absorbance at 280 nm. The proteins were eluted using a linear gradient from 0 to 0.75 M NaCl in column buffer in 16 h and fractions were collected at 10 min intervals. Those fractions with high Rubisco activity were combined and de-salted using a Sephadex G25 (Pharmacia) column 44 x 5 cm operated at 200 ml h^{-1} with fractions collected at 3 min intervals. Finally, fractions containing high amounts of protein were pooled together, the concentration of Rubisco was estimated as A_{280} x 0.61 mg ml^{-1} (Paulsen & Lane, 1966), and the solution dispensed into vials and freeze dried.

- For *N. tabaccum*, the pellet was re-suspended in 6 ml of column buffer (10 mM Tris pH 8.0 with 10 mM $MgCl_2$, 10 mM $NaHCO_3$, 1 mM EDTA and 1 mM KH_2PO_4) containing 1 mM each of DTT, PMSF, benzamidine and ε-amino-n-caproic acid. The suspension was then centrifuged to remove insoluble material. The supernatant liquid was layered onto step gradients from 1.2 to 0.4 M in sucrose in column buffer. Gradients were centrifuged at 50000 rpm for 120 min in a 70.1Ti rotor (Beckman, High Wycombe, UK). Fractions with high protein concentration were combined and applied to two 1 ml HiTrap Q HP columns (Amersham Biosciences, Fairfield, USA) connected in series, previously equilibrated with column buffer and operated at 1 ml min^{-1}. The proteins were eluted using a step gradient from 0 to 0.8 M NaCl in column buffer and fractions were collected in 1 ml intervals. Total soluble protein content in fractions was

confirmed using Bradford assay (Bradford, 1976). Those fractions with high protein (Rubisco) concentration were combined and stored at –70ºC.

3.2.10.2. Rubisco carboxylase activity measurements

Rubisco activity was measured at different stages of the purifications by adding 10 or 25 µl of solution containing protein to 0.2 ml of a solution containing 1 ml 0.1 M $NaH^{14}CO_3$, 0.5 μC_i per µmol, 5 ml 0.2 M bicine containing 40 mM $MgCl_2$ pH 8.2 and 4 ml H_2O. After 3 min 10 µl 20 mM RuBP was added and after a further 1 min the reaction was stopped by adding 0.1 ml of 10 M formic acid. To activate the slowly activating form of Rubisco present in solution after desalting, reaction mixtures, less the RuBP, were heated at 37ºC for 40 min and then cooled to room temperature before adding the RuBP and completing the assay. The acidified reaction mixes were dried down in an oven placed in a fume hood. After cooling, 0.4 ml of H_2O and 3.5 ml of Ultima Gold scintillation cocktail (Packard, Canberra, Australia) were added. ^{14}C in PGA (D-3-phosphoglycerate) was measured using a Scintillation Spectrometer.

3.2.10.3. Determination of leaf total soluble protein

Total soluble protein of leaf extracts was determined by the spectrophotometric assay of Bradford (1976). This method is based in the absorbance shift observed in an acidic solution of dye Coomassie Brilliant Blue G-250 (Bio-Rad, 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 dye has been assumed to bind to protein via electrostatic attraction of the dye's sulfonic groups to the protein. The bound points are primarily arginine residues, but the dye also binds to a lesser degree to hystidine, lysine, tyrosine, tryptophan and phenylalanine (Compton & Jones, 1985). The peak absorbance of the acidic dye solution changes from 465 to 595 nm when binding to protein occurs. Therefore, measuring the absorbance of the protein-dye complex at 595 nm allows an accurate quantification of the protein content of a sample.

3.2.10.4. Rubisco specificity factor determinations

For measurements at 25ºC, the freeze-dried Rubisco samples were dissolved and desalted by centrifugation through G25 Sephadex columns (Helmerhorst & Stokes, 1980) previously equilibrated with CO_2-free 0.1 M bicine pH 8.2 containing 20 mM $MgCl_2$. The desalted solutions were made 10 mM to $NaH^{14}CO_3$ and 0.4 mM to

orthophosphate. These mixtures were incubated at 37ºC for 40 min to activate the Rubisco. Reaction mixtures were prepared in an oxygen electrode by first adding a solution of 100 mM bicine pH 8.2, 10 mM $MgCl_2$ containing carbonic anhydrase and equilibrated with CO_2-free air at 25ºC. After adding $NaH^{14}CO_3$ the plug was fitted to the oxygen electrode vessel. Enough activated Rubisco was then added for the reaction to be completed within 5 min. The reaction was started by the addition of RuBP to give a 1 cm^3 total reaction volume. RuBP oxygenation was calculated from the oxygen consumption, and carboxylation from the amount of ^{14}C incorporated into PGA when all the RuBP had been consumed (Parry *et al*., 1989). A sequence of reaction mixtures containing pure wheat Rubisco were interspersed with those containing Rubisco from the test species, and the results normalised to the average value obtained from wheat Rubisco.

In addition, several species were selected for measurement of τ at 15ºC and 35ºC. From the slopes of the regressions between ln τ and 1000/RT, the difference in the free energy of activation to the transition state intermediates for the oxygenase and the carboxylase reactions of Rubisco ($\Delta G_o^{\ddagger}$-$\Delta G_c^{\ddagger}$) was calculated according to Uemura *et al*. (1997).

3.2.11. Rubisco molecular biology procedures

Since Rubisco S subunit from higher plants is encoded by 2-12 different nuclear genes, it was necessary to extract mRNA to obtain the amino sequence of the S subunits of the protein. After total RNA extraction from leaf samples, reverse transcription reaction was performed to get cDNA. Then, *rbc*S was obtained after a PCR using specific primers for *Caryophyllales*.

Regarding Rubisco L subunits encoding genes (*rcb*L), total DNA was extracted from leaf samples and then amplified by a PCR with specific primers for *Limonium* species.

Both PCR products, from *rbc*S and *rbc*L were purified, and multiples copies were obtained from cloning and transforming them in *E. coli*. Finally, plasmids with the amplified genes were purified and sequenced.

Chapter 4

SEED GERMINATION

GERMINATION CAPACITY AND TEMPERATURE DEPENDENCE IN MEDITERRANEAN SPECIES OF THE BALEARIC ISLANDS

4.1. SUMMARY

To test the germination capacity and its temperature dependence in Mediterranean plants, sixteen species representative of a diversity of habitats of the Balearic Islands were selected. The percentage of germination, the half-response time and the dormancy period of these species were studied at three alternating temperatures: 5-15ºC, 10-20ºC and 15-25ºC. Large differences were found among species in all three parameters studied. The influences of the temperature incubation in germination behaviour depended strongly on species, although the optimum temperature range to reach maximum germination was found to be 10-20ºC for most species. Although part of the endemic flora of the Balearic Islands currently has a limited and regressive distribution, no clear pattern was observed when comparing endemic and non-endemic species, although some of the endemics presented unfavourable characters, particularly a temperature independence of germination.

4.2. INTRODUCTION

The significance of seed stage capacity has long been recognized as one of the critical steps in species spatial and temporal establishment success (Harper, 1977; Silvertown & Lovett-Doust, 1993; Hilhorst & Toorop, 1997). The response pattern of seed germination is also regarded as a key characteristic in plant life history strategy (Angevine & Chabot, 1979; Mayer & Poljakoff-Mayber, 1989). Seed germination can be regulated not only through genotypic characteristics (Gutterman, 1993), but also by environmental conditions, being soil temperature the most important environmental factor controlling seed germination (Beardsell & Richards, 1987). Temperature can affect the germination capacity through its effects on seed deterioration, loss of dormancy and the germination process itself (Roberts, 1988).

The Mediterranean climate is characterised by its seasonality in temperature and precipitation, which leads to a hot drought period in summer and a cool wet period in winter (Joffre *et al*., 1999). This peculiarity of the Mediterranean climate has important implications on plant germination physiology, since dry summer conditions limit water availability and thus germination and growth, while cool winter temperatures also limit germination during the season with high water availability (Rundel, 1996). Spatial and temporal distribution of precipitation in these ecosystems is considered to be highly episodic and unpredictable (Terradas, 1991). The germination season, and for extension

the temperature requirements for germination, might be crucial for plant survival. For instance, it is obvious that a species with high, massive germination process relatively independent of temperature may not be favoured, because any short precipitation in late spring would cause its seeds to germinate, but seedlings will not be capable to overcome the following summer stresses (Lloret *et al*., 1999). Moreover, the presence of dormancy that delays germination is often advantageous in a competitive or seasonal environment (Harper, 1977; Vleeshouwers *et al*., 1995).

The Balearic Islands, located within the Mediterranean basin, are characterised by the richness of its endemic flora (Cardona & Contandriopoulos, 1979). Nevertheless, a substantial number of them have been described to have a limited and regressive distribution (Alomar *et al*., 1997). There are different lines of evidence showing that endemic species with a narrow distribution are less competitive than widespread species (Carlquist, 1970; Ehrendorfer, 1979; Givnish, 1998; Pattison *et al*., 1998; Walck *et al*., 1999; Durand & Goldstein, 2001; Gulías *et al*., 2002; Gulías *et al*., 2003). However, a comparative study of the germination capacity of endemic and non-endemic species is lacking in the literature, despite the fact that reduced germination capacity and viability is a likely candidate to explain the declining distribution of some of these species. Furthermore, a good understanding of the germination capacity and requirements of these species is essential for an optimal biodiversity conservation and management.

The main objective of this work was to compare the germination capacity and the ability to germinate under different temperature ranges, those typically occurring in the different seasons, of Mediterranean species of the Balearic Islands, belonging to different taxonomic, evolutionary and growth form groups.

4.3. MATERIAL AND METHODS

4.3.1. Plant species, seed collection and storage

Sixteen Mediterranean species inhabiting in the Balearic Islands were selected according to their evolutionary history (endemic and non-endemic species of the Balearic Islands) and growth form. Species were classified in couples of one endemic and one non-endemic species having similar growth form and naturally occurring in similar niches, although not necessary belonging to the same family (Table 4.1).

Table 4.1.

List of the species used in the present study with their family, evolutionary history, growth form and habitat characteristics and seed collection season. Species are listed in couples of one endemic and one non-endemic competitors.

Species	Evolutionary history	Growth form	Habitat	Seed collection season
Diplotaxis ibicensis Pau	Endemic	Annual herb	Coastal	May
Lavatera maritima Gouan	Non-endemic	Woody semi-deciduous	Coastal	June
Limonium magallufianum L. Llorens	Endemic	Woody evergreen	Coastal	September
Limonium gibertii (Sennen) Sennen	Non-endemic	Woody evergreen	Coastal	September
Beta maritima L. subsp. *marcosii* A. Juan & M. B. Crespo	Endemic	Perennial herb	Coastal	July
Beta maritima L. subsp. *maritima*	Non-endemic	Perennial herb	Coastal	July
Hypericum balearicum L.	Endemic	Woody evergreen	Shrubland	August
Pistacia lentiscus L.	Non-endemic	Woody evergreen	Shrubland	October
Phlomis italica L.	Endemic	Woody semi-deciduous	Shrubland	August
Cistus albidus L.	Non-endemic	Woody semi-deciduous	Shrubland	July
Urtica atrovirens subsp. *bianorii* (Knoche) Paira	Endemic	Annual herb	Ruderal	June
Urtica membranacea Poiret	Non-endemic	Annual herb	Ruderal	May
Lysimachia minoricensis J. J. Rodr.	Endemic	Perennial herb	Water sources	August
Mentha aquatica L.	Non-endemic	Perennial herb	Water sources	October
Pimpinella bicknelli Briq.	Endemic	Perennial herb	Ruderal	June
Kundmannia sicula (L.) D. C.	Non-endemic	Perennial herb	Ruderal	June

Seeds from at least 10 plants per species were collected in the field. From these seeds, 10 to 20 plants per species were obtained and grown at optimum conditions outdoors at the University of the Balearic Islands (Mallorca, Spain). Completely developed seeds from these plants were collected in 2002 and 2003 to perform the experiment. Seasonal seed collection depended of the phenology of each species (Table 4.1). When needed, seeds were manually separated from their capsules, except for the two *Beta* species, which are located in a hard capsule with 2-3 embryos per capsule. Healthy seeds were selected, placed in paper envelopes, and dry-stored in a refrigerator at 4°C until their use during the same season.

Seeds from *C. albidus* and *P. lentiscus* required pre-germination treatments. *C. albidus* presents dormancy due to the hardness and impermeability of its coat, which were broken with dry-heat pre-treatment, placing seeds spread on glass dishes in oven at 90ºC for 5 minutes (Thanos & Georghiou, 1988; Hanley & Fenner, 1998). In the case of *P. lentiscus*, fruit pulp was removed (García-Fayos & Verdú, 1998).

4.3.2. Germination tests

Seeds were placed in 9 cm diameter Petri-dishes on two layers of filter paper (Whatman no. 1) moistened to saturation with distilled water, so that germination was not limited by water. Additional water was added when needed. Care was taken not to inundate the seeds. Petri-dishes were covered with parafilm to minimise water loses.

For all species, 20 seeds were used per Petri-dish, excepting the two *Beta* species, with 15 seeds per dish, with 6 replicates (4 for *Beta* species) per each species. Germination tests were conducted in controlled environment chambers (Koxka, Spain). Petri-dishes were randomly distributed in germination chambers and their position was changed every 2-3 days. Seeds were incubated in continuous darkness at three alternating temperatures (12h-12h): 5-15, 10-20 and 15-25°C. These regimes were designed to simulate natural diurnal fluctuations of temperature in winter, spring/autumn and early summer, respectively. Germination was defined as the first emergence of the radicle. Newly germinated seeds were counted under green light every 2-3 days and subsequently removed from the Petri-dishes. Those seeds infected by fungi or bacteria were removed and not considered for the calculations. The experiment lasted 91 days. Non-germinated seeds were dissected to determine if they had embryo (full seeds), with the aid of a magnifying glass when required.

The percentage of cumulative seed germination (G) for each replicate was calculated at the end of the experiment as:

$$\%G = \frac{SG}{IS - ES} \cdot 100$$

Where: SG = number of germinated seeds.

ES = number of empty seeds.

IS = number of seeds initiated in each replicate.

The dormancy period (D) of a homogeneous group of seeds was determined as the number of days needed to observe the first seed germinated. In addition, the average time response (T_{50}) to germination conditions was determined as the number of days elapsed from the initial until germination of 50% of total germinated seeds.

4.3.3. Statistical analysis

All percent G values were arcsine square root transformed before analysis to normalize the variance (Zar, 1999). T_{50} and D values were logarithm transformed for

the same purpose. Statistical analysis of germination data was performed with the SPSS 11.5 software package (SPSS, Chicago, USA). A Tukey's multiple comparison test was then used to determine differences between species and temperatures for all parameter means ($P < 0.05$).

4.4. RESULTS

For a given temperature treatment, G largely diverged among species (Table 4.2). *D. ibicensis*, *L. magallufianum*, *L. gibertii*, *H. balearicum*, *U. membranacea* and *L. minoricensis* presented G values higher than 80%, at least for one temperature regime. By contrast, *L. maritima*, *B. vulgaris* subsp. *marcosii*, *B. vulgaris* subsp. *maritima*, *P. italica* and *U. atrovirens* subsp. *bianori* did not reach 40% of G at any temperature. Despite the large differences observed between species, G did not differ significantly between endemic and non-endemic species at any of the three temperatures ($P > 0.05$), however, four of the five species with G higher than 90% were endemics, but also three of the five with G lower than 40%.

Most of the species presented significantly different G values depending on the temperature treatment ($P < 0.05$) and different optimum temperature (i.e. the temperature regime at which G was maximal) (Table 4.2). Only *H. balearicum*, *P. italica*, *L. minoricensis* and *M. aquatica* showed no effect of temperature on G. In general, most of the species presented the lowest G at 15-25ºC and only *D. ibicensis* showed significant higher G values at 5-15ºC than at any other temperature regime ($P < 0.05$).

T_{50} ranged from 3.0 of *L. magallufianum* at 15-25ºC to 70.2 of *M. aquatica* at 5-15ºC (Table 4.2). In general, T_{50} was lower at 10-20ºC. For *L. maritima*, *L. magallufianum*, *L. gibertii*, *H. balearicum* and *U. membranacea* T_{50} was independent of the temperature treatment (Table 4.2). By contrast, *D. ibicensis* and *P. italica* germinated more slowly at higher temperatures, and both *Beta* species, *P. lentiscus*, *C. albidus*, *L. minoricensis*, *M. aquatica*, *P. bicknelli* and *K. sicula* had higher T_{50} at lower temperature regimes.

In respect to D, *D. ibicensis* and *L. magallufianum*, with 3 days at 15-25ºC treatment, had the lowest values. By contrast, *P. bicknelli*, with 55.3 days at 5-15ºC presented the highest D. Temperature did not have significantly effects ($P > 0.05$) on *P. italica* and *K. sicula* dormancy period (Table 4.2).

Table 4.2

Means of percentage of cumulative seed germination (G), average time response (T_{50}) and dormancy period (D) at the three alternating temperature treatments for each species. Different letters denote statistically significant differences by a Tukey's multiple comparison test ($P < 0.05$) within each parameter and species (small letters) or within each parameter and temperature range (capital letters).

	G (%)		
Species	**5-15ºC**	**10-20ºC**	**15-25ºC**
Diplotaxis ibicensis	97.9 ^{c,G}	84.4 ^{b,FG}	52.7 ^{a,DE}
Lavatera maritima	1.3 ^{a,A}	20.0 ^{b,AB}	2.0 ^{a,AB}
Limonium magallufianum	98.4 ^{b,G}	93.6 ^{ab,HI}	92.7 ^{a,F}
Limonium gibertii	91.2 ^{ab,G}	91.2 ^{b,HI}	73.6 ^{a,E}
Beta maritima subsp. *marcosii*	31.5 ^{b,CDE}	31.4 ^{b,ABC}	3.7 ^{a,A}
Beta maritima subsp. *maritima*	31.1 ^{b,CDE}	28.3 ^{b,ABC}	7.2 ^{a,AB}
Hypericum balearicum	97.7 ^{a,G}	97.8 ^{a,I}	98.6 ^{a,F}
Pistacia lentiscus	47.3 ^{b,EF}	39.3 ^{b,BCD}	4.7 ^{a,AB}
Phlomis italica	15.8 ^{a,ABC}	17.4 ^{a,A}	8.7 ^{a,AB}
Cistus albidus	41.3 ^{a,DE}	70.7 ^{b,EF}	37.0 ^{a,CD}
Urtica atrovirens subsp. *bianorii*	8.1 ^{a,AB}	36.1 ^{b,ABC}	20.4 ^{a,BC}
Urtica membranacea	34.7 ^{a,DE}	80.2 ^{b,FG}	20.9 ^{a,BC}
Lysimachia minoricensis	98.7 ^{a,G}	98.0 ^{a,I}	96.7 ^{a,F}
Mentha aquatica	67.3 ^{a,F}	70.9 ^{a,EF}	68.7 ^{a,E}
Pimpinella bicknelli	25.1 ^{b,BCD}	47.6 ^{c,CD}	14.4 ^{a,AB}
Kundmannia sicula	30.0 ^{a,CDE}	58.7 ^{b,DE}	20.7 ^{a,BC}

	T_{50} (days)			D (days)		
Species	**5-15ºC**	**10-20ºC**	**15-25ºC**	**5-15ºC**	**10-20ºC**	**15-25ºC**
Diplotaxis ibicensis	5.0 ^{a,A}	5.0 ^{a,A}	8.0 ^{b,AB}	5.0 ^{b,A}	5.0 ^{b,A}	3.0 ^{a,A}
Lavatera maritima	24.5 ^{a,EFG}	10.0 ^{a,B}	21.0 ^{a,BCDEFG}	24.5 ^{ab,BCD}	6.3 ^{a,AB}	21.0 ^{b,FG}
Limonium magallufianum	5.0 ^{a,A}	5.0 ^{a,A}	3.0 ^{a,A}	5.0 ^{b,A}	5.0 ^{b,A}	3.0 ^{a,A}
Limonium gibertii	9.0 ^{a,B}	8.3 ^{a,B}	9.0 ^{a,ABCD}	7.0 ^{b,A}	5.0 ^{a,A}	4.0 ^{a,AB}
Beta maritima subsp. *marcosii*	27.0 ^{g,G}	15.0 ^{a,CD}	12.6 ^{a,BCDEF}	19.3 ^{c,BCD}	9.5 ^{a,CDE}	13.7 ^{b,EF}
Beta maritima subsp. *maritima*	26.7 ^{b,FG}	14.7 ^{a,CD}	14.8 ^{a,BCEDF}	21.0 ^{c,CDE}	10.3 ^{a,DE}	14.0 ^{b,EF}
Hypericum balearicum	12.3 ^{a,BCD}	12.7 ^{a,C}	11.8 ^{a,ABCDE}	12.0 ^{b,B}	12.0 ^{b,E}	6.5 ^{a,BCD}
Pistacia lentiscus	42.2 ^{b,H}	34.0 ^{b,F}	16.5 ^{a,BCDEFG}	32.7 ^{b,EF}	23.7 ^{a,F}	21.0 ^{a,FG}
Phlomis italica	16.5 ^{ab,CDE}	13.0 ^{a,C}	18.8 ^{b,DEFG}	13.3 ^{a,BC}	12.0 ^{a,E}	13.0 ^{a,DEF}
Cistus albidus	57.5 ^{b,HI}	23.7 ^{a,E}	35.0 ^{a,EFG}	25.3 ^{b,DEF}	10.8 ^{a,DE}	22.0 ^{b,FG}
Urtica atrovirens subsp. *bianorii*	18.2 ^{b,DEF}	9.0 ^{a,B}	26.3 ^{b,DEFG}	16.4 ^{b,BCD}	7.7 ^{a,BC}	17.7 ^{b,EF}
Urtica membranacea	12.0 ^{a,BC}	9.0 ^{a,B}	26.0 ^{a,BCDEFG}	7.0 ^{a,A}	5.0 ^{a,A}	13.2 ^{b,DEF}
Lysimachia minoricensis	19.0 ^{b,EFG}	9.0 ^{a,B}	6.0 ^{a,ABC}	19.0 ^{c,BCD}	9.0 ^{b,CD}	6.0 ^{a,BC}
Mentha aquatica	70.2 ^{b,I}	17.3 ^{a,D}	13.0 ^{a,CDEFG}	36.8 ^{b,FG}	9.5 ^{a,CDE}	9.7 ^{a,CDE}
Pimpinella bicknelli	66.5 ^{c,I}	54.2 ^{b,G}	48.0 ^{a,G}	55.3 ^{b,G}	44.8 ^{a,G}	42.8 ^{a,H}
Kundmannia sicula	43.5 ^{b,H}	34.3 ^{a,F}	34.2 ^{a,FG}	33.5 ^{a,EF}	29.7 ^{a,F}	30.3 ^{a,GH}

4.5. DISCUSSION

As expected for Mediterranean ecosystems, where many species have been related to present different types and degrees of dormancy (Baskin & Baskin, 1998), large differences in G, D and T_{50} were found between species (Table 4.2). This wide range of variation could be partly explained by intraspecific differences, between populations and years, as already observed in some species, due to both environmental and genetic causes (Melzack & Watts, 1982; Baskin & Baskin, 1998; Gasque & García-Fayos, 2003). The production of seeds with different germinability is one of the most important survival strategies for species growing under unpredictable environment conditions (Gutterman, 1994; Kigel, 1995).

It was noteworthy the low percentage of viable seeds of some of the species, i.e. the percentage of non-geminated seeds at the end of the experiment (Table 4.2). Seven of the sixteen species did not reach 50% of germination at any of the temperature regimes analyzed. This is in agreement with other studies that have also reported low germination percentages in Mediterranean species, some of them included in this study, such as *P. italica* and *U. atrovirens* subsp. *bianorii* (Ayerbe & Ceresuela, 1982; Salvador & Lloret, 1995). This could be due to the presence of dormancy mechanisms in a large percentage of the seeds, as already described in *Cistus* species (Thanos & Georghiou, 1988; Delgado *et al*., 2001). Seed dormancy has been argued as a mechanism by which plants have adapted to unpredictable or seasonal environments (Bender *et al*., 2003), as the Mediterranean climate.

In general, the optimum temperature range to reach maximum G values was found to be 10-20ºC (Table 4.2). Only *D. ibicensis* presented an optimum temperature other than 10-20ºC. This optimum germination temperature range is similar to that reported for many other Mediterranean species (Ayerbe & Ceresuela, 1982; Lagarda *et al*., 1983; Baskin & Baskin, 1998; Barragán *et al*., 1999; Thanos, 2000). By contrast, Mitrakos (1981) showed that other Mediterranean species, such as *Nerium oleander*, *Ceratonia siliqua* and *Myrtus communis*, presented their optimum germination temperatures at 27.5ºC, which might be disadvantageous under Mediterranean climate, where high temperatures coincide with the dry season. Mitrakos (1981) suggested that this trait was acquired by these species before Mediterranean climate was originated in the late tertiary, when Mediterranean basin was characterized by a tropical climate.

Four species, *H. balearicum*, *P. italica*, *L. minoricensis* and *M. aquatica* (the former three, endemics) presented a temperature-independent germination behavior, with G values higher than 70%, regardless of temperature incubation (Table 4.2). This ability to germinate over a wide range of temperatures has been described to be an important characteristic of disturbed ecosystems (Santon, 1984) and has been also associated with species in which water supply is the main determinant of the timing of germination in the field (Grime *et al*., 1981). By contrast, in *L. maritima*, *B. maritima* subsp. *marcosii*, *B. maritima* subsp. *maritima*, *P. lentiscus*, *U. atrovirens* subsp. *bianorii*, *U. membranacea* and *K. sicula* maximum G doubled minimum G, suggesting a strong temperature-dependence of G. The low germination percentages of these species under some temperature regime could be due to the fact of significant degrees of secondary dormancy (Baskin & Baskin, 1998).

The high G of the extinct in the wild *L. minoricensis*, regardless of the temperature treatment, was in accordance with previous results reported by Rosselló & Mayol (2002), suggesting that seed viability was not the major cause of extinction of this species. Ayerbe & Ceresuela (1982), working with constant temperatures, reported different G values for *H. balearicum*, *U. atrovirens* subsp. *bianorii*, *P. bicknelli* and *P. italica*. Many other studies have shown different responses of G depending whether the incubation took place in constant or alternating temperatures (Beardsell & Richards, 1987; Del Monte & Tarquis, 1997).

Most of the species included in the present survey present their seed maturation and dispersal in late-spring or summer. Only *L. magallufianum*, *L. gibertii*, *P. lentiscus* and *M. aquatica* differ, with mature seeds occurring in autumn. This fact has ecological implications on seed germination, since under Mediterranean climate, favorable conditions for germination will start in early autumn, when precipitation usually initiates. Consequently, the presence of mechanisms that allow seeds to avoid germination under high temperature incubation would be positive in these environments. By contrast, high germination percentages and low dormancy period at 15-25ºC, together with mature seed cycle completed in late spring-summer might be disadvantageous in the Mediterranean climate, since any summer precipitation could lead to a massive germination, and seedlings will not be able to withstand summer water constraints. However, most of the species analyzed are likely to avoid this situation, since, at high temperatures (15-25ºC regime), although most of them decreased D and T_{50}, also decreased G to 20% or less (Table 4.2). *L. magallufianum*, *L. gibertii* and *M.*

aquatica presented high G values at the high temperature regime, but, as mentioned above, their seed maturation is not reached until autumn. *C. albidus* also maintained a relative high G at high temperatures, but a primary dormancy and capacity to establish a large seed soil bank balance is well-known in *Cistus* species (Thanos & Georghiou, 1988; Delgado *et al*., 2001).

Hence, only *D. ibicensis, H. balearicum* and *L. minoricensis* are likely to present a non-suitable germination behavior under the Mediterranean climate, with water limitation as the only environmental factor controlling germination. Nevertheless, in the present work the highest temperature range analyzed was 15-25ºC and it is obvious that higher temperatures are commonly reached in summer and might inhibit germination in these species. Furthermore, field emergence is affected by a number of other biological, physical and chemical factors (Hegarty, 1973; Egli & TeKrony, 1996; Weaich *et al*., 1996).

On the other hand, since the Mediterranean climate is characterized by highly episodic and unpredictable rainy events (Terradas, 1991), and therefore water availability in soil, it could be argued that a rapid and massive germination would constitute a positive adaptation to such environment. *D. ibicensis, L. magallufianum, L. gibertii*, *H. balearicum* and *L. minoricensis* may have reached this strategy, although assuming the intrinsic potential risks as a consequence of the negative effects drawn from the rapid changes of both temperature and humidity conditions under Mediterranean climate or the regularly presence of disturbances in this area (Noble & Gitay, 1996).

Because of wide range found on germination parameters among the species analyzed, none of the statistical analyses made after grouping species according to their evolutionary history resulted in significant differences. However, there are some qualitative aspects to be mentioned. For instance, endemic species generally present a higher germination capacity under any of the three alternate temperature incubations. Hence, at the 5-15ºC treatment, four of the five species that overcome 90% of G were endemics (Table 4.2). A similar pattern was found in the other temperature treatments analyzed, being endemics three of the four species with G higher than 90% at 10-20ºC, and only three endemic species were able to reach G of 90% at the 15-25ºC regime. Although a higher plasticity to temperature response is expected to be found in the more widespread and cosmopolitan species (Bouwmeester & Karssen, 1993), rather than among endemics, the only three species that overcome 90% of G despite of the

temperature incubation were all them endemics. By contrast, in some widespread species, such as *P. lentiscus* and *U. membranacea*, G, T_{50} and D highly depended on temperature. Indeed, endemics tend to reduce their dormancy period as the temperature increases to a larger extent than do non-endemic species. Hence, five endemic and two non-endemic species presented their lowest D at the 15-25ºC treatment, while three endemic and six non-endemic species after 10-20ºC incubation. Therefore, it can be argued that the reduced and declining distribution of some endemic species of the Balearic Islands are not due to a general deficient germination pattern of the endemic species. Moreover, the global change effects on the Mediterranean climate likely provide more frequent and longer drought periods, together with an increase of the temperature (Osborne *et al*. 2000). Consequently, germination capacity of some endemic species, such as *Limonium*, *H. balearicum* and *L. minoricensis*, will result less affected because of their capacity to germinate under moderate and high incubation temperatures.

In conclusion, this study shows a wide range of diversity in the temperature influences on the germination capacity of Mediterranean species, which might be related to different strategies adopted by these species as a consequence of the heterogeneity of habitats and climatic seasonality intrinsic to Mediterranean ecosystems. The endemic species of the Balearic Islands are likely to present a similar or a higher germination capacity and ability to germinate over a wide range of temperature than their relative widespread species. Therefore, at least for the species analyzed, other physiological characters than germination may be responsible for the limited and declining distribution of some of these endemic species.

Chapter 5

SEEDLING GROWTH

MODULATION OF RELATIVE GROWTH RATE AND ITS COMPONENTS BY WATER STRESS IN MEDITERRANEAN SPECIES WITH DIFFERENT GROWTH FORMS

5.1. SUMMARY

Effects of water availability on seedling growth were analysed in eight Mediterranean species naturally occurring in the Balearic Islands. Seedlings were grown outdoors during summer under two irrigation treatments: field capacity and 35% of field capacity. The relative growth rate (RGR) strongly depended on the growth form, from highest values in herbs to lowest in evergreen semi-shrubs. The main component associated with interspecific variation in RGR was the specific leaf area (SLA), and a quantitative grouping of the different growth forms appeared along the regression line between both parameters. The slow-growing species, i.e. evergreen shrubs had the lowest SLA and the fast-growing perennial herbs, the highest, while semi-deciduous shrubs appeared intermediate. Decreases in RGR due to water stress were analysed in terms of the relative contribution of the leaf mass ratio (LMR), SLA and the net assimilation rate (NAR). Pooling all species, the decrease in RGR caused by water deficit was mainly explained by decreases in SLA. However, this general pattern was strongly dependent of growth form. Thus, in the evergreen semi-shrubs, the decrease in RGR was accompanied by a 3-fold decrease in NAR which, however, increased in perennial herbs. SLA increased with decreasing water supply in evergreen semi-shrubs, and decreased in semi-deciduous shrubs and perennial herbs. Finally, decreases in LMR partly explained decreases in RGR in perennial herbs and evergreen shrubs. This different response of the different growth forms may reflect differences in seedling adaptation and surviving strategies to drought periods.

5.2. INTRODUCTION

The Mediterranean climate is characterised by a hot dry period in summer and a cool wet period in winter. From an ecophysiological point of view, the variability and unpredictability of precipitation imposes strong constraints on plants that could be extremely important for the survival of individuals (Joffre *et al*., 1999). In particular, the effects of water deficits during the summer severely influence the distribution and composition of vegetation in the Mediterranean basin. Moreover, the global change effects on the Mediterranean climate likely provide more frequent and longer drought periods (Osborne *et al*., 2000).

It is well known that seedling establishment is a critical developmental stage that may strongly depend on water availability (Moles & Westoby, 2004). Plant relative

growth rate (RGR) can be factorised into physiological and morphological components that determine the plant's carbon economy (Lambers *et al*., 1989). The physiological component is the net assimilation rate (NAR) and it is a measure of whole-plant daily net rate of change in plant carbon content (Poorter, 1989; McKenna & Shipley, 1999). NAR is generally correlated with the rate of photosynthesis per unit leaf area (Konings, 1989; Poorter & Van der Werf, 1998). The morphological component is related to the amount of leaf area per plant mass or the leaf area ratio (LAR), which, in turn, depends on two components: specific leaf area (SLA) and a measure of biomass allocation (leaf mass ratio, LMR) (Konings, 1989; Poorter & Van der Werf, 1998). The interspecific variation in seedling RGR is often strongly correlated with SLA in a large number of studies spanning a wide range of growth forms, originating from many habitats (Lambers & Poorter, 1992; Poorter & Van der Werf, 1998; Reich *et al*., 1998a; Wright & Westoby, 1999; Wright & Westoby, 2000). By contrast none generality has emerged for the relationships between RGR and LMR or between RGR and NAR (Lambers & Poorter, 1992; Poorter & Van der Werf, 1998). However, Shipley (2002) stated that the relative importance of SLA and NAR changes depending on irradiance, proposing a trade-off between these components as a function of daily irradiance.

Differences in RGR and its components among species and functional groups have been related to different adaptation patterns to the environment (Lambers & Poorter, 1992). For instance, early-successional species, as well as annual species, tend to be characterised by high RGRs, while late-successional species and perennials have, in general, a lower RGR (Poorter & Garnier, 1999). Even within a single functional group, differences in RGR due to, e.g., a different evolutionary history may lead to differences in competitiveness. In Hawaii, for instance, it has been shown that native species have a lower photosynthetic capacity, SLA and RGR than invasive species, and this has been related to the competitive and invasive ability of these species (Pattison *et al*., 1998; Baruch & Goldstein, 1999; Durand & Goldstein, 2001).

Water stress must be a major limiting factor for RGR in semi-arid climates, such as the Mediterranean, strongly constraining both growth and seedling survival during late spring and summer (Volaire *et al*., 1998; Moles & Westoby, 2004). Despite its importance, the number of analyses of the effects of water stress on RGR is surprisingly scarce, and limited to very few species, notably grasses (Retuerto & Woodward, 1993; Van den Boogaard *et al*., 1995; Kalapos *et al*., 1996; Van Splunder *et al*., 1996; Van den Boogaard *et al*., 1996; Van den Boogaard *et al*., 1997; Wang *et al*., 1998; Bargali &

Tewari, 2004). Traits such as greater allocation of biomass below than above ground, a lower evaporative surface, and a higher leaf mass per unit leaf area are common water-stress effects on biomass allocation (Ludlow, 1989). However, very few authors have factorised the causes of the variation in RGR due to water stress. Poorter & Nagel (2000), after reviewing the limited published data, attributed the decrease in RGR due to water stress to a decrease in NAR, and, to a lesser extent, to a decrease in SLA. However, Van den Boogaard *et al*. (1997) did not find significant differences in the contribution of LAR and NAR to variation in RGR between well-watered and water-stressed wheat cultivars. Similarly, Ball & Pidsley (1995) and Ball (2002) showed in two mangrove species that RGR decline with increase in salinity was due to decrease in both NAR and LAR. As far as we know, there is no comparative study including several species comprising different growth forms in which growth of plants at limiting water supply has been analysed in terms of RGR and its components. Moreover, the different growth forms (i.e. perennials *vs*. semi-deciduous and herbs *vs*. shrubs) differ in their morphological and physiological responses to water stress (Ludlow, 1989; Flexas *et al*., 2003). This makes it reasonable to hypothesise different strategies in the effects of drought on the relative contribution of NAR, SLA and LMR to the decrease in RGR across species comprising different growth forms. For instance, for species with a rapid leaf turnover such as deciduous plants and herbs, adjustments of RGR through changes in SLA and LMR in response to drought are likely to occur. By contrast, for woody evergreen species changes associated with morphological adjustments may be expected only at early stages of development, while in adult plants most changes may be expected to correlate with adjustments in NAR.

In this report we studied the effects of water stress on seedling establishment of 8 Mediterranean species comprising four growth forms. The objective of the present work was to determine the relative contribution of each of the underlying growth parameters to the decrease in RGR caused by water deficit across species comprising different growth forms.

5.3. MATERIALS AND METHODS

5.3.1. Plant material, environmental conditions and treatments

Eight Mediterranean species including different growth forms and occurring in different habitats were selected for this study (Table 5.1). Species were classified

depending on their growth form into: perennial herbs (PH), evergreen shrubs (ES), semi-deciduous shrubs (SDS) and evergreen semi-shrubs (ESS).

Plant material was obtained from seeds collected from natural populations of the selected species (Table 5.1). The smallest and the biggest seeds of those species showing high variability in the seed weight were discarded to homogenize initial seedling weight and increase the reliability of RGR estimates (Poorter & Garnier, 1996).

Table 5.1

Species analyzed and their characteristics.

Species	**Code**	**Family**	**Seed sources**	**Description**
Beta maritima L. subsp. *marcosii* A. Juan & M. B. Crespo	MC	Chenopodiaceae	Ses Bledes N 39º08'19'' E 02º57'41''	Perennial herb. Endemic of the Balearic Islands, inhabits in few very small islets subjected to saline spray and with high nutrient inputs.
Beta maritima L. subsp. *maritima*	MT	Chenopodiaceae	Cap Salines N 39º15'58'' E 03º03'02''	Perennial herb inhabiting coastal ecosystems. Widespread in temperate climates.
Phlomis italica L.	PI	Labiatae	Artà N 39º44'44'' E 03º20'55''	Semi-deciduous shrub up to 1 m, densely covered by hairs. Endemic of the Balearic Islands. The biggest populations are found 500 m above the sea level, where they compete with CA.
Cistus albidus L.	CA	Cistaceae	Artà N 39º43'58'' E 03º21'57''	Semi-deciduous shrub up to 1 m. Commonly found in the Mediterranean shrubland. As PI, their leaves are covered by hairs.
Limonium magallufianum L. Llorens	LM	Plumbaginaceae	Magalluf N 39º30'22'' E 02º32'46''	Woody evergreen semi-shrub, in cushion-like rosettes. Endemic of the Balearic Islands, inhabiting just in one coastal marsh located in Mallorca.
Limonium gibertii (Sennen) Sennen	LG	Plumbaginaceae	Es Carnatge N 39º32'39'' E 02º41'50''	Woody evergreen semi-shrub, in cushion-like rosettes. Occurring in West Mediterranean rocky and sandy coastal areas.
Hypericum balearicum L.	HB	Guttiferae	Mortitx N 39º52'25'' E 02º55'10''	Woody evergreen shrub up to 2 m, endemic of the Balearic Islands. The biggest populations are found in the shrubland 500 m above the sea level, where competes with PL.
Pistacia lentiscus L.	PL	Anacardiaceae	Mortitx N 39º52'25'' E 02º55'10''	Woody evergreen shrub up to 5 m, commonly found in the Mediterranean shrubland.

Seeds were germinated on filter paper moistened with deionized water in a controlled environment (germination chamber, at 18ºC in darkness). The experiment was performed in the first week of May 2002 for *Phlomis italica* (PI), *Cistus albidus* (CA), *Limonium magallufianum* (LM) and *Limonium gibertii* (LG), or 2003 for *Hypericum balearicum* (HB), *Pistacia lentiscus* (PL), *Beta vulgaris* subsp. *marcosii*

(MC), and *Beta vulgaris* subsp. *maritima* (MT). On the day following radicle emergence, seedlings were planted individually in pots (20 cm height, 4.1 L volume) containing a 40:40:20 mixture of clay-calcareous soil, horticultural substrate and perlite. For each species, seedlings of similar initial size were assigned to each treatment. Seedlings of each species and treatment were then randomly distributed under a shade cloth with a 30% light exclusion outdoors at the University of the Balearic Islands (Mallorca, Spain). Eventual instantaneous light measurements were performed to ensure that all seedlings were receiving equal amounts of irradiance.

The environmental conditions during the experiment were characteristic for the typical late spring-summer Mediterranean climate, with high temperatures and low relative humidity which generate considerable water losses by evapotranspiration. The maximum average temperature during the experiment in 2002 was recorded in July (24.1ºC), and the minimum in May (18.1ºC). 2003 was a somewhat hotter year, with a maximum average temperature of 27.7ºC in August and a minimum of 18.6ºC in May. By contrast, total irradiance during the experiment was similar between both years (2517 and 2674 MJ m^{-2}). Consequently, the mean monthly evapotranspiration was about 160 L m^{-2} in both years.

The transplanted seedlings were equally well watered during the first 40 days prior to exposure to water stress. In their habitats, seeds germinate and seedlings establish in early spring when soil moisture is adequate before experiencing water stress in summer. After that, seedlings were randomly assigned to two irrigation treatments: a) plants maintained at field capacity (control), and b) plants maintained at soil water deficit (35% of field capacity). Desired moisture levels were attained by allowing the soil to dry until close to the selected moisture level, as determined gravimetrically on each pot. The pots were weighed on alternate days, and the required amount of water was added in order to maintain the correct moisture level.

5.3.2. Growth parameters

In their habitats, these species typically endure three to five months of water stress. Therefore, growth of seedlings was monitored by harvesting 6 plants per species and treatment, 40 (H1), 80 (H2), 120 (H3) and 160 days (H4) after germination. Seedling mortality prior to the onset of the experiment obliged to reduce the number of replicates to 4 in two of the species, *P. lentiscus* and *H. balearicum*. After harvesting,

total plant leaf area (LA, m^2) was determined using an AM-100 leaf area meter (ADC, Herts, U. K.) and roots, stems and leaves were separated and dried in a ventilated oven at 60ºC until constant weight was reached. From these components, stem dry mass (SDM; g), leaves dry mass (LDM; g) and root dry mass (RDM; g) were determined. Based on these data, the following plant traits were calculated: total plant biomass (B; g), specific leaf area (SLA; $m^2\ kg^{-1}$), leaf mass ratio (LMR; $g\ g^{-1}$), leaf area ratio (LAR; $m^2\ kg^{-1}$), stem mass ratio (SMR; $g\ g^{-1}$) and root mass ratio (RMR; $g\ g^{-1}$). Average data from each species and treatment for two consecutive samplings were used to compute the partial net assimilation rate (NARp; $g\ m^{-2}\ day^{-1}$) and the partial relative growth rate (RGRp; $mg\ g^{-1}\ day^{-1}$) by the following equations:

$$NAR = \frac{(B_2 - B_1)\cdot(\log_e LA_2 - \log_e LA_1)}{(t_2 - t_1)\cdot(LA_2 - LA_1)}$$

$$RGR = \frac{\log_e B_2 - \log_e B_1}{t_2 - t_1}$$

where 1 and 2 refer to two consecutive harvests. The total NAR (NARt) and total RGR (RGRt) for the entire study period were calculated using the same formulae with t_1 and t_4. Leaf-related variables were calculated excluding the petiole weight.

Effects of growth form, species and moisture level on seedling growth parameters were analyzed by one-way ANOVA. All statistical analyses were performed using the SPSS package (SPSS, v10.0). To determine the change in each of the growth parameters (NARt, SLA and LMR), scaled with respect to the relative change in RGRt, we calculated the growth response coefficient (GRC) according to Poorter and Nagel (2000).

5.4. RESULTS

Table 5.2 shows mean values of total seedling biomass (B), leaf mass ratio (LMR), stem mass ratio (SMR), root mass ratio (RMR), leaf area (LA) and derived growth parameters. Both under control and water-stress conditions, the two *Beta* species were the species with the highest LA and growth rates. On the other hand, the evergreen shrubs HB and PL had the lowest. As common patterns, all species increased LA and reduced SLA with ageing, but their LAR and LMR decreased. Also, they tended to increase RMR with age. Apart from these general trends, the response of the remaining parameters with increase in plant age was strongly species-dependent. Most of them

showed their highest RGRp and NARp at early stages of development, but others, especially the evergreen shrubs, increased both parameters in the third harvest. These temporal variations in RGRp were mainly explained by changes in NARp, according to the strong correlation between both parameters (r ranged from 0.833 in PI to 0.993 in MC). Regarding to the effects of drought on these parameters, the most distinctive feature was the strong difference between growth forms (Table 5.2). For instance, even under water stress, perennial herbs had approximately a 10-fold greater LA than non-stressed evergreen shrubs in the fourth harvest.

Table 5.2

Mean values of total seedling biomass (B), leaf mass ratio (LMR), stem mass ratio (SMR), root mass ratio (RMR), leaf area (LA), specific leaf area (SLA), leaf area ratio (LAR), partial relative growth rate (RGRp) and partial net assimilation rate (NARp) for each species, treatment and harvest. The RGRp and NARp were calculated from two consecutive harvests. The periods for the different harvests were 40, 80, 120 and 160 days after germination. Drought treatment started after H1. The values given are means of 4 to 6 replicates per species, treatment and sampling period.

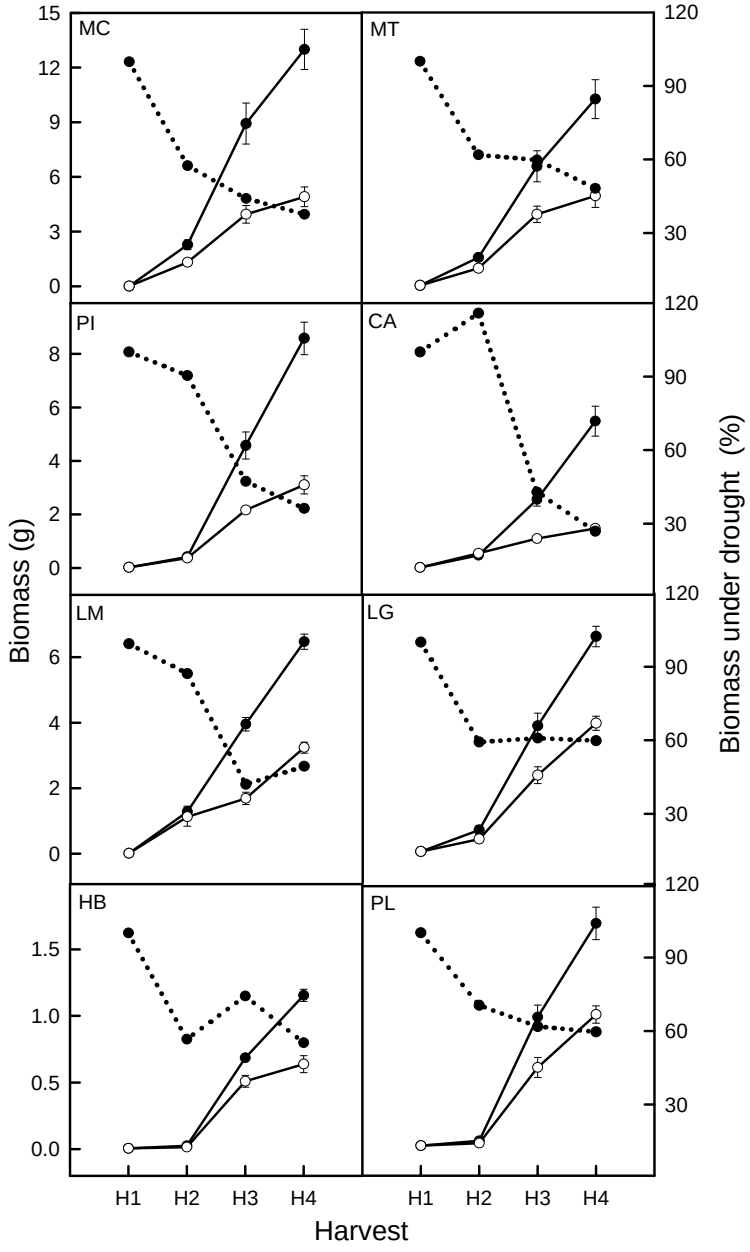
Species	Treatment	Harvest	B (g)	LMR	SMR	RMR	LA (m^2)	SLA (m^2 kg^{-1})	LAR (m^2 kg^{-1})	RGRp (mg g^{-1} d^{-1})	NARp (g m^{-2} d^{-1})
MC	Control	H1	0.01	0.65	0.06	0.28	0.03	46.2	29.7		
MC	Control	H2	2.28	0.66	0.06	0.28	4.62	31.8	21.2	137	6.4
MC	Water-stressed	H2	1.31	0.59	0.07	0.34	2.29	29.8	17.4	124	6.4
MC	Control	H3	8.93	0.55	0.08	0.38	12.85	26.7	14.5	34	2.0
MC	Water-stressed	H3	3.95	0.49	0.11	0.40	3.78	20.0	9.7	27	2.2
MC	Control	H4	12.99	0.54	0.07	0.39	16.70	24.1	13.0	10	0.8
MC	Water-stressed	H4	4.91	0.41	0.11	0.47	3.21	16.1	6.6	6	0.7
MT	Control	H1	0.02	0.79	0.07	0.14	0.06	46.3	36.4		
MT	Control	H2	1.55	0.65	0.06	0.30	2.98	31.0	20.1	112	5.1
MT	Water-stressed	H2	0.96	0.54	0.07	0.38	1.44	27.3	14.8	100	5.3
MT	Control	H3	6.56	0.52	0.10	0.39	8.46	26.0	13.5	36	2.4
MT	Water-stressed	H3	3.92	0.49	0.10	0.40	4.84	25.0	12.3	35	2.6
MT	Control	H4	10.24	0.51	0.07	0.42	12.61	24.7	12.6	11	0.9
MT	Water-stressed	H4	4.93	0.48	0.13	0.39	4.89	21.8	10.3	5	1.7
PI	Control	H1	0.02	0.63	0.10	0.26	0.05	35.2	22.3		
PI	Control	H2	0.41	0.53	0.12	0.34	0.31	13.9	7.4	73	6.7
PI	Water-stressed	H2	0.37	0.48	0.11	0.41	0.23	13.2	6.2	71	7.4
PI	Control	H3	4.58	0.49	0.18	0.32	3.35	14.8	7.3	61	8.3
PI	Water-stressed	H3	2.16	0.45	0.14	0.41	1.00	10.2	4.6	45	8.7
PI	Control	H4	8.58	0.41	0.15	0.44	5.00	14.2	5.9	16	2.6
PI	Water-stressed	H4	3.11	0.41	0.12	0.47	1.38	10.5	4.3	8	1.5
CA	Control	H1	0.02	0.69	0.12	0.18	0.04	37.1	25.8		
CA	Control	H2	0.47	0.41	0.15	0.45	0.47	24.9	10.1	83	6.3
CA	Water-stressed	H2	0.55	0.43	0.12	0.45	0.37	15.2	6.6	86	8.5
CA	Control	H3	2.56	0.44	0.14	0.41	1.52	13.5	6.0	42	5.8
CA	Water-stressed	H3	1.10	0.39	0.11	0.50	0.43	10.1	3.9	19	5.1
CA	Control	H4	5.49	0.29	0.20	0.51	2.32	14.0	4.1	19	3.4
CA	Water-stressed	H4	1.48	0.35	0.19	0.46	0.57	11.0	3.8	7	1.8

Species	Treatment	Harvest	B (g)	LMR	SMR	RMR	LA (m^2)	SLA (m^2 kg^{-1})	LAR (m^2 kg^{-1})	RGRp (mg g^{-1} d^{-1})	NARp (g m^{-2} d^{-1})
LM	Control	H1	0.01	0.79	0.00	0.21	0.03	29.3	22.9		
LM	Control	H2	1.28	0.65	0.05	0.29	1.16	14.1	9.3	117	10.6
LM	Water-stressed	H2	1.13	0.59	0.08	0.33	0.82	11.6	6.8	109	11.0
LM	Control	H3	3.95	0.56	0.11	0.33	1.90	8.6	4.9	29	4.7
LM	Water-stressed	H3	1.69	0.55	0.11	0.34	0.95	10.5	5.8	15	5.2
LM	Control	H4	6.47	0.49	0.06	0.44	2.71	8.5	4.2	12	2.8
LM	Water-stressed	H4	3.24	0.52	0.10	0.38	1.72	10.2	5.3	17	3.0
LG	Control	H1	0.01	0.77	0.00	0.23	0.03	31.3	24.5		
LG	Control	H2	0.68	0.67	0.09	0.24	0.76	17.5	11.9	96	7.2
LG	Water-stressed	H2	0.40	0.69	0.08	0.24	0.49	18.0	12.3	85	5.9
LG	Control	H3	3.86	0.56	0.08	0.36	2.80	13.1	7.3	46	5.4
LG	Water-stressed	H3	2.35	0.58	0.09	0.32	1.83	13.2	7.7	45	4.7
LG	Control	H4	6.58	0.49	0.10	0.41	3.02	9.4	4.6	14	2.8
LG	Water-stressed	H4	3.93	0.51	0.12	0.37	2.08	10.4	5.3	13	3.1
HB	Control	H1	0.01	0.63	0.13	0.24	0.01	23.9	15.1		
HB	Control	H2	0.03	0.56	0.11	0.33	0.02	14.4	8.1	40	3.9
HB	Water-stressed	H2	0.01	0.51	0.08	0.41	0.01	11.1	5.7	26	2.9
HB	Control	H3	0.69	0.36	0.25	0.40	0.29	11.9	4.2	83	16.4
HB	Water-stressed	H3	0.51	0.26	0.30	0.44	0.10	7.5	1.9	90	34.1
HB	Control	H4	1.15	0.30	0.28	0.41	0.35	10.1	3.1	13	3.9
HB	Water-stressed	H4	0.64	0.26	0.29	0.46	0.14	9.1	2.3	5	2.8
PL	Control	H1	0.02	0.75	0.04	0.21	0.03	18.1	13.6		
PL	Control	H2	0.06	0.68	0.17	0.14	0.08	18.0	12.3	22	1.7
PL	Water-stressed	H2	0.04	0.64	0.16	0.21	0.04	17.0	11.0	7	0.2
PL	Control	H3	0.99	0.52	0.26	0.22	0.42	8.2	4.3	71	12.0
PL	Water-stressed	H3	0.61	0.29	0.31	0.39	0.13	6.8	2.0	73	20.3
PL	Control	H4	1.69	0.45	0.31	0.24	0.46	6.1	2.8	14	4.3
PL	Water-stressed	H4	1.01	0.32	0.31	0.36	0.17	5.3	1.7	13	8.9

Figure 5.1 shows the evolution of plant biomass under well watered and water-stressed conditions and the evolution of the percentage of plant biomass under water-stressed in respect to the control values. The two *Beta* species were the species with the highest biomass at all harvests, both under well watered and water-stressed conditions, while the evergreen shrubs HB and PL had the lowest values. At the end of the experiment, the percentage of plant biomass under water-stressed in respect of the control values ranged between 30% and 60% for all species. However, that of evergreen semi-shrubs (LM and LG) and shrubs (HB and PL) was higher than 50%, while that of the remaining species was lower than 50%. For most of the species (MC, MT, LG, HB and PL) the effects of water stress on biomass reduction were more notorious early in the experiment, with strong reductions in plant biomass from H1 to H2. However, for PI, CA and LM the percentages of plant biomass under water-stressed in respect of the control values at H2 were still 90, 116 and 88%, respectively.

Figure 5.1

Biomass evolution along the four harvests under well watered (filled symbols) and water-stress conditions (empty symbols) and percentage of biomass under water-stressed conditions in respect to biomass under well watered conditions (dotted line) for each species. Species codes as in Table 5.1. The values for biomass evolution are means ± standard errors of 4 to 6 replicates per species, treatment and sampling period. The values for percentage of biomass under water-stressed conditions in respect to biomass under well watered conditions are means of 4 to 6 replicates per species, treatment and sampling period.

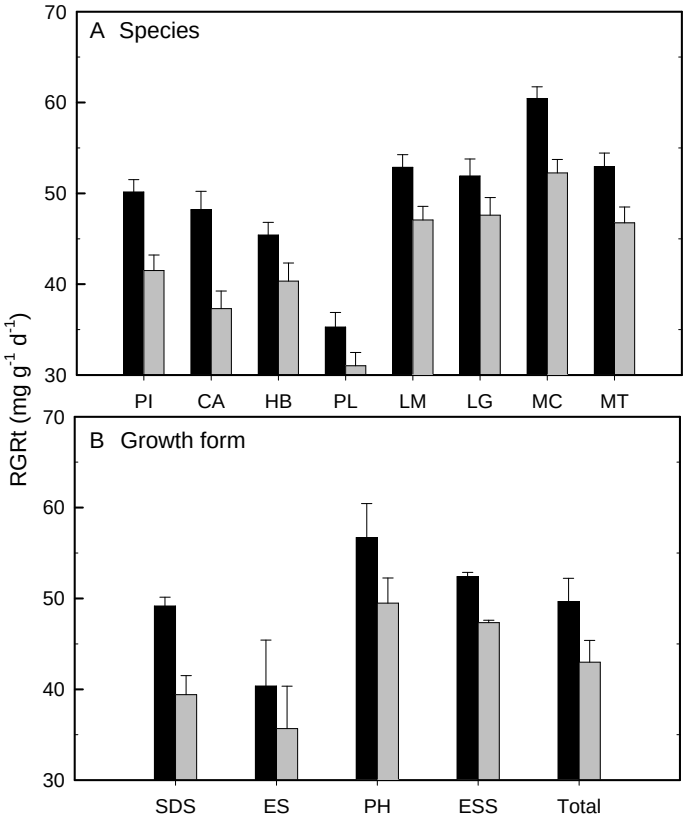


The highest RGRt both in control and stressed plants was that of MC (61 ± 1 and 52 ± 2 mg g^{-1} day^{-1}, respectively), while the lowest was that of PL (35 ± 2 and 31 ± 1 mg g^{-1} day^{-1}) (Fig. 5.2A). CA had the highest reduction in RGRt due to water stress

(23%) and LG the lowest (8%). Regarding growth form, perennial herbs had the highest RGRt, with 57 ± 3 and 50 ± 3 mg g^{-1} day^{-1}, under control and water stress, respectively (Fig. 5.2B). Evergreen shrubs had the lowest, 40 ± 5 and 36 ± 5 mg g^{-1} day^{-1}. The mean RGRt for all species together was 50 ± 3 mg g^{-1} day^{-1} in non-stressed plants and 43 ± 2 mg g^{-1} day^{-1} under water stress.

Figure 5.2

RGRt for each of the species studied (A), growth form and pooling all species together (B), under well watered (black bars) and water-stress conditions (grey bars). SDS (semi-deciduous shrubs), ES (evergreen shrubs), PH (perennial herbs) and ESS (evergreen semi-shrubs). Species codes as in Table 5.1. In Fig. 5.1A, the values are means ± standard errors of 4 to 6 replicates per species, treatment and sampling period. In Fig. 5.1B, the values are means ± standard errors for all the species belonging a particular group.

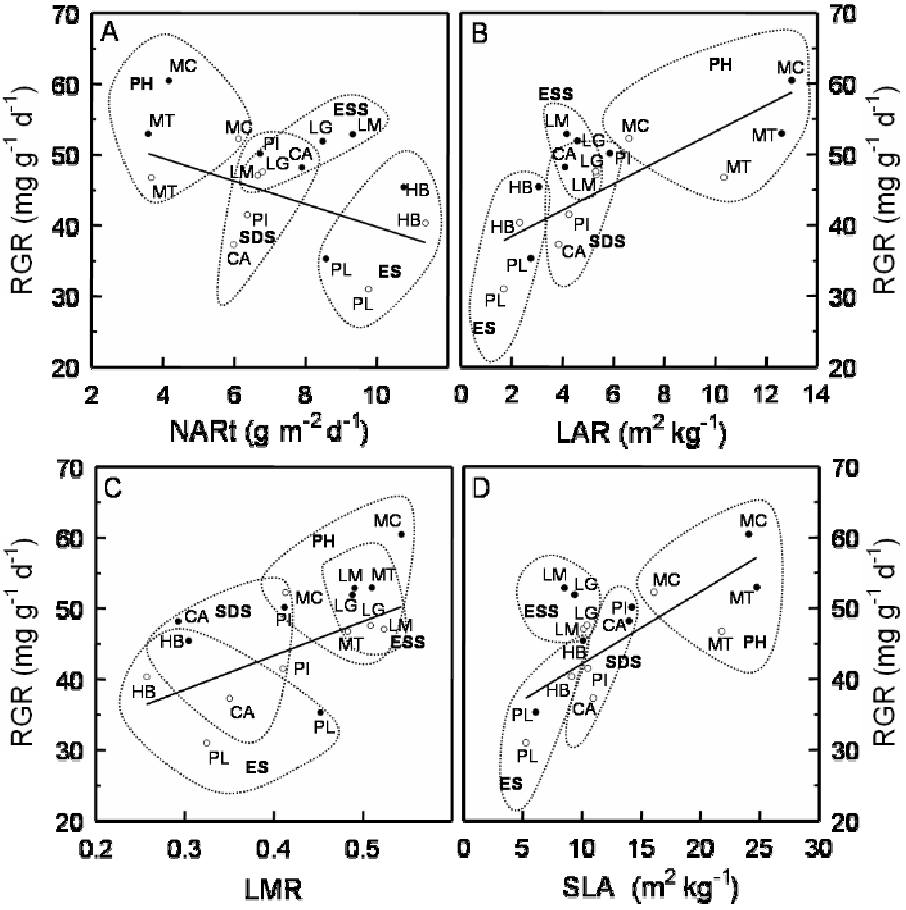


A regression analysis was performed to relate RGRt with each of its components (Fig. 5.3). ANCOVA revealed non-significant differences in the regression lines between well watered and water-stressed plants, for which only pooled plots are shown.

The highest RGRt values were found for those species with the smallest NARt (Fig. 5.3A). From the lowest to the highest NARt and from the highest to the lowest RGRt the following order was obtained: evergreen shrubs, semi-deciduous shrubs, evergreen semi-shrubs and perennial herbs. However, plotting together data of the final harvest from all species and treatments a non-significant relationship between NARt and RGRt was obtained (r = -0.466, $P > 0.05$) (Fig. 5.3A). By contrast, when plotting each species separately, including all harvests and both treatments (well watered and water stress), a significant, but positive relationship between RGRp and NARp was obtained for all species ($P < 0.01$) (data not shown).

Figure 5.3

Relationship between RGRt and NARt (A) and NAR (B), LMR (C) and SLA (D) at the end of the experiment for the species analysed under well watered (filled symbols) and water-stress conditions (empty symbols). SDS (semi-deciduous shrubs), ES (evergreen shrubs), PH (perennial herbs) and ESS (evergreen semi-shrubs). Species codes as in Table 5.1. The values are means of 4 to 6 replicates per species, treatment and sampling period.



The global positive relationship between final LAR and RGRt was found to be highly significant (r = 0.726, $P < 0.001$) (Fig. 5.3B) and, consequently, the relationships between LMR and SLA and RGRt were significant as well (r = 0.541, $P < 0.05$ and 0.715, $P < 0.01$, respectively) (Fig. 5.3C and 5.3D). Although it was not possible to establish statistically different groups of species, the four growth forms appeared again quantitatively in different regions of the relationship RGRt – SLA, with the same order than in Fig. 5.3A. *Limonium* species likely have a higher RGRt at a fixed SLA, pointing to a higher growth capacity. In all species but the two *Limonium*, SLA decreased when RGRt decreased due to water stress. When plotting each species separately, including all harvests and both treatments (well watered and water stress), also a significant, positive relationship between RGRp and LAR was obtained for all species ($P < 0.01$), except for *H. balearicum* and *P. lentiscus* (data not shown).

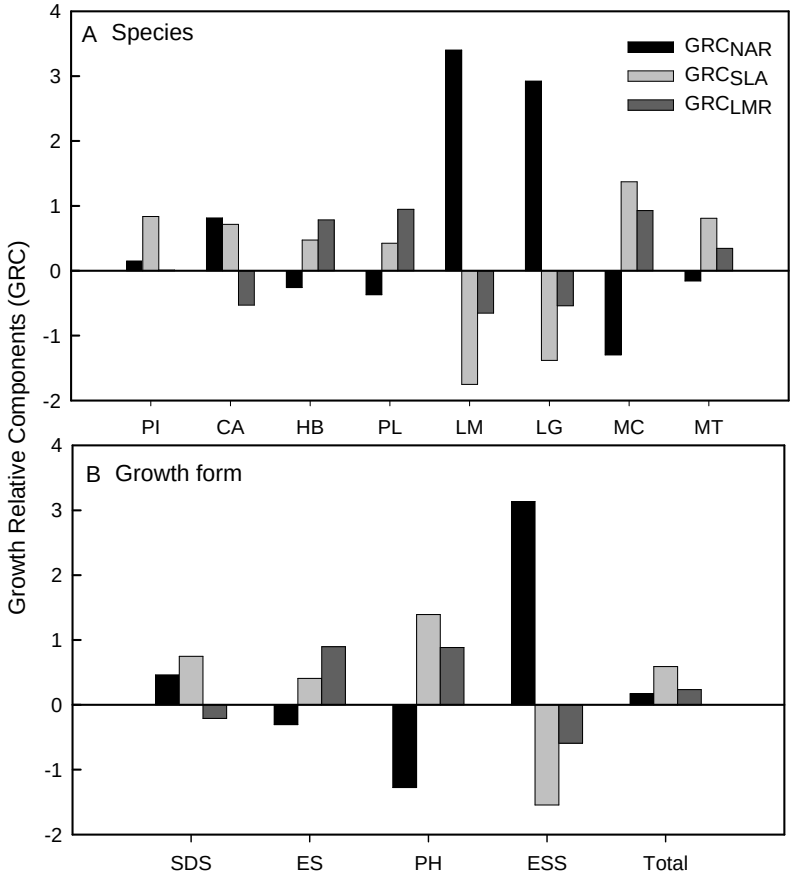
Figure 5.4A shows GRC for each species. Decreases in RGRt due to water stress in LM and LG were mostly associated with a decrease in NARt (GRC_{NAR} = 3.4 and 2.9, respectively), while LMR, and especially SLA, increased. In PI the only factor affected by the water-stress treatment and accounting for the decrease in RGRt was the decrease in SLA (GRC_{SLA} = 0.84). However, the other semi-deciduous shrub, CA, also decreased its NARt (GRC_{NAR} = 0.81), and therefore increased the LMR (GRC_{LMR} = -0.53). HB and PL mainly decreased LMR (GRC_{LMR} = 0.78 and 0.95, respectively). The decrease in MT RGRt was mainly associated with decreases in SLA (GRC_{SLA} = 0.81), but MC also decreased their LMR (GRC_{LMR} = 0.93) and so NAR increased (GRC_{NAR} = -1.3).

Figure 5.4B shows the relative contribution of each of the underlying growth parameters to the decrease of RGRt due to water deficit across the different growth forms. In semi-deciduous shrubs decreases in NARt (GRC_{NAR} = 0.46) and especially in SLA (GRC_{SLA} = 0.74) mostly explained the decrease in RGRt. In evergreen shrubs RGRt decreased due to water stress mainly because of decreases in LMR (GRC_{LMR} = 0.9). Perennial herbs strongly increased the physiological component (GRC_{NAR} = -1.28), and thus the morphological component was strongly decreased and responsible for the overall decrease in RGRt (GRC_{SLA} = 1.39 and GRC_{LMR} = 0.88). On the other hand, important decreases in NARt (GRC_{NAR} = 3.13), partially comensated by increases in SLA (GRC_{SLA} = -1.54) and in LMR (GRC_{LMR} = -0.59), were associated with the decreases in RGRt due to water stress in evergreen semi-shrubs.

Considering all species together, decreases in RGRt were accounted for by decreases in all three components underlying growth (GRC_{SLA} = 0.59, GRC_{LMR} = 0.24 and GRC_{NAR} = 0.17) (Fig. 5.4B).

Figure 5.4

Growth relative components (GRC) for each of the species studied (A), growth form and pooling all species together (B). WHS (semi-deciduous shrubs), ES (evergreen shrubs), PH (perennial herbs) and ESS (evergreen semi-shrubs). Species codes as in Table 5.1. Calculations were done from the average values for each species shown in Table 5.2.



5.5. DISCUSSION

Despite the fact that the most widespread soil-related stresses are nutrient shortage and drought (Poorter, 2002), only few studies, involving a limited number of species, have been published analyzing the causes of variation in RGR as affected by water availability (Poorter & Nagel, 2000). In this context, the main contribution of this study is that it analyses the causes of the decrease in RGR due to water stress in eight species comprising four different growth forms. The results obtained strongly suggest

that the influences of NAR, LAR, LMR and SLA on the decrease of RGR under drought depend on the species and growth form.

5.5.1. Changes in RGR with seedling age

In all the species analysed, the highest increase in total biomass occurred between H2 and H3 (Fig. 5.1). The change of RGR with increase in age, however, depended on growth form (Table 5.2). In those growth forms that invested more biomass in support structure, i.e. in evergreen and semi-deciduous shrubs, RGRp decreased slowly with increase in age. RGRp of the perennial woody shrubs still increased even at the third harvest. On the other hand, growth forms of smaller adult size, i.e. perennial herbs and evergreen semi-shrubs, had the highest RGRp at early stages of development, and this decreased strongly during plant development. This may be due to the different growth pattern of the species analyzed: *Beta* and *Limonium* species developed a high LA at early stages and hence self-shading appeared at relatively early developmental stage. By contrast, in shrubs LA development was slower, and self-shading was not significant at the third harvest. This is well related to NARp changes by a strong positive relationship between RGRp and NARp within each species with plant age, suggesting the physiological factor as the main component determining RGRp changes, in accordance with Antúnez *et al*. (2001). Reich (1998) hypothesized that differences in growth between species with short and long leaf life-span tend to disappear over time due to differences in the time necessary to develop a full canopy. In our case, *Beta* and, to a lesser extent, *Limonium* species, were able to develop their full canopy at the end of the experiment. On the other hand, shrubby species invest more in support structures from the early stages onwards, and thus their full canopy takes more time to develop an efficient structure to capture incident light. Regarding the morphological traits, in all plants an increase in age resulted in increased biomass allocation to roots (increased RMR), decreased biomass allocation to leaves (decreased LMR), increased leaf thickness (decreased SLA) and, as a consequence, decreased LAR.

Water stress decreased the overall growth of all species included in this study (Table 5.2). In all species, total biomass of water stressed at the end of the experiment was between 30% and 60% of irrigated plants, although the percent reduction tended to be lower in perennial species than in the other groups (Fig. 5.1). All species decreased

LA under water limited conditions, which has been related to the ability of species to tolerate and acclimate to water stress by morphogenetic plastic responses (Kozlowski *et al*., 1991). However, three of the species analyzed, CA, LM and LG, did not decrease the biomass allocation to leaves in relation to the total biomass (LMR).

5.5.2. Dependence of RGRt on morphological and physiological components

The same trend in growth-form dependence observed in the developmental changes of RGRp and NARp was observed for RGRt: RGRt decreased from perennial herbs to evergreen shrubs. Attending to the positive relationships between SLA and LMR with RGRt (Fig. 5.3C and 5.3D), this is due to a lower LAR or to a larger investment in supporting structures (Konings, 1989) (Fig. 5.3B).

The strong positive relationship found in this study between RGRt and SLA supports most of the studies available from the literature, which consider SLA as the prime factor associated with interspecific variation in RGRt (Lambers & Poorter, 1992; Wright & Westoby, 1999). Thus, a quantitative grouping of the different growth forms may be considered along the regression line between these parameters: the slow-growing species (i. e. evergreen shrubs) had the lowest SLA, and the fast-growing perennial herbs the highest, while semi-deciduous shrubs appeared intermediate (Fig. 5.3D). The growth form that lied more far from the regression line was that of evergreen semi-shrubs. A lower SLA is mainly due to a higher leaf mass density (Castro-Díez *et al*., 2000), and it has been shown to be inversely proportional to the internal gas conductance of leaves (g_i) (Syversten *et al*., 1995), and thus to the concentration of CO_2 in the chloroplast. This would imply that if, for given SLA, *Limonium* species have a higher RGRt, better carboxylation efficiency (i.e., photosynthetic gain per unit carbon available in the chloroplast) is likely found in *Limonium* than in the other species. This would be in accordance with the highest Rubisco specificity factor found in *Limonium* among C_3 species (Galmés *et al*., 2005).

The relationship between RGRt and the other morphological component, LMR was also positive (Fig. 5.3C). As a consequence, LAR exerted a positive influence on RGRt ($r = 0.726$, $P < 0.001$) (Fig. 5.3B). However, the lack of a positive relationship between RGRt and NARt (Fig. 5.3A) is in accordance with other authors (Dijkstra & Lambers, 1989; Khurana & Singh, 2000; Wright & Westoby, 1999). Some possible

explanations have been offered for this interrelation. First, Lambers & Poorter (1992) attributed this discrepancy to the resulting self-shading in plants with a high LAR. Secondly, since NARt is a complex function of photosynthesis, respiration and partitioning of biomass to photosynthetic and non-photosynthetic components (Lambers *et al*., 1989), fast respiration rates decrease NARt. Finally, Wright & Westoby (2000) concluded that the opposite trends in the leaf nitrogen productivity and the leaf nitrogen concentration with RGR explain the lack of relationship between NAR and RGR. Regarding the first hypothesis, species with lower NAR had the highest LAR ($r = -0.814$, $P < 0.001$) and self-shading events clearly appear in *Beta* and *Limonium* species. Moreover, when plotting RGRp *vs*. NARp considering all species and all harvests a positive relationship between these parameters was obtained ($r = 0.532$, $P < 0.05$), suggesting that NAR determined plant growth at early stages of development when self-shading effects still did not influence photosynthesis. Although leaf dark respiration is just a part of total plant respiration, the second hypothesis cannot be excluded. For instance, species with higher RGRt and higher LAR have a lower proportion of daily carbon gain used in respiration (Poorter *et al*., 1990; Reich *et al*., 1998b). The high proportion of biomass allocation to leaves, especially in the perennial herbs, must be at least considered as somewhat indicative of the high whole plant respiration rates of these plants. This weak negative relationship between RGRt and NARt when comparing different growth forms, however, turns into a high positive interaction between RGRp and NARp when considering all harvests within each species. This suggests that within a single species the physiological component becomes the main parameter determining RGRp. A negative relationship when pooling all the species comes, therefore, from compensatory responses of morphological components of RGRt.

5.5.3. Components of the decreased RGRt under water-limited conditions

Pooling all species, the decrease in RGRt is mainly explained by decreases in SLA (Fig. 5.4B), in contrast with the results of Poorter & Nagel (2000), who found in 15 observations from literature that decreases in SLA and especially in NAR were the causes of changes in RGRt by water stress. However, they mainly considered grasses and only one of the species, *Sinapis alba*, occurs in the Mediterranean climate.

The relative contribution of each of the underlying growth parameters to the decrease of RGRt caused by water deficit strongly depended on the species and growth forms (Fig. 5.4). As hypothesised, the fast growing perennial herbs, which present a rapid leaf turnover, decreased their RGRt under water stress mainly due to morphological adjustments, while NARt was indeed increased possibly due to a strong reduction in self-shading (Lambers & Poorter, 1992). Woody evergreens, however, showed two distinct patterns: in evergreen shrubs the decrease of RGRt under water stress was associated with morphological adjustments (decreased SLA and, especially, LMR), while in woody evergreen semi-shrubs it was associated with physiological adjustments (decreased NARt).

We had hypothesised that morphological adjustments may be expected in woody evergreens only at early stages of development, and this seems certainly the case in shrubs. These achieved a 30 cm height at the end of the experiment, while their adult size is typically up to 2 m (*H. balearicum*) and 5 m (*P. lentiscus*). Therefore, they can be considered being at early stages of development during the entire experiment, and their response to water stress fits the hypothesised behaviour. In fact, a well known typical adaptation of Mediterranean woody evergreens to drought is to increase the allocation of a greater biomass below than above ground, and to decrease the evaporating surface (Ludlow, 1989). In this study, evergreen shrubs decreased RGRt mainly because of a decrease in biomass allocation to leaves (Fig. 5.4B).

By contrast, evergreen semi-shrubs decreased their RGRt under water stress mostly due to a strong adjustment of NARt, a response that had been hypothesised mostly for adult evergreen semi-shrubs. In fact, because these plants achieve a very low height when adult (less than 20 cm), the studied plants at the end of the experiment were very close to their adult size. However, the great contribution of decreases in NARt to the decrease in RGRt in woody evergreen semi-shrubs may be attributed to the observed increase in LAR, which in turn may enhance the effects of drought on photosynthesis and respiration. In conditions where carbon gain is low, as in water-limited conditions, respiratory carbon use is proportionally a much greater fraction of carbon gain, and thus plays a larger role in determining NAR and RGR (Flexas *et al.*, 2005). For these species, in their natural habitats, the typical response of increasing allocation to roots (Ludlow, 1989) may not be of adaptive value, since they inhabit over rocky surfaces with no easy access to deeper water or with access only to marine water intrusions.

Finally, as a group semi-deciduous shrubs showed an intermediate response, reducing RGRt under drought through a combination of morphological (decreased SLA) and, to a lesser extent, physiological (decreased NARt) adjustments (Fig. 5.4B). However, the two species belonging to this group presented significantly different patterns. In PI only decreases in SLA are responsible for decreased RGRt under water stress, while in CA decreased NARt contributed to a similar extent than decreases in SLA (Fig. 5.4A). These results suggest that the importance of several morphologic and physiological adjustments of RGRt to water stress may depend not only on the growth form but also, in some cases, on the species within a given growth form. These species are known to loose part of their foliage in the dry season (Flexas *et al.*, 2003). In the present experiment they certainly decreased LA strongly under water stress (Table 5.2), although this was more likely due to a reduction in leaf appearance. Nevertheless, these changes in the biomass allocation to leaves did not correlate significantly with the observed decreases in RGRt, which was mostly dependent on decreased SLA. These species are indeed known to produce leaves with a higher leaf mass per unit area under drought conditions (Margaris, 1981).

In conclusion, the present study showed that the influences of NAR, LMR and SLA on the decrease of RGR imposed by drought strongly depend on the species and growth forms. This might reflect differences in response and adaptation to environmental constraints.

Chapter 6

WATER RELATIONS AND STOMATAL REGULATION

ECOPHYSIOLOGICAL RESPONSES TO WATER STRESS AND RECOVERY IN MEDITERRANEAN PLANTS WITH DIFFERENT GROWTH FORMS. I. WATER RELATIONS AND STOMATAL CONDUCTANCE

6.1. SUMMARY

The stochastic behaviour of rainfall in Mediterranean areas implies that plants with different phenologies and growth forms have to endure temporary drought. The aim of this study was to extent the range of knowledge about water relations and stomatal responses to water stress to ten Mediterranean plants with different growth forms.

Plants were grown in pots and subjected to four different levels of water stress and a treatment of recovery. Soil water content, stomatal attributes (stomatal density, SD), stomatal conductance and leaf water relations (pre-dawn and midday leaf water potential, Ψ_{PD} and Ψ_{MD}; leaf specific hydraulic conductance, K_L; bulk modulus of elasticity, ε) were determined. Stomatal responsiveness to water stress (SR) was assessed as the initial slope between g_s and Ψ_{PD}.

A wide range of water relations and stomatal characteristics was found, largely independent of the plant growth form. Only SD and K_L, but not Ψ_{PD} or ε, provided general relationships with SR including both isohydric and anisohydric species. The extent of g_s recovery after drought depended mostly of K_L recovery.

It is concluded that a high variability is present among Mediterranean plants reflecting a continuum of leaf water relations and stomatal behaviour in response to water stress, which is independent of the growth form.

6.2. INTRODUCTION

Summer drought is considered the main environmental constraint for plant growth and survival in Mediterranean type ecosystems, a constraint that is even increasing as a result of climate change (Houghton *et al*., 2001). In Mediterranean environments, natural vegetation has developed an array of adaptations to drought, resulting in a high diversity of growth forms. The resulting vegetation consists mostly of deep rooted evergreen sclerophyll trees and shrubs, which maintain green leaves during the summer drought period, semi-deciduous shrubs, which lose a part of their leaves during summer, and geophytes and winter annual herbs, which escape drought by finishing their annual cycle before summer (Ehleringer & Mooney, 1983).

Under these conditions, the physiological regulation of water use in response to soil water depletion may strongly determine species survival, productivity, distribution and competitive relationships (Joffre *et al*., 1999; Chaves *et al*., 2002). This

appreciation has led researchers to explore the variability of plant responses to water stress in Mediterranean species. The physiological characteristics that have received more attention include the regulation in response to drought of leaf water relations (Davis & Mooney, 1986; Lo Gullo & Salleo, 1988; Abrams, 1990; Salleo & Lo Gullo, 1990; Terradas & Savé, 1992; Damesin *et al*., 1998) and stomatal conductance (Acherar *et al*., 1991; Duhme & Hinckley, 1992; Savé *et al*., 1999; Mediavilla & Escudero, 2003, 2004).

Keeping cell water content within an operational range is essential for plant metabolism and survival. Therefore, the regulation of plant water relations in response to soil water depletion may be crucial in Mediterranean species. The evolution of leaf water potential and/or leaf relative water content during the season has been analysed, and important differences between species have been observed (Hinckley *et al*., 1980; Davis & Mooney, 1986; Rhizopoulou & Mitrakos, 1990; Abril & Hanano, 1998; Salleo & Nardini, 2000; Serrano *et al*., 2005). Generally speaking, drought semi-deciduous species attain lower leaf water potentials and contents during summer than evergreen sclerophylls (Correia & Catarino, 1994; Werner *et al*., 1999). However, important differences are also found among species within a single growth form. For instance, in a classical study by Lo Gullo & Salleo (1988), three different evergreen sclerophyll shrubs were shown to present different strategies regarding the regulation of leaf water relations. Thus, *Olea oleaster* presented a drought-tolerant behaviour, enduring substantial day water losses during the dry season, even below the turgor loss point. In contrast, *Ceratonia siliqua* and *Laurus nobilis* behaved as drought-avoidant species, the former compensating large water losses with high water uptake and the latter reducing water losses through a strong seasonal adjustment of the leaf bulk modulus of elasticity. Similar differences have been described between species within a single genus, as in *Quercus* (Salleo & Lo Gullo, 1990; Savé *et al*., 2000; Corcuera *et al*., 2002; Savé *et al*., 2003). While differences in leaf water relations among species are supposed to be of adaptive value, a comparison between Mediterranean species including a broad range of growth forms has not been performed. Moreover, whether these differences are important in terms of, for instance, water saving or recovery after drought, has seldom been analysed.

Stomatal closure in response to soil water shortage is a common response among plants, but the extent and velocity of this response may differ among species (Schulze & Hall, 1982). Stomatal aperture appears to be controlled by complex mechanisms,

including chemical and hydraulic signalling from roots, shoots and leaves (Tardieu & Simmoneau, 1998; Comstock, 2002). These mechanisms operate in a coordinated manner to maintain a balance between CO_2 uptake and water vapour loss, thus preventing leaf desiccation. Therefore, understanding the morphological and physiological traits leading to the regulation of stomatal closure may be of key interest in the study of drought-adaptation to semi-arid conditions (Poole & Miller, 1975; Duhme & Hinckley, 1992; White *et al*., 2000; Mediavilla & Escudero, 2003, 2004). Some general features have emerged regarding the factors involved in regulation of stomatal conductance in species belonging to different growth form groups (Schulze & Hall, 1982; Mediavilla & Escudero, 2003). For instance, stomatal size is smaller and stomatal density is greater in species typical of xeric environments (Dunlap & Stettler, 2001; Pearce *et al*., 2006). Also, isohydric and anisohydric behaviours have been discussed in terms of its underlying physiological mechanisms (Tardieu & Simmoneau, 1998; Schultz, 2003), as well as regarding their adaptive value under drought conditions. For instance, anisohydric species may behave as drought-tolerant plants capable of maintain higher stomatal conductances at lower leaf water potentials than drought-intolerant species (Bunce *et al*., 1977; Abrams, 1988). Isohydric species, by contrast, may be considered as drought-avoiders, keeping their leaf water potential within narrow limits. In Mediterranean species, the relationship between the stomatal conductance and plant water relations has been analysed mostly in woody evergreen and winter deciduous species (Duhme & Hinckley, 1992; Abril & Hanano, 1998; Mediavilla & Escudero, 2003; Vilagrosa *et al*., 2003). As with water relations, a general analysis of the plant traits involved in the stomatal response to drought, including Mediterranean species with broad range of growth forms, is lacking.

Therefore, the vast majority of studies about water relations and stomatal regulation in Mediterranean plants have focused strictly on woody species, and particularly in evergreen sclerophylls and winter deciduous, whose leaves stay green during summer and therefore withstand the most severe drought conditions of the Mediterranean region. However, the stochastic distribution of rainfall in this region leads to frequent episodic drought events in whatever season of the year. Thus, the capacity of withstanding a drought period and the capacity for rapid recovery after rainfall may be adaptive to Mediterranean plants regardless of their growth forms.

The aim of the present study was to undertake a comparative analysis of water relations and stomatal responses to drought, including a wide variety of Mediterranean

species with different growth forms. To compare the physiological responses of plants belonging to different functional groups the study was performed in potted plants growing under the same conditions and subjected to similar extents of drought. Some specific questions were addressed: (1) which are the ranges in water relations parameters within Mediterranean species?; (2) do water relations and/or stomatal morphology traits determine the extent and velocity of stomatal closure in response to soil drying?; (3) what determines the extent of recovery of leaf water status and stomatal conductance after a drought period?; and (4) do these characteristics differ between species belonging to different growth forms?

6.3. MATERIAL AND METHODS

6.3.1. Plant material

Ten Mediterranean species naturally occurring in the Balearic Islands, some of them endemic to these islands, were selected for this study (Table 6.1). Special care was taken in the selection of the species, in order to include taxons representative of different growth forms: two evergreen sclerophyll shrubs (*Pistacia lentiscus* and *Hypericum balearicum*), two evergreen sclerophyll semi-shrubs (*Limonium gibertii* and *L. magallufianum*), three summer semi-deciduous shrubs (*Lavatera maritima, Phlomis italica* and *Cistus albidus*), two perennial herbs (*Beta maritima* subsp. *maritima* and *Beta maritima* subsp. *marcosii*) and an annual herb (*Diplotaxis ibicensis*). Seeds of each species were collected in the field from natural populations and taken from several parent plants to obtain a representative sample of populations in the nature. Seeds were germinated on filter paper moistened with deionized water in a controlled environment (germination chamber, at 18ºC in darkness). After germination and emergence of one true leaf, ten seedlings were transplanted into pots (25 L volume, 40 cm height) containing a 40:40:20 mixture of clay-calcareous soil, horticultural substrate (peat) and pearlite (granulometry A13). Plants were grown outdoors at the University of the Balearic Islands (Mallorca, Spain). The experiment was performed in five rounds, each one with one couple of species at the same time. Four weeks before starting the experiment, plants were placed in a controlled growth chamber with a 12h photoperiod (26ºC day/20ºC night) and a photon flux density at the top of the leaves of about 600 $\mu mol\ m^{-2}\ s^{-1}$.

Table 6.1

List of species considered for study with their family and a brief description. The number of plants used was 10 per species, and the age differed because of the different phenology of the species selected. Plants of *P. lentiscus*, *H. balearicum*, *C. albidus*, *P. italica* and *L. maritima* were three years old, plants of *M. aquatica*, *L. minoricensis*, *L. magallufianum* and *L. gibertii* were a year and half old and plants of *D. ibicensis*, *B. maritima* subsp. *marcosii* and *B. maritima* subsp. *maritima* were six months old at the onset of the experiments.

Species	**Code**	**Family**	**Description**
Diplotaxis ibicensis Pau	DI	Brassicaceae	Annual herb, endemic of the Balearic Islands and inhabiting a few coastal locations.
Beta maritima L. subsp. *marcosii* A. Juan & M. B. Crespo	MC	Chenopodiaceae	Perennial herb. Endemic of the Balearic Islands, inhabiting a few small islets subjected to strong saline spray.
Beta maritima L. subsp. *maritima*	MT	Chenopodiaceae	Perennial herb inhabiting coastal ecosystems. Widespread in Mediterranean and temperate climates.
Lavatera maritima Gouan	LA	Malvaceae	Semi-deciduous shrub up to 2 m, densely covered by hairs. Inhabits in coastal locations.
Phlomis italica L.	PI	Labiatae	Semi-deciduous shrub up to 1 m, densely covered by hairs. Endemic of the Balearic Islands. The biggest populations are found 500 m above the sea level, where they co-exist with *Cistus albidus*.
Cistus albidus L.	CA	Cistaceae	Semi-deciduous shrub up to 1 m. Commonly found in the Mediterranean garrigue. Its leaves are densely covered by hairs.
Hypericum balearicum L.	HB	Guttiferae	Woody evergreen shrub up to 2 m, endemic of the Balearic Islands. The biggest populations are found in the garrigue 500 m above the sea level, where competes with PL.
Pistacia lentiscus L.	PL	Anacardiaceae	Woody evergreen shrub up to 5 m, commonly found in the Mediterranean garrigue.
Limonium magallufianum L. Llorens	LM	Plumbaginaceae	Woody evergreen semi-shrub, in cushion-like rosettes. Endemic of the Balearic Islands, inhabiting just in one coastal marsh located in Magalluf, Mallorca.
Limonium gibertii (Sennen) Sennen	LG	Plumbaginaceae	Woody evergreen semi-shrub, in cushion-like rosettes. Occurring in West Mediterranean rocky and sandy coastal areas.

Plants were daily fertitirrigated with 100% Hoagland's solution. Measurements corresponding to control treatments were made during the first day of the experiment, when all the plants were well watered. Thereafter, irrigation was stopped in five plants for each species. Pots were weighted every day to determine the amount of water loss. To obtain different degrees of drought, measurements were made on days 4, 8 and 13-17 after the last irrigation, when plants were subjected to mild, moderate and severe drought intensities. Each drought experiment was stopped when g_s was close to zero (for more details see Gas Exchange Measurements), 13-17 days after water withholding, depending on species. Once such g_s values were achieved, pots were again irrigated at

field capacity, and considered for the re-watering treatment measurement on the next day. Control plants were watered daily during all the experiment and eventually measured to ensure that they maintained constant values of each parameter during the experiment.

6.3.2. Plant water status

Leaf pre-dawn (Ψ_{PD}) and midday (Ψ_{MD}) water potentials were determined with a Scholander chamber (Soilmoisture Equipment Corp., USA). For three of the species, *C. albidus*, *L. maritima* and *H. balearicum*, because their very short petiole difficulted measuring single leaves, water potentials were measured in small apical branches including two or three leaves. Four replicates per species and treatment were done.

The relative water contents at pre-dawn (RWC_{PD}) and midday (RWC_{MD}) were determined as follows: RWC = (Fresh weight - Dry weight) / (Turgid weight – Dry weight) × 100. To determine the turgid weight of the samples, these were kept in distilled water in the darkness at 4°C to minimise respiration losses, until they reached a constant weight (full turgor, typically after 12 h). Their dry weight was obtained after 48 h at 70ºC in an oven. Four replicates per species and treatment were obtained.

Pressure – volume (P-V) curves were not determined in this experiment. However, plotting for each species the inverse of the Ψ against RWC, including all the data collected during the experiment, was taken as a surrogate for P-V curves. The highest values of leaf RWC were obtained from well-watered plants, while the lowest values were obtained from the severe drought treatment. Values from the re-watering treatment were not considered. These plots allowed getting some insights into the tissue-water relations parameters typically evaluated using pressure-volume curves: osmotic potential at full ($\Psi_{\pi 100}$) and at zero turgor ($\Psi_{\pi 0}$), and the relative water content at zero turgor (RWC_0) (Sobrado, 1986; Turner, 1988). Leaf volumetric elastic modulus (ε) at 100% of RWC (RWC_{100}) was estimated assuming the linear relationship between turgor potential (Ψ_p) and RWC:

$$\varepsilon = \Psi_{p100}\, RWC_0 / RWC_{100} - RWC_0$$

where Ψ_{p100} is the leaf water potential at full turgor and RWC_0 is turgor loss point (Johnson *et al.*, 1985; Nguyen & Croy, 1984; Savé *et al.*, 1993). These parameters should to be considered as 'average' for the species during the entire experiment. Since these were short-term experiments (i.e., about 2 weeks), we believe that changes in leaf

hydraulic properties, as occur along the season under field conditions (Lo Gullo & Salleo, 1988; Serrano *et al*., 2005), did not happen.

6.3.3. Specific leaf weight

Specific leaf weight (SLW) was calculated in four leaves per species under the well-watered treatment, as the ratio of leaf dry mass to leaf area. First, the leaf area was determined with an AM-100 Area Meter (Analytical Development Company, Herts, UK). Then, the dry mass of these leaves was determined after oven drying for 48 h at 60ºC.

6.3.4. Gas exchange measurements

Instantaneous determinations of stomatal conductance (g_s) and transpiration rate (E) at saturating light (1500 μmol photon m^{-2} s^{-1}), 25ºC and 400 μmol mol^{-1} CO_2 were performed at mid-morning, using a Li-6400 (Li-Cor Inc., Nebraska, USA) in one leaf of four different plants per treatment and species. Relative humidity was kept at 50 ± 5% during measurements. The IRGA was calibrated every day, according to manufacturer's recommendations.

For each one of the species, g_s was plotted against Ψ_{PD}, and the initial slope of the relationship was calculated as an indicator of stomatal responsiveness to water stress (SR) (Mediavilla & Escudero, 2003).

6.3.5. Leaf specific hydraulic conductance

Leaf-specific hydraulic conductance (K_L) was estimated from the slope of the relationship between leaf transpiration rate (E, mmol H_2O m^{-2} s^{-1}) and Ψ, and was calculated as $E/(\Psi_{MD}-\Psi_{PD})$ (Schultz, 2003; Sperry & Pockman, 1993).

6.3.6. Stomatal density and size

Fully exposed mature leaves were detached from each plant. Stomatal density (SD) was determined using the silicon leaf impression method (Weyers & Johansen, 1985) on the abaxial lamina immediately to the right of the mid-vein. The three pubescent species (i.e., *L. maritima*, *P. italica* and *C. albidus*) were not included in this analysis, since we were not able to remove trichomes to prepare microscopic impressions. All the species analysed were hypostomatic. The numbers of stomata were

counted with a microscope at 400 × magnification on four different vision fields of separate impressions of the lamina obtained from four different leaves of four well-watered plants per species (i.e., sixteen different vision fields). Guard cell length was measured on sixteen randomly selected stomata from the same impressions used for SD determinations. Stomatal area index (SAI) was calculated by taking the product of the mean stomatal length and the SD according to Ashton & Berlyn (1994), and expressed in mm stomata × number of stomata mm^{-2} leaf.

6.3.7. Statistical analysis

Regressions coefficients were calculated with the 8.0 Sigma Plot software package (SPSS). Differences between means were revealed by Duncan analyses ($P < 0.05$) performed with the SPSS 12.0 software package (SPSS, Chicago, USA).

6.4. RESULTS AND DISCUSSION

6.4.1. Water relations in response to water stress

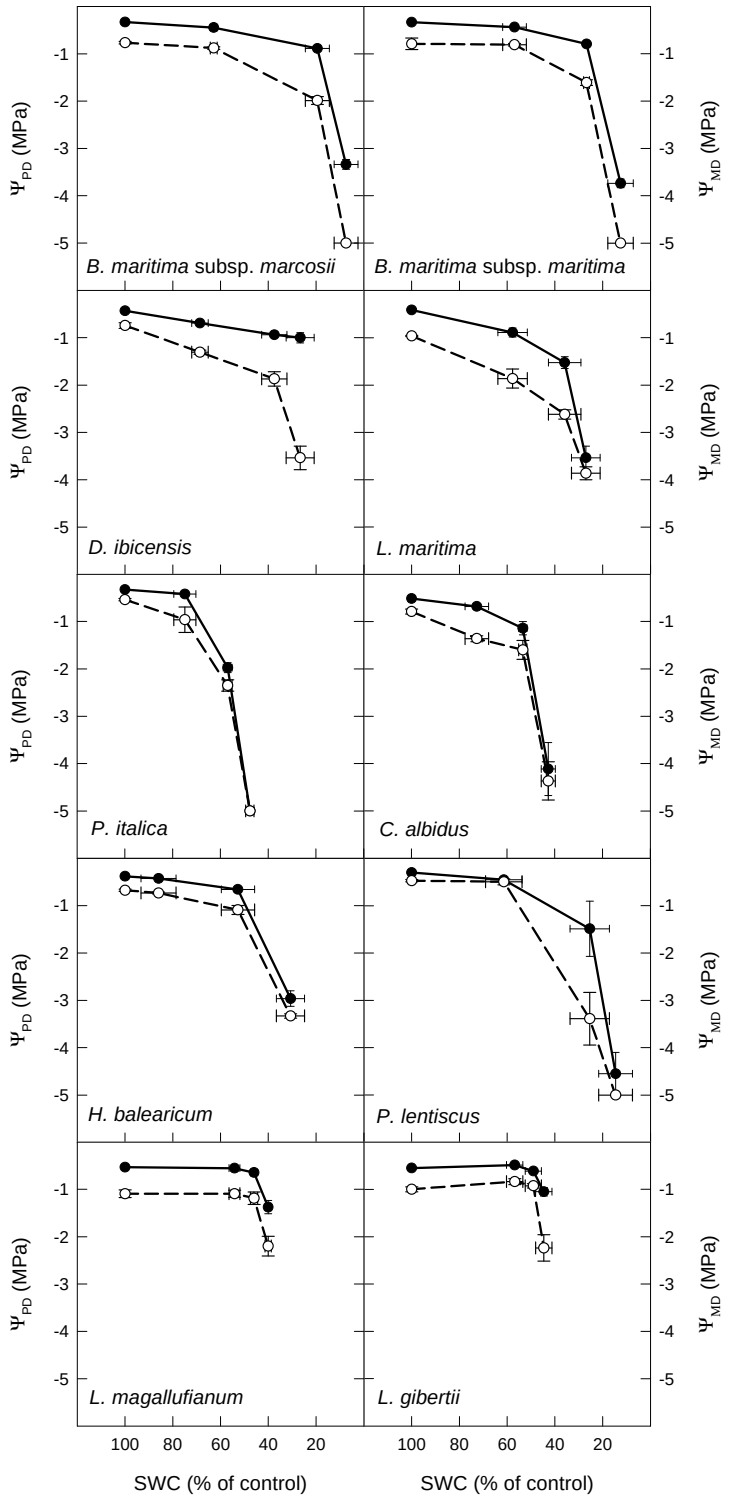
Among Mediterranean plants, the response of leaf water relations and stomatal conductance to water stress has been studied in woody species, mostly in evergreen sclerophylls and winter deciduous shrubs and trees and, to a lesser extent, in summer semi-deciduous shrubs (Hinckley *et al*., 1980; Davis & Mooney, 1986; Rhizopoulou & Mitrakos, 1990; Abril & Hanano, 1998; Serrano *et al*., 2005). In the present study, we include an annual and two perennial herbs, three summer semi-deciduous shrubs and four evergreen sclerophylls, in order to compare water relations and their response to drought in Mediterranean species with a broader range of growth forms. The different species did not reach the same water stress level in terms of soil water content at the end of the drought period, which was probably due to differences in transpiration rate and/or leaf area among species. However, all species presented similar stomatal conductance values, i.e. close to zero, in the last day of measurements, which permits comparisons in the water relations among species. Maximum pre-dawn leaf water potential ranged between -0.30 MPa and -0.55 MPa for all the species analyzed, but there was a much larger variation under water stress, the lowest values ranging from -1 MPa in *D. ibicensis* to -5 MPa in *P. lentiscus* (Fig. 6.1). The latter differences were not reflecting differences in soil water availability between species, since a significant correlation between soil water content and Ψ_{PD} was found only for Ψ_{PD} values above - 1.5 MPa,

i.e., from mild to moderate water stress ($r^2 = 0.55$, $P < 0.01$). Further decreases of Ψ_{PD} during severe stress were associated to small decreases in SWC, and may therefore reflect differences between species in their ability to explore all soil volume in the pots or to adjust their osmotic potential to continue extracting available water from the soil. Clearly, the minimum water potential achieved during water stress did not depend on specific growth forms. For instance, the highest Ψ_{PD} under severe stress was found in the annual herb *D. ibicensis* as well as in the two evergreen semi-shrubs *L. gibertii* and *L. magallufianum*. Low values (i.e., below -3.5 MPa) were found in the perennial herb *B. maritima* subsp. *maritima*, in the three summer semi-deciduous shrubs and in the evergreen sclerophyll *P. lentiscus*. Previous studies have shown that, under field conditions during the dry season, summer deciduous and semi-deciduous shrubs usually attain lower Ψ_{PD} than evergreens (Duhme & Hinckley, 1992; Correia & Catarino, 1994; Werner *et al*., 1999; Mediavilla & Escudero, 2003). However, comparing the physiological responses of plants belonging to different functional groups under field conditions is confusing, since plants can be exploring different soil depths. The present results, obtained in plants growing under identical conditions and exploring at least more similar soil volume, suggest that the differences between functional groups in minimum leaf water potential observed under field conditions may be due to the fact that evergreens are more deep-rooted as compared with summer deciduous species, and not because they possess specific drought-avoiding mechanisms at the leaf level.

Only the two *Limonium*, among the species analyzed in the present study, presented a clear isohydric behaviour, i.e., they maintained midday leaf water potentials invariable during early stages of water stress development, until water stress was severe (Fig. 6.1). *H. balearicum* and *P. lentiscus* presented an intermediate pattern, maintaining Ψ_{MD} constant only from well-watered to mild stress conditions, and decreasing thereafter (Fig. 6.1). The perennial herb *B. maritima* subsp. *maritima* presented a pattern similar to that of *P. lentiscus*, while all the other species showed clear anisohydric patterns. The isohydric behaviour of the two *Limonium* species enable them to keep their leaf relative water content above 60% through the entire experiment, while the other eight species attained values of 50% or less (data not shown). Therefore, an anisohydric behaviour was predominant among the species analyzed here, as described in many other Mediterranean species (Rhizopoulou & Mitrakos, 1990; Correia & Catarino, 1994; Serrano *et al*., 2005).

Figure 6.1

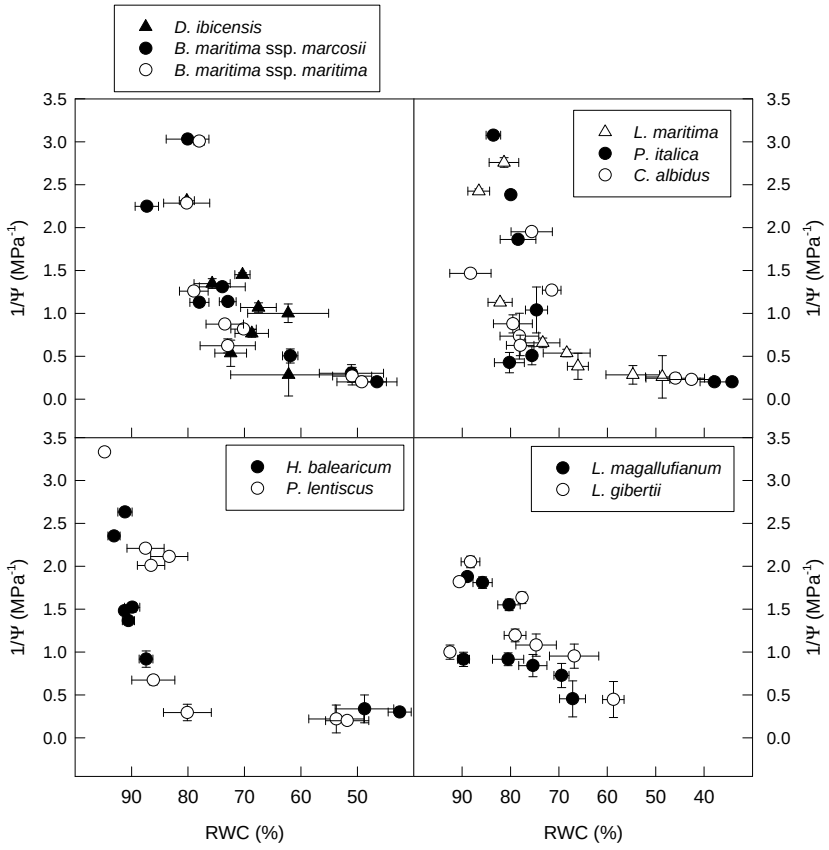
Relationship between pre-dawn (Ψ_{PD}, filled symbols) and midday (Ψ_{MD}, empty symbols) leaf water potential and soil water content (SWC, % respect to control values) for the ten selected species. Values represent mean ± standard error of four replicates.



An anisohydric behaviour favours keeping a water potential difference between leaves and soil as soil dries, therefore increasing the driving force for water uptake. However, increased access to declining soil water may be achieved at the risk of reaching the turgor loss point. The turgor loss point of a given species can be assessed by measuring pressure-volume (P-V) curves (Sobrado, 1986; Lo Gullo & Salleo, 1988). Although we did not perform P-V curves in the present study, we plotted all the data of $1/\Psi$ and RWC collected during the experiment as a surrogate of P-V curves (Fig. 6.2). From these plots, a series of water relation parameters, such as osmotic potential at full ($\Psi\pi_{100}$) and zero turgor ($\Psi\pi_0$), the relative water content at turgor loss (RWC_0), and the bulk elastic modulus (ε), were calculated (Table 6.2). These should be considered as 'average' for the species during the entire experiment. Since these were short-term experiments (i.e., about 2 weeks), we assume that changes in leaf ε, as occur along the season under field conditions (Lo Gullo & Salleo, 1988; Serrano *et al*., 2005), should be irrelevant. From these relationships, it is clear that all the species except the two *Limonium* (i.e., the anisohydric but not the isohydric species) reached turgor loss point during the experiment, most of them under severe drought. These anisohydric species may be considered as drought-tolerant, since they all survived and recovered leaf water potential to some extent after re-watering. In contrast, the two isohydric *Limonium* may be considered as drought-avoidant species, since they did not show the typical inflexion in the $1/\Psi$ *vs*. RWC curve, and therefore they overcome the experimental conditions above the turgor loss point. Clearly, *Limonium* species were able to keep leaf water potential and RWC at higher values than the other species despite similar soil water depletion.

Figure 6.2

Relationship between the reciprocals of leaf water potential (1/Ψ) and relative water content (RWC). These are not true P-V curves, but surrogates obtained including data both from predawn and midday for all treatments except recovery. Values are means ± standard errors of four replicates.



For the eight species that achieved turgor loss point, values for ε were obtained, being of around 2 MPa in herbs, 3-4 MPa in semi-deciduous shrubs, and higher than 6 MPa in evergreens (Table 6.2). The relative water content at the turgor loss point (RWC_0) ranged from 62% in *B. maritima* subsp. *marcosii* to 86% in *H. balearicum* (Table 6.3). These data are consistent with values already published, particularly for evergreen Mediterranean species (Lo Gullo & Salleo, 1988; Savé *et al*., 1999; Serrano *et al*., 2005). Differences between species in osmotic potential at full turgor and turgor loss point were less important than differences in ε (Table 6.2). Bulk modulus of elasticity was closely correlated ($P < 0.001$) with specific leaf weight (Fig. 6.3), as already described (Salleo & Lo Gullo, 1990; Groom & Lamont, 1997; Salleo *et al*., 1997). Therefore, it is clear that ε values followed a pattern that was to some extent dependent on growth forms, particularly on the degree of sclerophylly. However, although it was not possible to calculate ε for the two *Limonium* species with the data

available, it is clear from Fig. 6.2 that their ε would be the lowest of all the species analyzed, lying somewhere between 0 and 2 MPa. We have adopted an arbitrary value of 1.5 MPa for both species, just to make visual in Fig. 6.3 that these two species did not follow the general SLW- ε relationship found for the others. That not all Mediterranean species fit this general relationship has been already pointed out by Salleo & Nardini (2000). A similar conclusion was reached by Zobel (1996) for temperate trees of the Appalachian Mountains. Whatever the true values of ε for the two *Limonium*, it becomes then clear that the observed inter-specific differences in water status response to water stress were not related to ε – or other P-V derived parameters – in a single way.

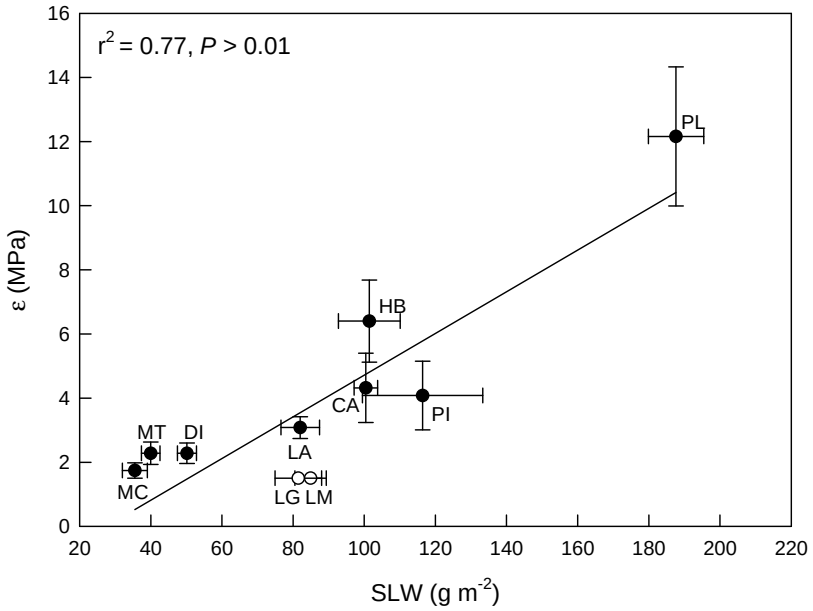
Table 6.2

Parameters derived from water potential *vs*. relative water content curves for the 10 species subjected to study, reflecting the relationship between the reciprocals of the leaf water potential and the relative water content (Fig. 6.2). Osmotic potential at full turgor ($\Psi\pi_{100}$), at zero turgor ($\Psi\pi_0$), relative water content at zero turgor (RWC_0) and leaf bulk elastic modulus (ε). Data obtained both from predawn and midday measurements were considered, excepting values from re-watering treatment. Values are means ± standard errors of four different plants per species.

Species	$\Psi\pi_{100}$ (MPa)	$\Psi\pi_0$ (MPa)	RWC_0 (%)	ε (MPa)
D. ibicensis	-0.91 ± 0.21	-1.05 ± 0.31	71.5 ± 4.1	2.28 ± 0.32
B. maritima subsp. *marcosii*	-1.07 ± 0.13	-2.00 ± 0.08	61.9 ± 1.4	1.74 ± 0.24
B. maritima subsp. *maritima*	-1.06 ± 0.12	-1.61 ± 0.06	68.3 ± 3.1	2.28 ± 0.35
L. maritima	-1.58 ± 0.25	-2.39 ± 0.18	66.1 ± 2.1	3.08 ± 0.34
P. italica	-1.52 ± 0.24	-1.76 ± 0.25	72.9 ± 2.6	4.08 ± 1.07
C. albidus	-1.71 ± 0.12	-2.20 ± 0.14	71.7 ± 1.6	4.32 ± 1.08
H. balearicum	-1.08 ± 0.10	-1.21 ± 0.11	85.6 ± 0.7	6.40 ± 1.28
P. lentiscus	-2.39 ± 0.25	-2.75 ± 0.20	83.6 ± 2.1	12.16 ± 2.17
L. gibertii	n.d.	n.d.	<61.4 ± 0.1	n.d.
L. magallufianum	n.d.	n.d.	<66.5 ± 3.8	n.d.

Figure 6.3

Relationship between leaf bulk elastic modulus (ε) and specific leaf weight (SLW) for the species analysed. An arbitrary value of 1.5 MPa for ε was assigned to the two *Limonium* species (empty symbols; the true value cannot be calculated precisely, but range between 0 and 2 MPa). Values are means ± standard errors of four replicates. Species codes as in Table 6.1.



6.4.2. Stomatal traits and stomatal conductance responsiveness to water stress

Stomatal conductance (g_s) strongly differed among species and growth forms, approximately in a ten-fold range (Table 6.3). *L. maritima* showed the highest g_s values under well-watered conditions (1.022 mol H_2O m^{-2} s^{-1}), and *P. lentiscus*, the lowest (0.122 mol H_2O m^{-2} s^{-1}). All the species decreased g_s to values between 0 and 0.06 mol H_2O m^{-2} s^{-1} as water limitation increased. The maximum g_s was significantly higher for herbaceous and semi-deciduous shrubs than for evergreens, except *H. balearicum* that presented similar values to the semi-deciduous *P. italica* and *C. albidus* (Table 6.3). A non-significant relationship was found between the maximum g_s and the degree of sclerophylly, approached as the SLW (Fig. 6.4A). Although non-significant, the negative trend of such relationship is consistent with the typically described water-saving behaviour of Mediterranean evergreen sclerophylls (Ehleringer & Mooney, 1983; Savé *et al*., 2003). Stomatal morphology was analysed in seven of the ten species. Stomatal density (SD), ranged from 60 stomata per mm^2 in *L. magallufianum* to 420

stomata per mm^2 in *P. lentiscus*. The latter was the only of the seven species analysed for which previous determinations of SD were available, differing between 287 stomata per mm^2 (Meister & Bolhar-Nordenkampff, 2001) and 325 stomata per mm^2 (Gratani & Varone, 2004). The present results show that there is a high inter-specific variability in SD. A highly significant negative relationship was found between stomatal length and stomatal density (Fig. 6.4B), while a positive relationship was obtained between SD and SAI (Fig. 6.4C), as usually described (Larcher, 1995; Hetherington & Woodward, 2003; Gratani & Varone, 2004; Pearce *et al*., 2006). The maximum stomatal conductance, was to some extent positively correlated to SD (Fig. 6.4D), as already shown for Mediterranean (Gratani & Varone, 2004) and non-Mediterranean species (Pearce *et al*., 2006). Only *P. lentiscus* behaved as an outlier for this relationship.

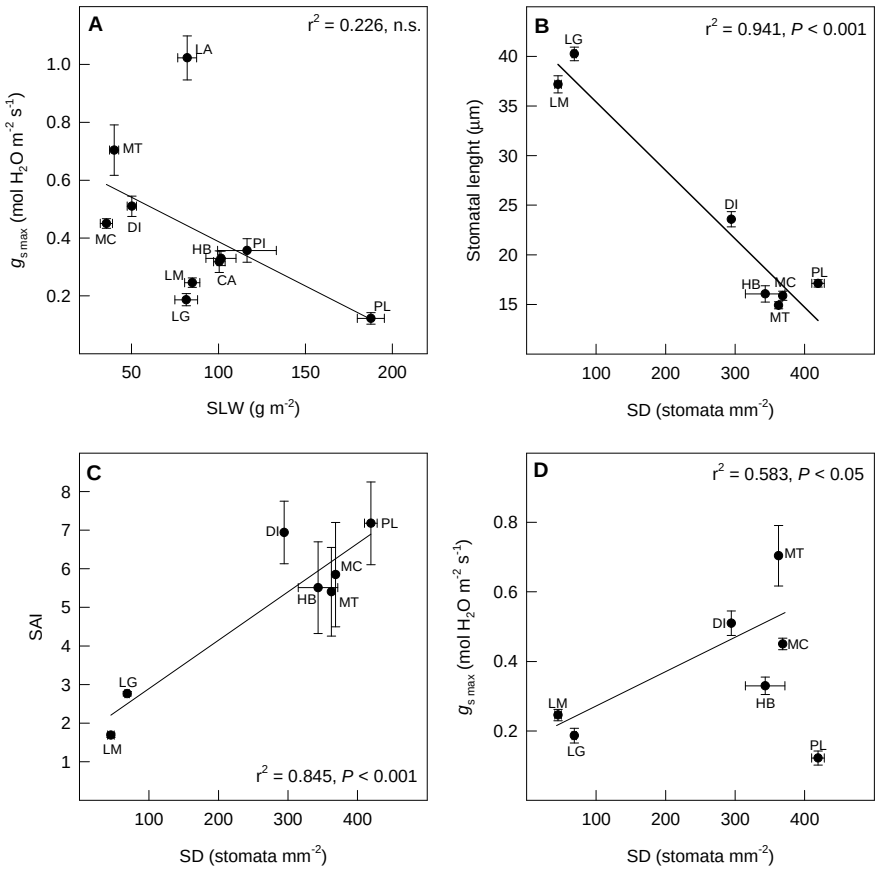
Table 6.3

Maximum (measured in irrigated plants), minimum (under severe drought conditions) and range of variation of stomatal conductance (g_s) for the ten species analysed. Values are means ± standard error of four replicates.

Specie	g_s (mol H_2O m^{-2} s^{-1})		
	Maximum	**Minimum**	**Range**
D. ibicensis	0.510 ± 0.035	0.059 ± 0.012	0.451
B. maritima subsp. *marcosii*	0.450 ± 0.017	0.009 ± 0.040	0.442
B. maritima subsp. *maritima*	0.704 ± 0.087	0.008 ± 0.002	0.696
L. maritima	1.022 ± 0.076	0.052 ± 0.010	0.970
P. italica	0.357± 0.041	0.016 ± 0.001	0.341
C. albidus	0.318 ± 0.037	0.022 ± 0.004	0.296
H. balearicum	0.330 ± 0.025	0.023 ± 0.004	0.307
P. lentiscus	0.122 ± 0.020	0.014 ± 0.002	0.109
L. magallufianum	0.246 ± 0.016	0.017 ± 0.005	0.229
L. gibertii	0.187 ± 0.021	0.029 ± 0.007	0.158

Figure 6.4

(A) Relationship between the maximum stomatal conductance ($g_{s\ max}$) and the specific leaf weight (SLW). (B) Relationship between the stomatal length and the stomatal density (SD). (C) Relationship between the stomatal area index (SAI) and the stomatal density (SD). (D) Relationship between the maximum stomatal conductance ($g_{s\ max}$) and the stomatal density (SD). Values represent means ± standard errors of four replicates for $g_{s\ max}$ and sixteen replicates for the stomatal characters. Regression coefficients and significance of each relationship are shown in the correspondent figure. The regression coefficient of Fig. 6.4D was obtained excluding *P. lentiscus*. Species codes as in Table 6.1. n.s.: non-significant.



During drought stress, stomatal conductance decreased proportionally to soil water content (not shown) and Ψ_{PD} (Fig. 6.5) in all the species analysed. The relationship was biphasic, and the slope of the initial phase was taken as an indicator of stomatal responsiveness (SR) to water stress (Acherar *et al*., 1991; Mediavilla & Escudero, 2003). A large variability was found in SR, which was not related to growth forms. For instance, both the maximum (-1.92 and -1.70 in *L. magallufianum* and *L. gibertii*, respectively) and the minimum (-0.08, *P. lentiscus*) values were found in evergreen sclerophyll species. Among semi-deciduous species, *L. maritima* presented

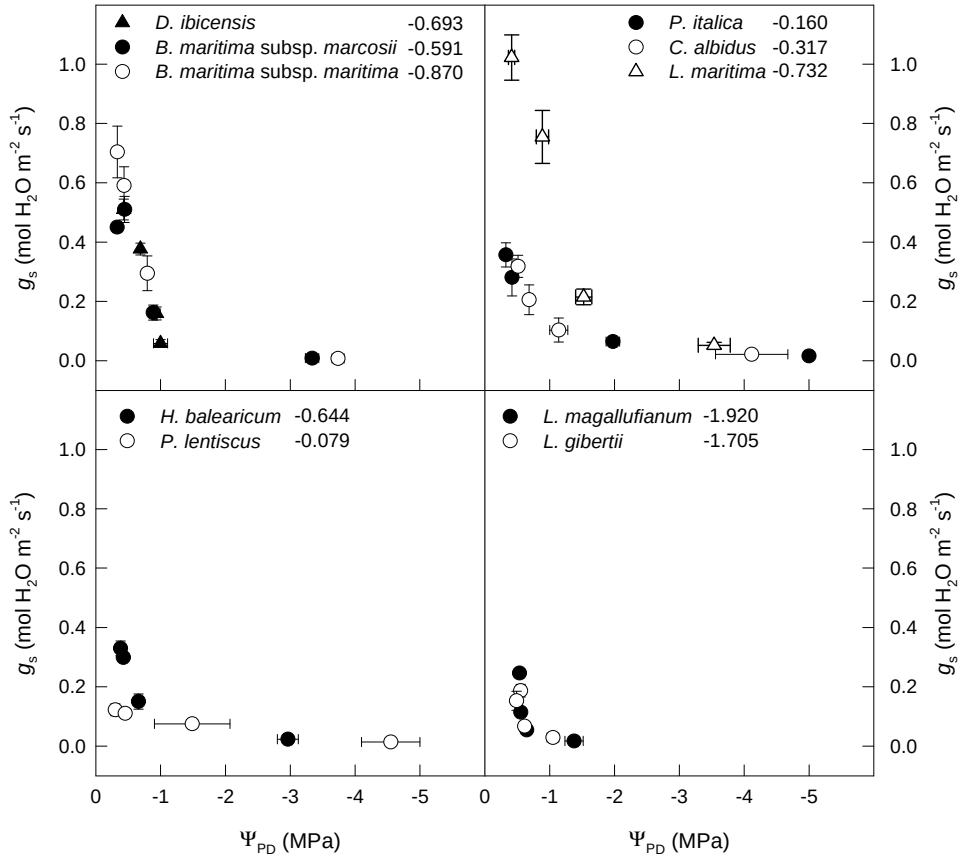
one of the highest values (-0.73) found among all the species, while *P. italica* showed one of the lowest (-0.16) and *C. albidus* displayed an intermediate value (-0.32). Herbaceous species presented consistently high values of SR and on average their SR did not differ from that of woody species due to the large variability of the latter. The fact that the two *Limonium* species had the highest SR is consistent with and explains that they were the only among the ten species analysed showing an isohydric behaviour. In previous surveys including the same pool of species, the two *Limonium* already showed some other ecophysiological characteristics that make them different from the other species. For instance, they displayed the highest Rubisco specificity factor among higher C_3 plants, which may allow them to sustain a somewhat higher photosynthesis with their stomata almost totally closed (Galmés *et al*., 2005a). Similarly, *Limonium* spp. were the only in which drought-induced decreased relative growth rate was strongly associated to decreased net assimilation rate, and not to morphological adjustments as in the other species (Galmés *et al*., 2005b). It would be interesting to perform further studies in these species to understand how different ecophysiological traits combine to provide specific adaptations to adverse environments.

The above results do not support the idea that Mediterranean evergreen sclerophylls have a higher stomatal control than malacophyll species or annuals (Gratani & Varone, 2004), but rather that a high variability is present among Mediterranean plants reflecting a continuum of stomatal behaviour in response to water stress that is independent of the growth form (Joffre *et al*., 1999). In fact, a lower SR in evergreen oaks as compared to winter deciduous and malacophyll species has already been observed (Acherar *et al*., 1991; Mediavilla & Escudero, 2003, 2004). This is corroborated by the fact that no general correlation was found between SR and either SLW (Fig. 6.6A) or ε (Fig. 6.6B). While these relationships were significant – although weak – for eight of the studied species, the two *Limonium* were clearly outliers. The fact that SR and ε were correlated when only anisohydric species were considered, but not when isohydric species were included, is surprising since it is usually reported that the anisohydric behaviour does not depend on leaf hydraulics while it is the isohydric behaviour which results from an interaction between hydraulic and chemical factors (Tardieu & Simmoneau, 1998). In addition, it is remarkable that the relationship between SR and ε was negative, i.e. the higher the ε the lower the SR. These data contradict the general assumption that, because stomatal closure is activated by the loss

of cellular turgor, the greater the ε the quicker the stomatal closure (Corcuera *et al.*, 2002). The opposite is observed, which suggests that mechanisms other than the direct loss of cellular turgor in leaves were responsible for stomatal closure in these species.

Figure 6.5

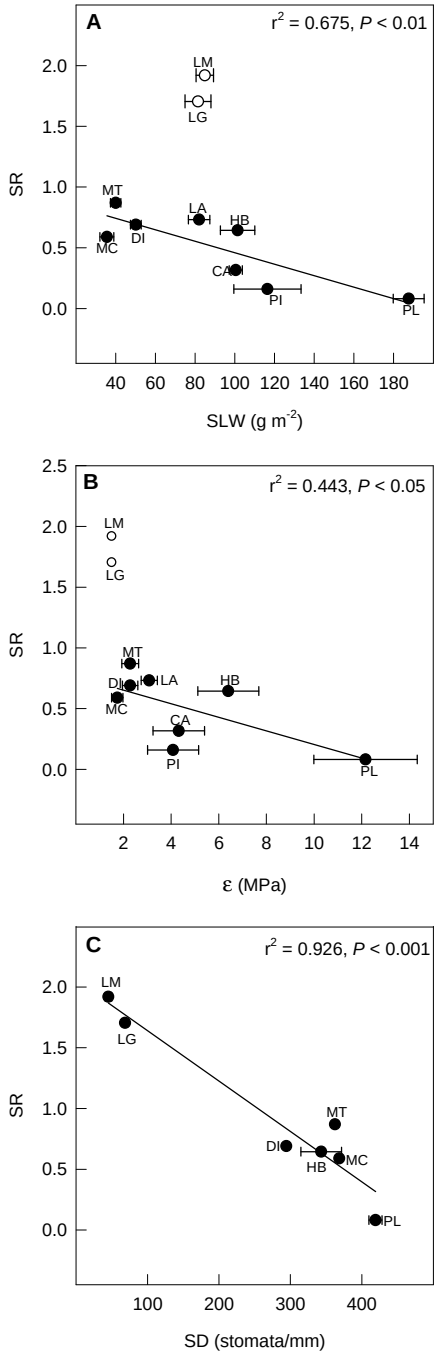
Relationship between the stomatal conductance (g_s) and the predawn leaf water potential (Ψ_{PD}) along the drought experiment for the ten selected species. Values represent means ± standard errors of four replicates. The numbers represent the slope of the initial phase of g_s declining *vs.* Ψ_{PD} for each species, which is taken as a measurement of stomatal responsiveness to water stress (SR).



In contrast to SWC and ε, a general, highly significant negative relationship was found between SR and SD for the seven species where the latter parameter was calculated (Fig. 6.6C). Contrary to what is usually assumed (Larcher, 1995), a higher SD did not result in a higher stomatal control, but the opposite may be true. To the best of our knowledge this is the first time that a direct relationship among different species is described between SD and a parameter reflecting stomatal responsiveness to water stress.

Figure 6.6

Relationship between the stomatal responsiveness to water stress (SR) and (A) specific leaf weight (SLW), (B) leaf bulk elastic modulus (ε) and (C) stomatal density (SD). Values represent means ± standard error of four replicates for SLW and ε and sixteen replicates for SD. The standard error of SR was not calculated since this parameter was obtained from means for each one of the species. The regression coefficients and significance of each relationship are shown. The regression coefficients of Fig. 6.8A and 8b were obtained excluding both *Limonium* species (empty symbols). In Fig. 6.8B an arbitrary value of 1.5 MPa for ε was assigned to the two *Limonium* species (the true value cannot be calculated precisely, but ranges between 0 and 2 MPa). Species codes as in Table 6.1.

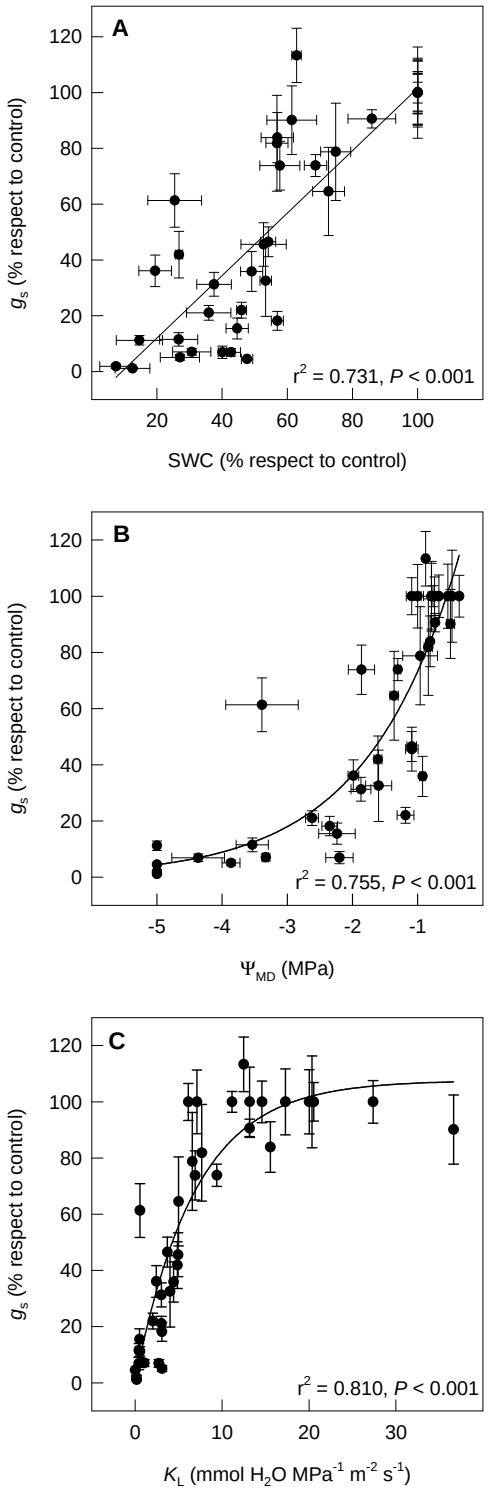


It is under discussion whether stomata close under drought in response to soil-to-leaf chemical signals (Davies & Zhang, 1991; Tardieu & Simmoneau, 1998; Christmann *et al*., 2005), leaf water potential (Comstock & Mencuccini, 1998; Mencuccini *et al*., 2000) or leaf specific hydraulic conductivity (Salleo *et al*., 2000; Cochard *et al*., 2002; Brodribb & Holbrook, 2003; Schultz, 2003). While we did not measure leaf contents in abscisic acid or other chemical signals in the present study, root-to-shoot chemical-induced stomatal closure would be reflected in a strong correlation between stomatal conductance and soil water content regardless of leaf water potential (Gollan *et al*., 1985; Gollan *et al*., 1986). A significant correlation (r^2 = 0.731, $P < 0.001$) was found between g_s (expressed as % of control values) and SWC plotting all the species together (Fig. 6.7A), but an equally strong correlation (r^2 = 0.755, $P < 0.001$) was observed between g_s and Ψ_{MD} (Fig. 7.7B). Therefore, we could not conclude whether root-to-shoot chemical signals or leaf water potential were the main factors inducing stomatal closure during drought in Mediterranean species.

Leaf specific hydraulic conductivity (K_L) ranged between 6 and 27 mol m^{-2} s^{-1} MPa in irrigated plants, the two extremes corresponding to the evergreen sclerophylls *L. magallufianum* and *H. balearicum*, respectively. K_L declined during drought in all the species, and it correlated with changes in g_s more strongly (r^2 = 0.810, $P < 0.001$) than SWC or Ψ_{MD} (Fig. 7.7C). Up to a K_L of 8 mmol m^{-2} s^{-1} MPa^{-1} the relationship was linear, as described by Schultz (2003) in grapevines. Further increases in K_L were not related to g_s, since there was no correlation between maximum g_s and maximum K_L in these species (not shown). The present results strongly support that g_s variations during water stress are largely determined by hydraulic conductance in Mediterranean species, as already suggested (Salleo *et al*., 2000; Serrano & Peñuelas, 2005).

Figure 6.7

Relationship between the stomatal conductance (g_s, in percentage respect to control values) and (A) the soil water content (SWC, in percentage respect to control values), (B) midday leaf water potential (Ψ_{MD}), and (C) leaf specific hydraulic conductance (K_L). Values represent means ± standard errors of four replicates. The standard error of K_L was not calculated since this parameter was obtained from means for each one of the species. The regression coefficients and significance of each relationship are shown.



6.4.3. Recovery of leaf water relations and stomatal conductance after re-watering

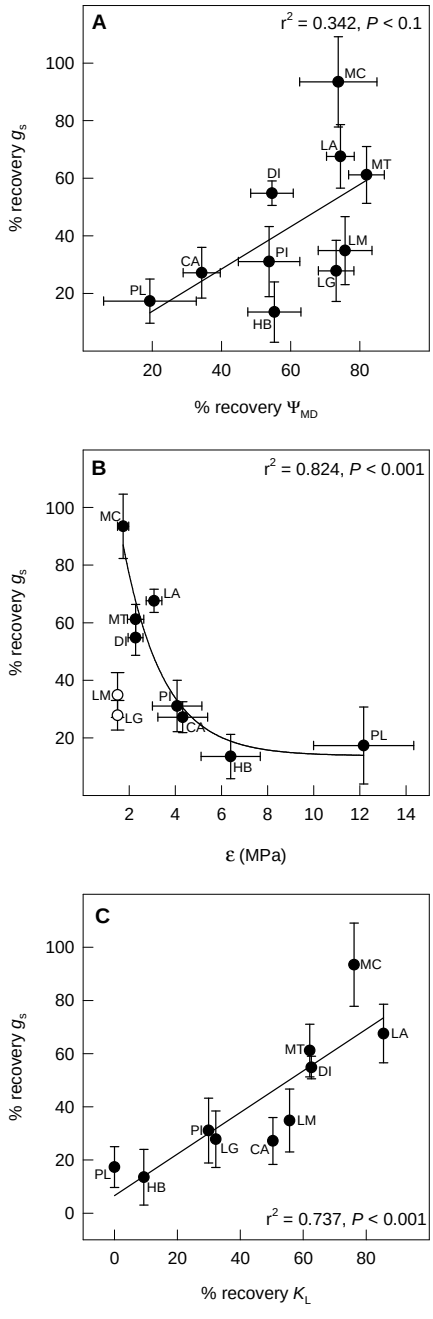
After severe drought stress, plants were re-watered at field capacity (i.e., SWC was restored to 100%), and water relations and g_s were determined after 24 h to assess recovery. Ψ_{PD} recovered to values ranging from 11% (*P. lentiscus*) to 78% (*B. maritima* subsp. *maritima*) of the initial (data not shown). The extent of Ψ_{MD} recovery ranged from 20% to 82%, the extremes corresponding to the same species as for Ψ_{MD} (Fig. 6.8A). No clear pattern of water relations recovery was observed among growth forms. For instance, maximum recovery (70-80%) was achieved by the two perennial herbs (*Beta*), a semi-deciduous shrub (*L. maritima*) and two evergreens (*Limonium*).

The range of stomatal conductance recovery was similar to that of leaf water potential (Fig. 6.8A), from 13% (*H. balearicum*) to 93% (*B. maritima* subsp. *marcosii*). In this case, a certain effect of growth form and was observed, with herbs showing the highest recovery, the semi-deciduous showing an intermediate recovery, and evergreens showing the lowest recovery. A similar trend for recovery was proposed by Gratani & Varone (2004) for sclerophyll versus malacophyll shrubs. However, this trend cannot be generalised since, as shown in Fig. 6.8A, the semi-deciduous *L. maritima* aligns with the herbs and the two evergreens *Limonium* align with the semi-deciduous shrubs.

However, the relationship between the extent of g_s and Ψ_{MD} recovery was only marginally significant ($P < 0.1$, Fig. 6.8A). A significant, negative relationship between the extent of g_s recovery and ε was found when considering the anisohydric species only, but again the two *Limonium* did not follow the same trend (Fig. 6.8B). The negative relationship found between g_s recovery and ε contradicts the idea that low cell-wall elasticity would allow a rapid recovery after stress (Corcuera *et al.*, 2002).

Figure 6.8

Relationship between the percentage of recovery of the stomatal conductance (g_s) and (A) the percentage of recovery of midday leaf water potential (Ψ_{MD}), (B) the leaf bulk elastic modulus (ε), and (C) the percentage of recovery of the leaf specific hydraulic conductance (K_L). Values represent means ± standard errors of four replicates. The standard error of K_L was not calculated since this parameter was obtained from means for each one of the species. The regression coefficients and significance of each relationship are shown. The regression coefficient of Fig. 6.8B was obtained excluding both *Limonium* species (empty symbols). In this figure, an arbitrary value of 1.5 MPa for ε was assigned to the two *Limonium* species (the true value cannot be calculated precisely, but range between 0 and 2 MPa). Species codes as in Table 6.1.



As occurred with SR to water stress, the extent of g_s recovery showed a general, highly significant relationship with the extent of K_L recovery (Fig. 6.8C). This correlation strongly suggests that stomatal regulation in Mediterranean plants is mostly governed by changes in K_L not only in response to water stress but also during recovery. To the best of our knowledge, the present results are the first showing the involvement of K_L in the inter-specific differences in the extent of g_s recovery after a drought period. The regulation of K_L depend on cavitation and recovery of xylem vessels, from which leaf veins seem the most sensitive (Cochard *et al*., 2002; Brodribb & Holbrook, 2003). The mechanisms leading to K_L recovery after cavitation are not fully understood and constitute an active area of research. Recent reports suggest the involvement of aquaporins in K_L regulation (Morillon & Chrispeels, 2001; Nardini *et al*., 2005). A better knowledge about these mechanisms may deserve better attention, and the present results suggest that it would be crucial for the understanding of stomatal regulation in response to water stress in Mediterranean plants.

6.4.4. Concluding remarks

The general aim of the present study was to increase the range of growth forms and for the broad comparative analysis of water relations and stomatal responses to drought in Mediterranean plants. This was achieved by including two evergreen sclerophyll shrubs, two evergreen sclerophyll semi-shrubs, three summer semi-deciduous shrubs, two perennial herbs and an annual herb, all growing under the same conditions and exploring the same soil volume.

Regarding the first specific objective, i.e., analysing the ranges in water relations parameters within Mediterranean species, the results show that both iso- and anisohydric behaviors associated to drought-tolerant and drought-avoidant strategies, respectively, were present in the group of species sampled. Leaf hydraulic properties, like the modulus of elasticity (ε) or the leaf specific hydraulic conductivity (K_L), were largely variable, not only between groups but also within the evergreen sclerophylls. With respect to the second specific objective, i.e., understanding the factors determining the stomatal response to water stress, among the constitutive traits only stomatal density (but not SLW or ε) was related to stomatal responsiveness (SR), as determined from the slope of g_s to Ψ_{PD}. Among the physiological parameters, K_L showed the highest correlation with g_s plotting all the species together. Also the extent of g_s recovery after

re-watering, which was the third objective of the present work, depended more strongly on the recovery of K_L than on that of Ψ_{MD}. These results confirm that g_s variations during water stress are largely determined by hydraulic conductance in Mediterranean species, as already suggested (Salleo *et al*., 2000; Serrano & Peñuelas, 2005).

Finally, regarding the fourth objective, i.e., assessing the influence of growth form on the observed water relations and stomatal responses to water stress, none of the traits analysed were clearly associated to specific growth form groups. Rather, the present results show that there is a large variability in responses to water stress among Mediterranean plants, which is independent of the growth form and that likely reflects the fact that any species in this environment has to endure temporary drought periods, which has lead to an array of different adaptive strategies.

Chapter 7

PHOTOSYNTHETIC LIMITATIONS

ECOPHYSIOLOGICAL RESPONSES TO WATER STRESS AND RECOVERY IN MEDITERRANEAN PLANTS WITH DIFFERENT GROWTH FORMS. II. PHOTOSYNTHETIC LIMITATIONS.

7.1. SUMMARY

Ten Mediterranean species belonging to different growth forms were grown in pots and subjected to four different levels of water stress, the most severe followed by re-watering. A quantitative limitation analysis was applied to estimate the effects of drought on stomatal (S_L) and non-stomatal limitations (NS_L) to light-saturated net photosynthesis, relative to the maximum rates obtained under conditions of optimal soil water content. Furthermore, based on combined gas exchange and chlorophyll fluorescence measurements, NS_L was partitioned into a diffusive (due to a decrease in mesophyll conductance, MC_L) and a biochemical component (due to a decrease in carboxylation capacity, B_L). Results confirmed that a general pattern of photosynthetic response to drought exist among C_3 plants when g_s is used as a reference parameter. As g_s values dropped from a maximum to about 0.05 mol H_2O m^{-2} s^{-1}, the total photosynthetic limitation rose from 0 to about 70%, and this was due to a progressive increase of both S_L and MC_L limitations, while B_L remained negligible. When lower values of g_s were achieved (i.e., total photosynthetic limitations from 70% to 100%), the contribution of S_L declined, while MC_L still increased and B_L contributed significantly (20% to 50%) to the total limitation. Finally, when photosynthetic recovery from severe drought was analysed after re-watering, a different pattern was observed, consisting in a dominant role of MC_L irrespective of the degree of photosynthesis recovery, which differed by ca 60% among species. B_L recovered fast and to a great extent after re-watering.

7.2. INTRODUCTION

Low water availability is considered the main environmental factor limiting plant growth and yield worldwide, but especially in semi-arid areas (Boyer, 1982). The limitation to plant growth imposed by low water availability is mainly due to reductions of plant carbon balance, which is dependent on the balance between photosynthesis and respiration. Therefore, the response of photosynthesis to water stress received considerable attention in the past, and a long-standing controversy was maintained regarding which was the primary limitation to photosynthesis, stomatal closure or metabolic impairment (Boyer, 1976; Sharkey, 1990; Chaves, 1991; Lawlor, 1995; Cornic & Massacci, 1996). In the recent years, an effort has been done towards generalising the response of photosynthetic parameters to water stress in higher plants

(Cornic & Fresneau, 2002; Flexas & Medrano, 2002a; Lawlor & Cornic, 2002; Chaves *et al*., 2003; Bota *et al*., 2004; Grassi & Magnani, 2005; Osório *et al*., 2006). As a result of such effort, there is now considerable consensus that diffusion limitations to photosynthesis under most water stress situations are predominant, but these involve not only stomatal closure but also decreased mesophyll conductance to CO_2 (g_i), an important but sometimes neglected process (Roupsard *et al*., 1996; Flexas *et al*., 2002; Black *et al*., 2005; Ennahli & Earl, 2005). Regardless of the species analyzed, down-regulation of photochemistry and biochemistry starts when daily maximum stomatal conductance (g_s) drops below 0.15 mol H_2O m^{-2} s^{-1}, and a general failure of metabolism can occur only eventually when g_s drops below 0.05 mol H_2O m^{-2} s^{-1} (Flexas *et al*., 2004a; Grassi & Magnani, 2005).

However, this general pattern has been tested mostly in crops, and only few data are available including natural vegetation of different origins. Because natural environments offer a big range of microhabitats and ecological niches, it is likely that particular adaptations can be found, therefore opening the possibility of finding notable exceptions to general rules (Schulze, 1988). The Mediterranean climate is characterized by a hot dry period in summer and a cool wet period in winter, as well as by high inter-annual variability. From an ecophysiological point of view, the variability and unpredictability of precipitation impose strong constraints on plants and could represent an important factor of evolutionary pressure (Joffre *et al*., 1999). As a consequence, natural vegetation from the Mediterranean area seems an appropriate genetic background to search for particular adaptations that may represent exceptions to the above described general pattern of photosynthesis response to water stress. In fact, it is well known that the natural vegetation of Mediterranean areas has developed an array of adaptations to drought, resulting in a high diversity of growth forms and growth forms. The resulting vegetation consists, among other life forms, of deep rooted evergreen sclerophyll trees and shrubs, which tolerate and/or avoid water stress and maintain green leaves during the summer drought period, semi-deciduous shrubs, which lose a part of their leaves during summer, and geophytes and winter annual and biennial herbs, which escape drought by finishing their annual cycle before summer (Ehleringer & Mooney, 1983). In addition to this diversity of morpho-phenological forms, we have observed in Mediterranean plants a strong diversity in ecophysiological traits that are likely of adaptive value, such as the specificity factor of Rubisco (Galmés *et al*., 2005a), the response of relative growth rate and its components to water stress (Galmés *et al*.,

2005b) or leaf water relations and stomatal control (Chapter 6). Therefore, a primary objective of the present study was to test the generality of the described pattern of photosynthetic response to water stress, using the natural plant diversity of the Mediterranean area. A gas exchange analysis of photosynthetic limitations under drought in Mediterranean plants has been already performed, but this focused mostly on evergreen sclerophyll shrubs (Tenhunen *et al*., 1985; Harley *et al*., 1986; Harley *et al*., 1987a; Gulías *et al*., 2002; Peña-Rojas *et al*., 2004) an one summer semi-deciduous shrub, *Cistus salvifolius* (Harley *et al*., 1987b), and none of the studies took g_i variations into account, for which conclusions regarding biochemical limitations must be viewed with care.

On the other hand, the carbon balance of a plant enduring a drought period may depend as much on the velocity and degree of photosynthetic recovery as much as it depends on the degree and velocity of photosynthesis decline during water depletion. In general, plants subjected to severe water stress recover only 40-60% of the maximum photosynthesis rate during the next day after re-watering (De Souza *et al*., 2004; Flexas *et al*., 2004b). Slow recovery of photosynthesis after severe drought is usually accompanied by a slow recovery of g_s and increased antioxidant activities in the leaves (Sofo *et al*., 2004; De Souza *et al*., 2004; Miyashita *et al*., 2005). De Souza *et al*. (2004) also showed that recovery of photosynthesis required changes in the photochemical apparatus that lasted for several days, which were evidenced by a decline in the maximum photochemical efficiency of photosystem II (F_v/F_m) after re-watering, followed by a recovery to control values. However, while many studies have addressed different aspects of photosynthetic limitations during drought imposition, an analysis of the photosynthetic limitations during photosynthetic recovery after drought stress has not yet been performed (Miyashita *et al*., 2005; Flexas *et al*., 2006), with the exception of an early study by Kirschbaum (1987, 1988), which suggested that photosynthesis during recovery was co-limited by an incomplete stomatal opening and a metabolic component. Therefore, another objective of the present work was to perform an analysis of photosynthetic limitations the day next to re-watering in different species having endured severe drought. In particular, in view of the recently highlighted importance of decreased g_i in the regulation of photosynthesis during drought, we test the hypothesis that limited recovery of g_i after re-watering may contribute to incomplete recovery of photosynthesis.

7.3. MATERIAL AND METHODS

7.3.1. Plant material

Selected species and treatments as in Chapter 6.

7.3.2. Chlorophyll fluorescence measurements

Chlorophyll fluorescence parameters were measured on attached leaves using a portable pulse amplitude modulation fluorometer (PAM-2000, Walz, Effeltrich, Germany). For each sampling time and treatment, four measurements were made on different plants.

A measuring light of about 0.5 μmol photon m^{-2} s^{-1} was set at a frequency of 600 Hz to determine, at pre-dawn, the background fluorescence signal (F_o), the maximum fluorescence (F_m), and the maximum quantum efficiency of PSII ($F_v/F_m = (F_m-F_o)/ F_m$). At mid-morning the same leaves analysed at pre-dawn were measured with a photon flux density around 1500 μmol m^{-2} s^{-1}, obtained using the halogen lamp of the PAM-2000, measuring steady state fluorescence signal (F_s). To obtain the steady-state maximum fluorescence yield (F_m'), saturation pulses of about 10000 μmol photon m^{-2} s^{-1} and 0.8 s duration were applied. The PSII photochemical efficiency ($\Delta F/F_m$', Genty *et al*., 1989) was then calculated as:

$$\Delta F/F_m' = (F_m'-F_s)/ F_m'$$

and used for the calculation of the relative linear electron transport rate (ETR) according to Krall & Edwards (1992):

$$ETR = \Delta F/F_m' \cdot PPFD \cdot \alpha \cdot \beta$$

where PPFD is the photosynthetically active photon flux density α is the leaf absorptance, and β is a factor that assumes equal distribution of energy between the two photosystems (the actual factor has been described to be between 0.4 and 0.6; Laisk & Loreto 1996). Leaf absorptances were determined for all ten species in ten replicates on leaves of well-irrigated plants with a spectroradiometer coupled to an integration sphere (UniSpec, PP-Systems, USA). A value of 0.84 was obtained for all species, except for *C. albidus* and *P. italica*, which presented leaf absorptance values of 0.74 and 0.77, respectively. Potential changes in leaf absorptance with drought were not assessed but, because changes in chlorophyll content were small or non-significant, depending on the

species (not shown), they were assumed to be small, inducing no important biases in the calculations of ETR.

7.3.3. Gas exchange measurements

Light-saturated net CO_2 assimilation rates (A_N) and stomatal conductance (g_s) were measured at mid-morning on one attached and fully developed young leaf of four plants per species and treatment with a gas exchange system (Li-6400, Li-Cor Inc., Nebraska, USA) equipped with a light source (6200-02B LED, Li-Cor). Environmental conditions in the chamber used for leaf measurements consisted in a photosynthetic photon flux density of 1500 μmol photon m^{-2} s^{-1}, a vapour pressure deficit of 1.0.-1.5 kPa, an air temperature of 25ºC and an ambient CO_2 concentration (C_a) of 400 μmol mol air^{-1}.

After inducing steady-state photosynthesis, photosynthesis response curves to varying sub-stomatal CO_2 concentration (C_i) were performed. First, the C_a was lowered stepwise from 360 to 50 μmol mol^{-1} and then fixed again at 360 μmol mol^{-1} until reaching a steady-state value similar to that obtained at the beginning of the curve. Then, C_a was increased stepwise from 360 to 1500 μmol mol^{-1}. Gas exchange measurements were determined at each step after maintaining the leaf for at least 5 min at the new C_a. Measurements consisted in 12-13 measurements for each curve. A_N-C_i curves were transformed to A_N-C_c curves as described in the next section.

7.3.4. Estimations of CO_2 concentration at the site of carboxylation and mesophyll conductance

From combined gas-exchange and chlorophyll fluorescence measurements, the CO_2 concentration in the chloroplasts (C_c) was calculated according to Epron *et al*. (1995). This model works in the assumption that all the reducing power generated by the electron transport chain is used for photosynthesis and photorespiration, and that chlorophyll fluorescence gives a reliable estimate of the quantum yield of electron transport. Thus, the electron transport rate (ETR) measured by chlorophyll fluorescence can be divided into two components: $ETR = ETR_A + ETR_P$, where ETR_A is the fraction of ETR used for CO_2 assimilation, and ETR_P is the fraction of ETR used for photorespiration. ETR_A and ETR_P can be solved from data of A_N, R_L and ETR, and from the known stochiometries of electron use in photosynthesis and photorespiration, as

follows (Epron *et al*., 1995; Valentini *et al*., 1995): $ETR_A = 1/3\ [ETR + 8\ (A_N + R_L)]$; $ETR_P = 2/3\ [ETR - 4\ (A_N + R_L)]$.

The ratio ETR_A to ETR_P is related to the C_c/O ratio in the chloroplast (where O represents the oxygen molar fraction at the oxygenation site) through the Rubisco specificity factor (τ), as follows (Laing *et al*., 1974): $\tau = (ETR_A / ETR_P) / (C_c/O)$. Using the values of τ previously determined *in vitro* for each species (Galmés *et al*., 2005a), and assuming O to be equal to the molar fraction in the air, the above equation was solved for C_c. The mesophyll conductance to CO_2 was then calculated as:

$g_i = A_N / (C_i - C_c)$.

7.3.5. Quantitative limitation analysis

At ambient CO_2 concentration, light-saturated photosynthesis is generally limited by substrate availability, which was verified by A_N-C_i curves in the present data for each species and treatment (not shown). Under CO_2-limited conditions, photosynthesis can be expressed as (Farquhar *et al*., 1980):

$$A_N = \frac{V_{c,\max} \cdot C_c}{C_c + K_c \cdot (1 + O/K_o)} \cdot \left(1 - \frac{\Gamma^*}{C_c}\right) - R_L$$

where $V_{c,max}$ is the maximum rate of carboxylation of Rubisco, K_c and K_o are the Michaelis–Menten constants for CO_2 and O_2, respectively, Γ^* is the CO_2 compensation point in the absence of mitochondrial respiration, and R_L is the rate of non-photorespiratory CO_2 evolution in the light. Estimations of $V_{c,max}$ were derived from fitting A_N-C_c curves, according to the 'one point method' proposed by Wilson *et al*. (2000), i.e. from the measure of assimilation and C_c at ambient CO_2 and the treatment average of Γ^* for the species. This method requires that photosynthesis is substrate-limited (i.e. that C_c is on the Rubisco-limiting portion of the curve), which was the case in all our data-set according to the full A_N-C_c curves. The treatment average of Γ^* for the species was obtained, according to Brooks & Fraquhar (1985), as:

$$\Gamma^* = \frac{0.5O}{\tau}$$

from specific τ values for each one of the species (Galmés *et al*., 2005). Finally, R_L was calculated for the A_N-C_i curve on the same treatment, as in Grassi & Magnani (2005).

To compare relative limitations to assimilation due to drought, photosynthetic limitations were partitioned into their functional components following the approach proposed by Grassi & Magnani (2005). This approach, which requires the measurement of A_N, g_s, g_i and $V_{c,max}$, makes it possible to partition photosynthesis limitations into components related to stomatal conductance (S_L), mesophyll conductance (MC_L) and leaf biochemical characteristics (B_L), assuming that a reference maximum assimilation rate can be defined as a standard. The maximum assimilation rate, concomitantly with g_s and $V_{c,max}$, was reached under well-watered conditions, therefore the control treatment was used as a reference. Finally, non-stomatal limitations were defined as the sum of the contributions due to mesophyll conductance and leaf biochemistry (NS_L = MC_L + B_L), while diffusive limitations were the sum of stomatal and mesophyll conductance components (D_L = S_L + MC_L).

It should be noticed that g_i calculations (and, therefore, $V_{c,max}$ calculations) may be impaired if heterogeneous stomatal closure, which has been sometimes described as a response to drought (Laisk, 1983; Beyschlag *et al*., 1992; Gunasekera & Berkowitz, 1993), significantly affects C_i calculations. This may impair the application of limitation analysis. However, it has been shown that this effect is negligible for g_s values above 0.03 mol H_2O m^{-2} s^{-1} (Buckley *et al*., 1997; Grassi & Magnani, 2005). In the present study, lower values were achieved only under severe drought and in some of the species analysed (see Results and Discussion). Even in those cases, g_i estimations were considered a good approximation of actual values because (i) C_c calculations are unaffected by C_i in the model of Epron *et al*. (1995), contrary to other methods (Harley *et al*., 1992); and (ii) at low values of g_i the results are much less affected by errors in C_i. For instance, under severe drought treatment, with a g_s of 0.017 mol H_2O m^{-2} s^{-1}, *L. magallufianum* showed an A_N of 1.6 μmol CO_2 m^{-2} s^{-1}, an ETR of 148 μmol electrons m^{-2} s^{-1}, and a C_i of 222 μmol mol^{-1} (Table 7.1). Even in the case of 50% overestimation or 150% underestimation of the measured C_i, the differences between g_i were no longer than 0.015 mol H_2O m^{-2} s^{-1}, very low if compared to control values (of about 0.120 mol CO_2 m^{-2} s^{-1}), which may suppose only a 15% difference in the calculated MC_L, S_L and B_L (Table 7.1).

7.3.6. Statistical analysis

Regressions coefficients between g_s and A_N, ETR, g_i and $V_{c,max}$ were calculated with the 8.0 Sigma Plot software package (SPSS). Differences between means were revealed by Duncan analyses ($P < 0.05$) performed with the SPSS 12.0 software package (SPSS, Chicago, USA).

Table 7.1

Net photosynthetic rate (A_N, μmol CO_2 m^{-2} s^{-1}), stomatal conductance (g_s, mol H_2O m^{-2} s^{-1}), electron transport rate (ETR, μmol e^- m^{-2} s^{-1}) and substomatal CO_2 concentration (C_i, μmol mol air^{-1}), observed for *L. magallufianum* under severe drought treatment, with the corresponding mesophyll conductance (g_i, mol CO_2 m^{-2} s^{-1}) and photosynthetic limitations depending on the C_i estimation. S_L = stomatal limitation, MC_L = mesophyll conductance limitation, B_L = biochemical limitation. A possible underestimation of C_i by 50% and overestimation by 150% due to heterogeneous stomatal closure was considered to analyse how much it would affect limitation calculations.

	A_N	g_s	ETR	C_i
	1.6	0.017	148	222
	g_i	S_L	MC_L	B_L
C_i measured	0.010	28	33	28
C_i 50%	0.025	37	18	33
C_i 150%	0.006	20	46	23

7.4. RESULTS AND DISCUSSION

7.4.1. Photosynthetic limitations during drought imposition

The response of net photosynthesis (A_N) to gradual drought was determined in ten different Mediterranean species belonging to different growth forms (Fig. 7.1). The species included comprised an array of different leaf hydraulic properties and water relations response to drought, including both iso- and anisohydric responses (see Chapter 6). Despite of this, all ten species showed a gradual decline of A_N as drought intensified, starting at mild drought except for the two *Beta* (Fig. 7.1). $V_{c,max}$ followed a different pattern, consisting in maintaining values similar to irrigated plants under mild to moderate drought, depending on the species, and declining thereafter (Fig. 7.1).

Both stomatal (g_s) and mesophyll (g_i) conductances to CO_2 declined progressively as drought intensified (Fig. 7.2). Remarkably, under irrigation g_i was

equal to or lower than g_s for all the species analysed, although the differences became smaller as drought intensified. A g_i higher than g_s is sometimes described in woody plants (Miyazawa & Terashima, 2001; Hanba *et al*., 2002; Centritto *et al*., 2003; De Lucia *et al*., 2003; Peña-Rojas *et al*., 2004; Warren *et al*., 2004; Warren *et al*., 2006) - although not in all cases (Epron *et al*., 1995) - and it is rarely observed in herbaceous plants (Loreto *et al*., 1992; De Lucia *et al*., 2003; Warren *et al*., 2006). This has been interpreted in terms of the leaf mesophyll anatomy effects on g_i (Syversten *et al*., 1995; Hanba *et al*., 1999). However, the present data suggest that g_i may be more limiting for photosynthesis than g_s in different Mediterranean plants, regardless of their growth form and leaf anatomy. Other factors than leaf anatomy are possibly involved in g_i, among which aquaporins have been recently reported to play an important role (Terashima & Ono, 2002; Uehlein *et al*., 2003; Hanba *et al*., 2004).

To see whether these data fitted the photosynthetic response pattern usually described for C_3 plants (Flexas *et al*., 2002; 2004a; Medrano *et al*., 2002), the above parameters, as well as the electron transport rate (ETR), were plotted against g_s pooling all species together (Fig. 7.3). For all the range of g_s, a decline of g_s resulted in a proportional decline of A_N, and a strong relationship was found between both variables (Fig. 7.3A). The ETR plot presented larger scattering due to a large variability in maximum ETR values among species (Fig. 7.3B). Despite of this, the general trend suggested that ETR was largely unaffected by water stress for g_s values above 0.20 mol H_2O m^{-2} s^{-1}, which is in accordance with what is usually observed in C_3 plants, and is due to the compensatory increase in photorespiration as A_N declines during the early stages of drought (Flexas & Medrano, 2002c).

Figure 7.1

Net photosynthetic rate (A_N) and maximum velocity of the carboxylation rate ($V_{c,max}$) under different irrigation treatments: control (CO), mild drought (MiD), moderate drought (MoD), severe drought (SD) and re-watering (RW). Values are means ± standard error of three to five replicates per species and treatment.

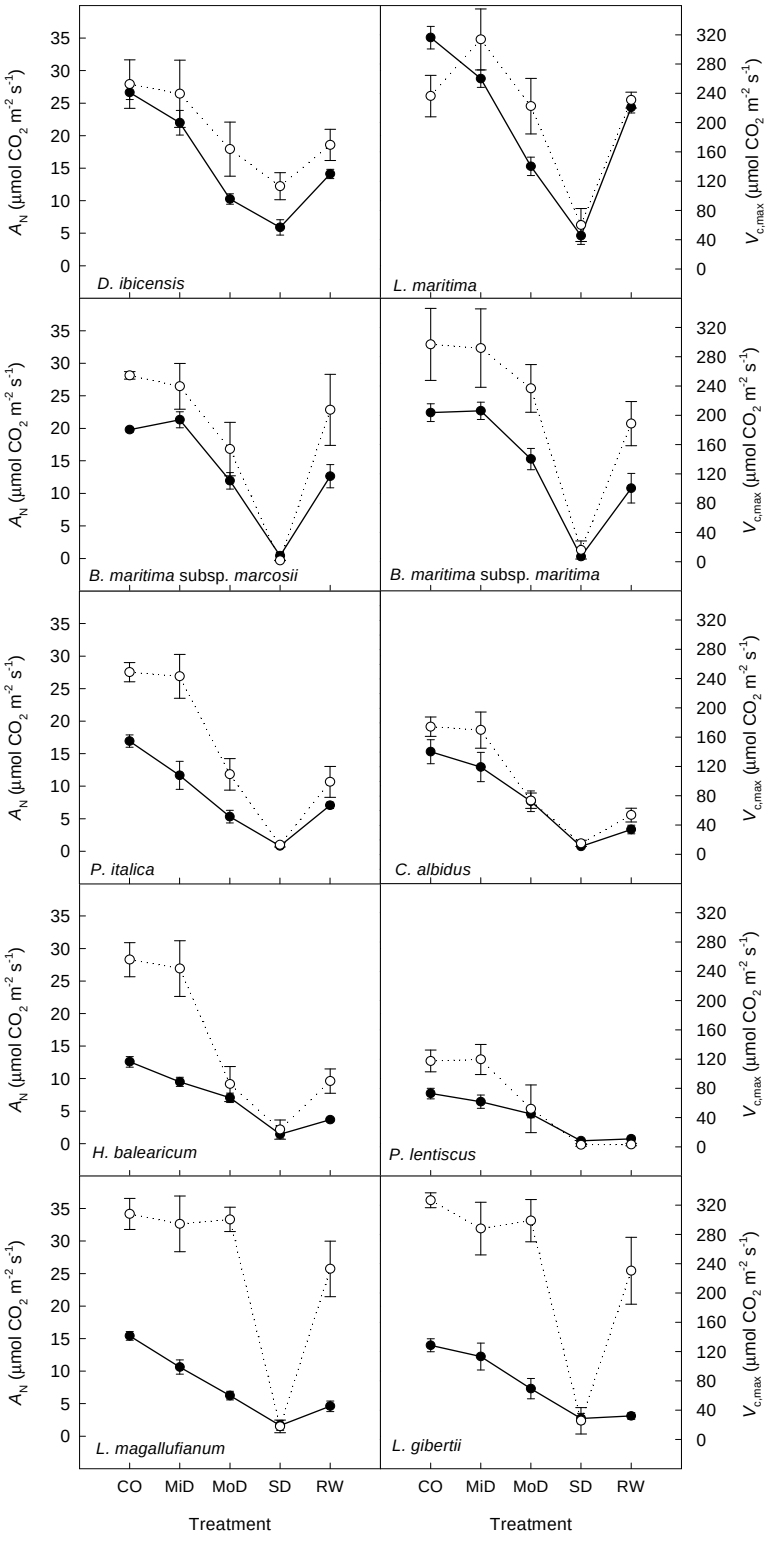
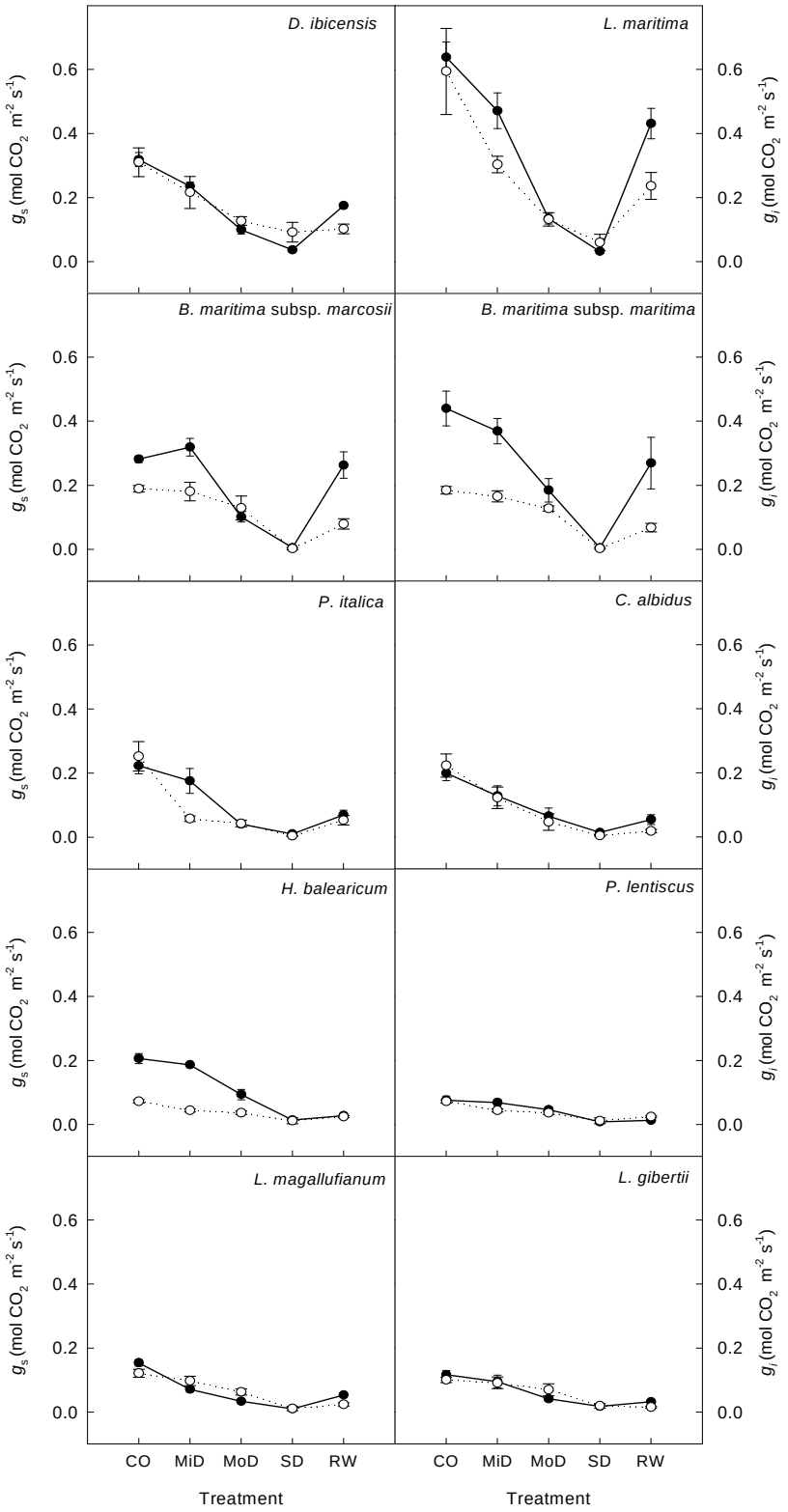


Figure 7.2

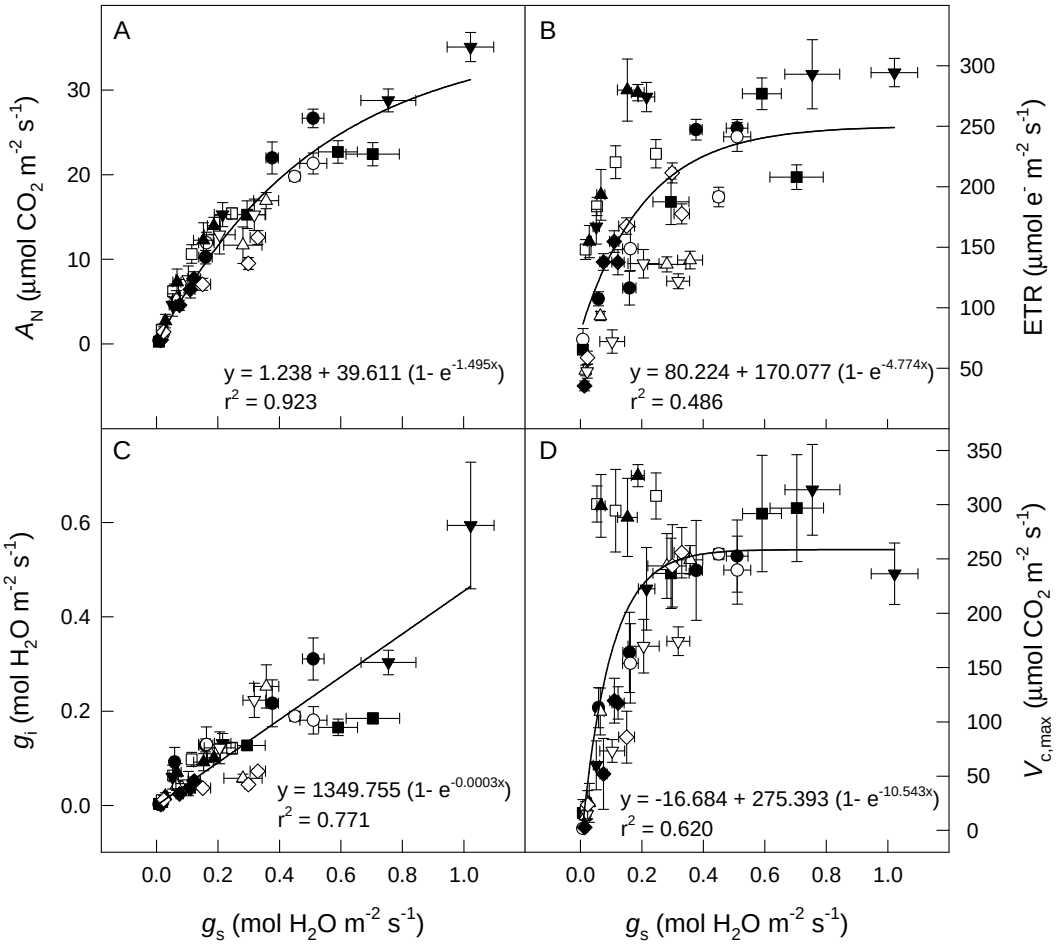
Stomatal conductance (g_s) and mesophyll conductance (g_i) under different irrigation treatments: control (CO), mild drought (MiD), moderate drought (MoD), severe drought (SD) and re-watering (RW). Values are means ± standard error of three to five replicates per species and treatment.



The mesophyll conductance to CO_2 (g_i) was linearly related to g_s when pooling all the species together, although *B. maritima* subsp. *marcosii* seem to follow a slightly different, curvilinear pattern (Fig. 7.3C). Whether the relationship between g_i and g_s is linear or curvilinear is an unresolved question (Flexas *et al*., 2004a; Warren *et al*., 2006), which is of importance for the understanding of g_i effects on photosynthetic nitrogen and water use efficiency under drought or salinity (Warren *et al*., 2006). The present results, along with those of Centritto *et al*. (2003), suggest that linear relationships may be more common, but a curvilinear relationship may be found in some species, such as *B. maritima* subsp. *marcosii* or *Vitis vinifera* (Flexas *et al*., 2002). The actual implications of these differences remain to be established. Regarding $V_{c,max}$ (Fig. 7.3D), the pattern somewhat resembled that of ETR, except that inter-specific differences in the maximum values were not so large. None of the species analysed presented a decline in $V_{c,max}$ until g_s dropped below ca. 0.10-0.15 mol H_2O m^{-2} s^{-1}, and in some species (both *Limonium*) even lower g_s values were required. Again, this is very consisting with the pattern usually described in C_3 plants. Therefore, although slight differences have been observed between species, they all follow roughly a general pattern consisting in an early phase of drought-induced A_N decline associated to g_s and g_i reductions, followed by a second phase in which $V_{c,max}$ and ETR are also reduced to some extent (Medrano *et al*., 2002; Flexas *et al*., 2004a).

Figure 7.3

Relationship between (A) net photosynthetic rate (A_N), (B) electron transport rate (ETR), (C) mesophyll conductance (g_i) and (D) maximum rate of carboxylation ($V_{c,max}$) and stomatal conductance (g_s). Values from re-watering treatment were not included. The regression coefficients and significance of each relationship are shown. Values are means ± standard error of three to five replicates per species and treatment. Symbols and species as follows: ● *D. ibicensis*, ○ *L. maritima*, ■ *L. magallufianum*, □ *L. gibertii*, ▲ *B. maritima* subsp. *marcosii*, Δ *B. maritima* subsp. *maritima*, ▼ *P. italica*, ∇ *C. albidus*, ♦ *H. balearicum*, ◊ *P. lentiscus*.



The above described responses relate *qualitatively* drought-induced variations in some photosynthetic parameters to drought-induced reductions in A_N. A *quantitative* relationship can be obtained through a limitation analysis (Jones, 1985; Grassi & Magnani, 2005). The results are shown in Table 7.2. At mild drought (as well as at moderate drought in *L. maritima* and the two *Limonium*), the biochemical limitations (B_L) were negligible, and the sum of stomatal (S_L) and mesophyll conductance (MC_L) limitations accounted for the entire photosynthetic limitation. In some species, such as

L. maritima and the two *Limonium*, S_L was much more important than MC_L at mild to moderate drought. In other species, such as *C. albidus*, *H. balearicum* and *P. lentiscus* (i.e., the most sclerophyll species), MC_L was much larger than S_L. In the rest of species, both limitations were of similar magnitude. At moderate to severe drought, only in *L. maritima* S_L was still the most important limitation to photosynthesis. In most species, MC_L was the most important limitation at severe drought, although in some of them (*D. ibicensis*, *B. maritima* subsp. *marcosii* and subsp. *maritima*, and *L. magallufianum*) B_L was of similar magnitude. Therefore, in all the species regardless of their growth form and leaf type, there was a shift from mostly CO_2 diffusion limitations at mild to moderate drought to a combination of diffusion and biochemical limitations at severe drought (Tenhunen *et al*., 1985; Harley *et al*., 1986; Harley *et al*., 1987a, b; Gulías *et al*., 2002; Lawlor & Cornic, 2002; Flexas *et al*., 2004a; Peña-Rojas *et al*., 2004). As already shown (Grassi & Magnani, 2005), the evolution of these limitations with drought closely correlated with g_s (Fig. 7.4A), and B_L become detectable only when g_s dropped below 0.05-010 mol H_2O m^{-2} s^{-1}, a situation where MC_L was the most important limitation to photosynthesis. Therefore, the present data also highlight the importance of g_i as a limiting factor for photosynthesis in Mediterranean plants (Niinemets *et al*., 2005), particularly under drought conditions (Roupsard *et al*., 1996; Flexas *et al*., 2002, 2004a), which has been hypothesized as one of the possible causes for the observed discrepancies between measured water use efficiency and that estimated with current gas exchange models in Mediterranean ecosystems (Reichstein *et al*., 2002).

Figure 7.4

Relationship between limitations of A_N (%) and the stomatal conductance (g_s) considering all ten species. (A) Values obtained form mild, moderate and severe drought treatments. (B) Values obtained from re-watering treatment. The regression coefficient and significance of the relationship between total limitations and stomatal conductance is shown. B_L = biochemical limitation, MC_L = mesophyll conductance limitation, S_L = stomatal limitation, T_L = total limitations.

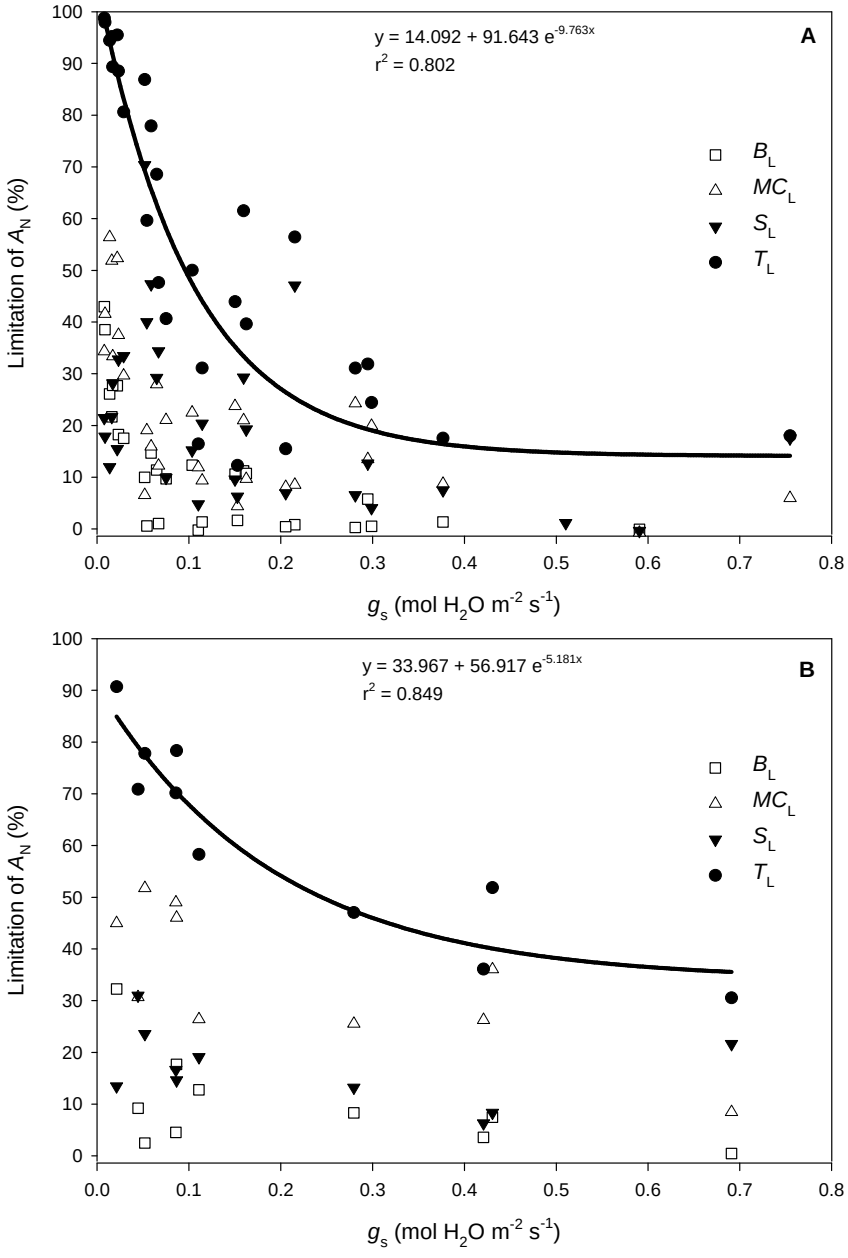


Table 7.2

Limitations of A_N, expressed as %, under different irrigation treatments: mild drought (MiD), moderate drought (MoD) and severe drought (SD).

Treatment	Total limitations (T_L)	Stomatal limitation (S_L)	Mesophyll conductance limitation (MC_L)	Biochemical limitation (B_L)	Non-stomatal limitations (NS_L)	Diffusional limitations (D_L)
D. ibicensis						
	T_L	S_L	MC_L	B_L	NS_L	D_L
MiD	17	7	9	1	10	16
MoD	61	29	21	11	32	50
SD	78	47	16	15	31	63
B. maritima subsp.*marcosii*						
	T_L	S_L	MC_L	B_L	NS_L	D_L
MiD	0	0	0	0	0	0
MoD	40	19	10	11	21	29
SD	99	18	42	39	81	60
B. maritima subsp *maritima*						
	T_L	S_L	MC_L	B_L	NS_L	D_L
MiD	0	0	0	0	0	0
MoD	32	13	13	6	19	26
SD	98	21	34	43	77	55
L. maritima						
	T_L	S_L	MC_L	B_L	NS_L	D_L
MiD	24	18	6	0	6	24
MoD	57	47	9	1	10	56
SD	87	70	7	10	17	77
P .italica						
	T_L	S_L	MC_L	B_L	NS_L	D_L
MiD	31	7	24	0	25	31
MoD	68	29	28	11	39	57
SD	96	22	52	22	74	74
C. albidus						
	T_L	S_L	MC_L	B_L	NS_L	D_L
MiD	15	7	8	0	9	15
MoD	49	15	22	12	34	37
SD	95	15	52	28	80	67
H. balearicum						
	T_L	S_L	MC_L	B_L	NS_L	D_L
MiD	24	4	20	0	20	24
MoD	45	10	24	11	35	34
SD	89	33	38	18	56	71
P. lentiscus						
	T_L	S_L	MC_L	B_L	NS_L	D_L
MiD	17	5	12	0	12	17
MoD	41	10	21	10	31	31
SD	94	12	56	26	82	68
L. magallufianum						
	T_L	S_L	MC_L	B_L	NS_L	D_L
MiD	30	20	9	1	10	29
MoD	60	40	19	1	20	59
SD	89	28	33	28	61	61

	L. gibertii					
	T_L	S_L	MC_L	B_L	NS_L	D_L
MiD	12	6	4	2	6	10
MoD	47	34	12	1	13	46
SD	81	33	30	18	48	63

7.4.2. Limitations to photosynthesis recovery after a drought period

Contrary to photosynthetic limitations during drought development, which have been intensely studied along the past 30 years, the photosynthetic limitations when recovering after a drought period have been much less explored. Usually, the photosynthesis recovery after a mild drought (i.e., whenever g_s is maintained above 0.15 mol H_2O m^{-2} s^{-1}) is rapid (one day after re-watering) and almost complete (Flexas *et al*., 2004b, 2006). By contrast, after severe water stress the recovery of photosynthesis is progressive and slow (lasting from days to weeks) and sometimes incomplete (Flexas *et al*., 2004b, 2006; De Souza *et al*., 2004; Miyashita *et al*., 2005). In the latter case, it would be interesting to know which are the factors limiting recovery in the short term. However, with the exception of early studies by Kirschbaum (1987, 1988), which did not take into account mesophyll conductance limitations, a detailed photosynthetic limitations analysis, including S_L, MC_L and B_L has not yet been performed.

In the present study, we analysed the recovery of photosynthesis 24h after re-watering severely drought stressed plants, in which g_s and A_N were severely depressed. It has been shown that plants subjected to severe water stress usually recover 40-60% of the maximum photosynthesis rate during the next day after re-watering (De Souza *et al*., 2004, Flexas *et al*., 2004b). In the present study, the range was broader, from a recovery of photosynthesis to less than 10% of control values in *P. lentiscus* to almost 70% in *L. maritima* (Table 7.3). In general, and with the exception of *L. maritima*, herbs showed the largest recovery (49-64%), semi-deciduous an intermediate recovery (21-42%), and evergreens the lowest recovery (10-29%). This may reflect different adaptations to water stress periods under Mediterranean conditions. For instance, herbs may experience short drought periods during the favourable season, and therefore a capacity for rapid recovery may be of importance to ensure their carbon balance requirements before ending their life cycle in late spring. On the contrary, evergreens suffer less from short dry periods during the favourable season, because of their large root system (Rambal, 1984; Canadell *et al*., 1996), but may have to endure a long drought period in

summer during which they may rely on more permanent physiological changes precluding rapid recovery (Mittler *et al*., 2001).

Table 7.3

Limitations of A_N, expressed in %, 24 h after re-watering plants to field capacity.

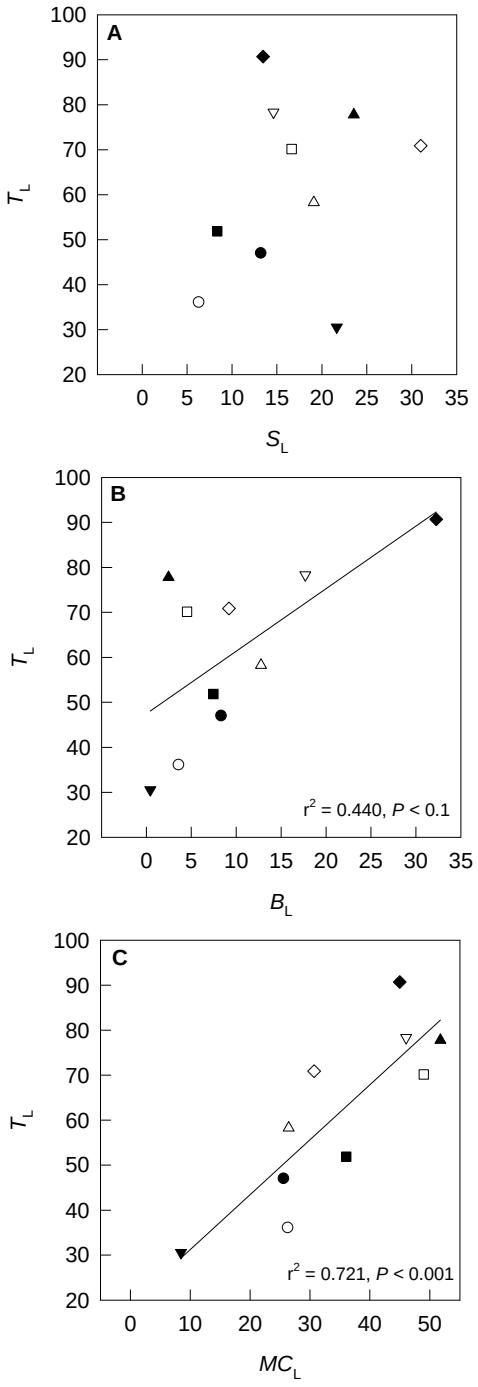
Species	Total limitations (T_L)	Stomatal limitation (S_L)	Mesophyll conductance limitation (MC_L)	Biochemical limitation (B_L)	Non-stomatal limitations (NS_L)	Diffusional limitations (D_L)
D. ibicensis	47	13	26	8	34	39
B. maritima subsp. *marcosii*	36	6	26	4	30	32
B. maritima subsp. *maritima*	51	8	36	7	43	44
L. maritima	30	22	8	0	8	30
P .italica	58	19	26	13	39	45
C. albidus	79	15	46	18	64	61
H. balearicum	71	31	31	9	40	62
P. lentiscus	90	13	45	32	77	58
L. magallufianum	71	17	49	5	54	66
L. gibertii	78	24	52	2	54	76

Regarding the mechanisms limiting photosynthetic recovery after severe drought, the different extents of recovery of A_N were accompanied by different extents in the recovery of either g_s, g_i or $V_{c,max}$ (Figs. 7.1 and 7.2). However, the limitation analysis revealed that MC_L was, by far, the strongest limitation to photosynthesis recovery in all the species analysed, with the exception of *L. maritima*, the species showing the largest recovery. Contrary to what is usually assumed (Flexas *et al*., 2004a), the recovery of biochemical limitations after severe drought was generally large. Only in *P. lentiscus* B_L still accounted for 32%, but even so, it contributed only to one third of the total limitation. Remarkably, the relationship between photosynthetic limitations and g_s during recovery was not the same as during water stress imposition (Fig. 7.4B). While there was still a highly significant relationship between total limitation and g_s (i.e., A_N and g_s maintained their co-regulation), MC_L was the most important limitation at any given g_s, while S_L and B_L were of similar magnitude through the entire range. That limited recovery of g_i was the most important limitation to photosynthetic recovery in these species was further highlighted by comparing the relationships between total photosynthetic limitation and partial limitations after re-watering. While the relationship between T_L and S_L was non-significant (Fig. 7.5A), and

that between T_L and B_L was only marginally significant (Fig. 7.5B), that between T_L and MC_L was highly significant pooling all the species together (Fig. 7.5C).

Figure 7.5

Relationship between total limitation of photosynthesis (T_L) 24 h after re-watering plants and (A) stomatal limitation (S_L), (B) biochemical limitation (B_L), and (C) mesophyll conductance limitation (MC_L). The regression coefficients and significance of each relationship are shown. Symbols as in Fig. 7.3.



Therefore, contrary to water stress imposition, in which two phases of photosynthetic regulation have been shown, the first one consisting of combined stomatal and mesophyll diffusion limitations and the second including an important biochemical limitation, the main photosynthetic limitation during photosynthesis recovery after a severe stress seems to be mesophyll conductance. These results represent the first photosynthetic limitation analysis performed during recovery from drought, and highlight even more the importance of mesophyll conductance in photosynthesis.

7.4.3. Concluding remarks

In summary, these data, including Mediterranean plants with a wide variety of growth forms, confirm that a general pattern of photosynthetic response to drought exist among C_3 plants when g_s is used as a reference parameter (Flexas *et al*., 2002; 2004a; Medrano *et al*., 2002). As g_s values drop from a maximum (which may largely differ among species) to about 0.05 mol H_2O m^{-2} s^{-1}, the total photosynthetic limitation rises from 0 to about 70%, and this is due to a progressive increase of both stomatal and mesophyll conductance limitations, while biochemical limitations remain negligible (i.e., contributing to less than 10% of the total limitation). When lower values of g_s are achieved (i.e., total photosynthetic limitations from 70% to 100%), the contribution of S_L declines, while MC_L still increases and B_L finally contributes significantly (20% to 50%) to the total limitation. Interestingly, once g_i and its effects on $V_{c,max}$ are taken into account, the same pattern is obtained using biochemical measurements (Flexas *et al*., 2004a), a qualitative (Medrano *et al*., 2002) or a quantitative (Grassi & Magnani, 2005) gas-exchange/chlorophyll fluorescence analysis. Therefore, the previously highlighted differences in the response pattern depending on the methodology used (Lawlor & Cornic, 2002) seem mostly due to the fact that g_i was traditionally neglected in gas exchange studies (Ethier & Livingston, 2004; Warren *et al*., 2006).

By contrast, when photosynthetic recovery from severe drought was analysed after re-watering, a different pattern was observed, consisting in a dominant role of MC_L irrespective of the degree of photosynthesis recovery, which differed by ca 60% among species. Biochemical limitations recovered fast and to a great extent after re-watering, therefore resulting a less critical factor for recovery than usually assumed (Flexas *et al*., 2004a). To the best of our knowledge, this is the first report showing that limited

recovery of g_i is the most important factor limiting photosynthesis recovery after a severe drought, which highlights even more the role of g_i in controlling photosynthesis and stresses the needs for a better understanding of the physiological and molecular mechanisms underlying the regulation of g_i.

Chapter 8

PHOTOINHIBITION AND PHOTOPROTECTION

ECOPHYSIOLOGICAL RESPONSES TO WATER STRESS AND RECOVERY IN MEDITERRANEAN PLANTS WITH DIFFERENT GROWTH FORMS. III. PHOTOINHIBITION AND PHOTOPROTECTION

8.1. SUMMARY

In the present study, the diversity of photoprotection and photoinhibition responses to a short-term drought treatment was studied in Mediterranean plants. Ten Mediterranean species belonging to different growth forms were grown in pots and subjected to four different levels of water stress, the most severe followed by re-watering. On these plants leaf pigment composition was analysed, along with gas exchange (stomatal conductance) and chlorophyll fluorescence measurements (maximum quantum efficiency of PSII photochemistry and thermal energy dissipation). In addition, from combined gas exchange and chlorophyll fluorescence techniques, photorespiration rates were also estimated. The results show that Mediterranean plants, regardless of their growth form, are substantially resistant to drought-induced photoinhibition. However, although all the species analysed achieved photoprotection by a combination of photochemical (photorespiration) and non-photochemical (thermal dissipation) mechanisms, the mechanisms and / or pigments involved in the latter may differ among species, in a manner that is independent of the plant growth form. Similarly, the velocity of photosystem II recovery from photoinhibition also differed among species.

8.2. INTRODUCTION

Summer drought is considered the main environmental constraint for plant growth and survival in Mediterranean-type ecosystems. In these environments, natural vegetation has developed an array of adaptations to drought, resulting in a high diversity of life habits and growth forms. The resulting vegetation consists mostly of deep rooted evergreen sclerophyll trees and shrubs, which maintain green leaves during the summer drought period, semi-deciduous shrubs, which lose a part of their leaves during summer, and geophytes and winter annual herbs, which escape drought by finishing their annual cycle before summer (Ehleringer & Mooney, 1983). Low soil water availability during summer is accompanied by high temperature and excessive radiation, which imposes a multiple stress to plants (Di Castri, 1973). The combination of these stresses can lead to photoinhibition and photodamage of the photosynthetic apparatus, which may result in decreased photosynthetic capacity and, eventually, in plant death (Chaves *et al*., 2002; Peñuelas *et al*., 2004). Because of this, and taking into account the large variability of habitat microclimates in the Mediterranean, as well as the stochastic distribution of

rainfall, it is likely that Mediterranean plants may have evolved a large diversity of photoprotective mechanisms to cope with excess light, particularly during the summer drought period.

Many photoprotective mechanisms have been actually described in higher plants (Björkman & Demmig-Adams, 1994; Niyogi, 1999), including reducing light absorption through leaf or chloroplast movements, decreased chlorophyll contents, or reflective structures such as hairs; regulation of energy dissipation through photochemical (e.g. photorespiration) and non-photochemical (e.g. safety thermal dissipation of excess light, associated to the xanthophylls cycle) mechanisms; scavenging of reactive oxygen species formed due to excess light; and repair and synthesis of photodamaged components of the photosynthetic apparatus (e.g., the D1 protein). In Mediterranean plants, in particular, many of these mechanisms have been described. For instance, steep leaf angles have been described as efficient structural photoprotective features in Mediterranean perennial grasses like *Stipa tenacissima* (Valladares & Pugnaire, 1999), semi-deciduous shrubs like *Cistus albidus* and *C. monspeliensis*, and evergreen sclerophyll shrubs like *Arbutus unedo* and *Quercus coccifera* (Werner *et al*., 1999; Werner *et al*., 2001). This mechanism is more important for photoprotection in semi-deciduous than in evergreens (Werner *et al*., 1999). Some semi-deciduous shrubs, like *Phlomis fruticosa*, *Cistus salviifolius*, *C. creticus*, *Rosmarinus officinalis* and *Melissa officinalis* present another mechanism to reduce light absorption during summer, consisting of partial leaf loss in parallel to a substantial loss of chlorophyll in the remaining leaves (Kyparissis *et al*., 1995; Kyparissis *et al*., 2000; Munné-Bosch & Alegre, 2000a, b). In *P. fruticosa*, chlorophyll loss during summer is not accompanied by decreased photochemical capacity, which suggests it being a photoprotective feature (Kyparissis *et al*., 1995). In the tussock grass *Stipa tenacissima*, which inhabits more arid environments than *Phlomis*, substantial loss of chlorophyll is accompanied by a large reduction in photochemical capacity and a marked decrease in leaf water content, but leaves totally recover after autumn rainfalls. This has been interpreted as a poikilohydric-type response allowing for a greater tolerance to water shortage in the most extreme Mediterranean environments (Balaguer *et al*., 2002). Reduced light absorption through accumulation of red carotenoids in leaf surfaces has also been recently described as a particular photoprotective mechanism of the evergreen shrub *Buxus sempervirens* (Hormaetxe *et al*., 2005). Mechanisms leading to reactive oxygen scavenging and antioxidant protection have also been described in

Mediterranean plants, particularly in evergreen and semi-deciduous shrubs. These mechanisms include carotenoids (Munné-Bosch & Alegre, 2000b; Munné-Bosch & Peñuelas, 2003), isoprene (Affek & Yakir, 2002), tocopherols (Munné-Bosch *et al*., 1999; Munné-Bosch & Peñuelas 2004), diterpenes (Munné-Bosch *et al*., 1999; Munné-Bosch *et al*., 2001) and enzymatic antioxidants (Kyparissis *et al*., 1995; Faria *et al*., 1996; Alonso *et al*., 2001).

Besides these mechanisms, thermal energy dissipation in the pigment bed, associated to the so-called xanthophyll cycle, is usually regarded as the most important photoprotection mechanism in higher plants (Demmig *et al*., 1987; Björkman & Demmig-Adams, 1994; Niyogi, 1999), and the xanthophyll accumulation has been shown to be directly related to light tolerance in British plant species (Johnson *et al*., 1993). The operation of the xanthophylls cycle has been confirmed in many Mediterranean plants. The first evidences that drought stress increased de-epoxidation of the xanthophyll cycle were indeed described in the Mediterranean evergreen sclerophylls *Nerium oleander* (Demmig *et al*., 1988) and *Arbutus unedo* (Demmig-Adams *et al*., 1989). Since then, substantial evidence has been accumulated for increased de-epoxidation of the xanthophyll cycle during summer in Mediterranean evergreens (Faria *et al*., 1996; 1998; García-Plazaola *et al*., 1997; Gulías *et al*., 2002; Peñuelas *et al*., 2004), semi-deciduous (Munné-Bosch & Alegre, 2000a, b, c; Munné-Bosch *et al*., 2003) and perennial herbs (Balaguer *et al*., 2002). However, a decline in the total xanthophyll pool during summer is also usually observed (García-Plazaola *et al*., 1997; Faria *et al*., 1998), although not in all the species (Munné-Bosch & Alegre, 2000a; Munné-Bosch *et al*., 2003). In *Quercus* sp. and other Mediterranean species, a lutein epoxy cycle has been described, and a similar function to that of the xanthophyll cycle proposed (García-Plazaola *et al*., 2002, 2003, 2004; Llorens *et al*., 2002).

The xanthophyll cycle is usually assumed to photoprotect plants through its involvement in thermal energy dissipation in the pigment bed, but xanthophylls also act as membrane stabilizers leading to reduced oxidative stress (Havaux, 1998; Havaux *et al*., 2000). Thermal energy dissipation can be routinely assessed with modulated fluorimeters as non-photochemical quenching of chlorophyll fluorescence (qN or NPQ) or as a decrease in PSII intrinsic efficiency (F_v'/F_m'). In crops (Brestic *et al*., 1995; Saccardy *et al*., 1998; Medrano *et al*., 2002) and plants from temperate regions (Demmig-Adams & Adams, 1996; Demmig-Adams, 1998), good correlations are generally found between $1-F_v'/F_m'$ or NPQ and the de-epoxidation state (DPS) of the

xanthophylls cycle during drought, suggesting that xanthophylls are mainly involved in thermal dissipation under these conditions. However, when looking at the literature in Mediterranean vegetation, such a relationship is often unclear. For instance, good correlations between DPS and midday NPQ or F_v'/F_m' have been described in *Lavandula stoechas* (Munné-Bosch & Alegre, 2000a), *Melissa officinalis* (Munné-Bosch & Alegre, 2000c), *Pinus halepensis* (Martínez-Ferri *et al*., 2000) and *Olea europaea* (Faria *et al*., 1998). By contrast, no correlation between the two parameters was found in *Quercus ilex* (Faria *et al*., 1998; Martínez-Ferri *et al*., 2000), *Quercus coccifera* (Martínez-Ferri *et al*., 2000) or *Stipa tenacissima* (Balaguer *et al*., 2002). In *Quercus suber*, good correlations were found in some studies (García-Plazaola *et al*., 1997) but not in others (Faria *et al*., 1998). In a study with six species, Gulías *et al*. (2002) suggested a good correlation for the most desiccation-avoidant species (*Quercus* sp. and *Pistacia* sp.) and no correlation for desiccation-tolerant species (*Rhamnus* sp.). Similarly, good correlations have been described between pre-dawn DPS and F_v/F_m in some species, but not in others (Demmig *et al*., 1988; Martínez-Ferri *et al*., 2000; Gulías *et al*., 2002). Finally, the relationship between the extent of operation of the xanthophyll cycle and the avoidance of photoinhibition is also unclear. For instance, with similar NPQ and DPS, *Olea europea* was more photoinhibited than *Quercus* sp. (Faria *et al*., 1998). Similarly, *Rhamnus alaternus*, the species showing the highest DPS, also showed the largest reductions in F_v/F_m in a comparison of six species (Gulías *et al*., 2002).

In summary, it can be concluded that, although there is large evidence of the involvement of the xanthophyll cycle in photoprotection in Mediterranean plants, its particular relationship with thermal dissipation and photoinhibition remains unclear. Moreover, most of the studies have focused on evergreen shrubs and trees and semi-deciduous shrubs, while much less information is available for semi-shrubs or perennial herbs. On the other hand, most of these studies have analysed the variation of photoprotective mechanisms during the season. The short term response (i.e., days to weeks), which may be also relevant due to the abundance of episodic water stress periods in Mediterranean areas, has been less evaluated, particularly in relation to recovery after re-watering. In the present study, we assessed the relationship between the xanthophyll cycle and thermal dissipation (measured as non-photochemical quenching of chlorophyll fluorescence) and photoinhibition during short term water stress and recovery in Mediterranean plants with different and growth forms. The objectives were: (i) to ascertain whether the correlation between NPQ and DPS is a

particular feature of one or several functional plant groups; (ii) to study how photoprotection relates to photoinhibition in these plants; and (iii) to investigate the variability in the recovery of photoprotection and photoinhibition after re-watering.

8.3. MATERIAL AND METHODS

8.3.1. Plant material

Selected species and treatments as in Chapter 6.

8.3.2. Gas exchange measurements

Net CO_2 assimilation rate (A_N) and stomatal conductance (g_s) were measured at mid-morning on one attached and fully developed young leaf of four plants per species and treatment as explained in Chapter 7.

For dark respiration measurements, four leaf samples per treatment and species were collected during the light period and stored 20 min in the dark in 0.2 mM $CaCl_2$ for membrane stabilization. O_2 uptake rates were measured in the dark, using a liquid-phase oxygen electrode (Hansatech Instruments Ltd., England) in ambient air-equilibrated 10 mM Mes buffer (pH 5.7), as previously described (Delieu & Walker, 1981; Azcón-Bieto *et al*., 1994). Leaf 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, except at low O_2 concentrations. To avoid oxygen-limiting conditions inside the cuvette, all measurements were determined with O_2 concentration above 60% of saturation. Respiration measurements were performed with the oxygen electrode technique to avoid the gasket-related leak with the CO_2 gas exchange measurements (Long & Bernacchi, 2003; Hurry *et al*., 2005). It is well known that the precision of the oxygen electrode techniques for respiration measurements are much higher than techniques based on CO_2 gas-exchange measurements (Hurry *et al*., 2005).

8.3.3. Chlorophyll fluorescence measurements

Chlorophyll fluorescence parameters were measured on attached leaves using a portable pulse amplitude modulation fluorometer (PAM-2000, Walz, Effeltrich, Germany). For each sampling time, treatment and species, four measurements were made on different plants.

A measuring light of about 0.5 μmol photon m^{-2} s^{-1} was set at a frequency of 600 Hz to determine, at pre-dawn, the background fluorescence signal (F_o), the maximum fluorescence (F_m), and the maximum quantum efficiency of PSII ($F_v/F_m = (F_m-F_o)/F_m$). At mid-morning the same leaves analysed at pre-dawn were measured with a photon flux density around 1500 μmol m^{-2} s^{-1}, obtained using the halogen lamp of the PAM-2000, measuring steady state fluorescence signal (F_s). To obtain the steady-state maximum fluorescence yield (F_m'), saturation pulses of about 10000 μmol photon m^{-2} s^{-1} and 0.8 s duration were applied. The Stern-Volmer non-photochemical quenching (NPQ) at mid-morning was calculated using the expression $NPQ = (F_m - F_m')/F_m'$. The electron transport rate (ETR) was calculated as in Chapter 7.

8.3.4. Photorespiration estimations

From combined gas-exchange and chlorophyll fluorescence measurements, the photorespiration rate (P_r) was calculated according to Valentini *et al*. (1995). This model works in the assumption that all the reducing power generated by the electron transport chain is used for photosynthesis and photorespiration, and that chlorophyll fluorescence gives a reliable estimate of the quantum yield of electron transport. Thus, P_r can be solved from data of A_N, R_L and ETR, and from the known stochiometries of electron use in photorespiration, as follows (Valentini *et al*., 1995): $P_r = 1/12$ [ETR - 4 ($A_N + R_L$)]. Rates of respiration in the light (R_L) were obtained from A_N-C_i curves on the same treatment, using the values calculated in Chapter 7.

8.3.5. Pigment analyses

Immediately after chlorophyll fluorescence measurements (at predawn and midday), discs were punched from leaves of the same plants showing the same orientation as those used for fluorescence measurements and submersed into liquid nitrogen. Four samples per treatment and species were taken from different plants (four leaves per sample). Pigments were extracted by grinding leaf tissue in a mortar with acetone in the presence of sodium ascorbate. Pigments were identified and quantified by high performance liquid chromatography according to Abadía & Abadía (1993), with modifications as described in Larbi *et al*. (2004).

8.3.6. Statistical analysis

Regressions coefficients were calculated with the 8.0 Sigma Plot software package (SPSS). Differences between means were revealed by Duncan analyses ($P < 0.05$) performed with the SPSS 12.0 software package (SPSS, Chicago, USA).

8.4. RESULTS AND DISCUSSION

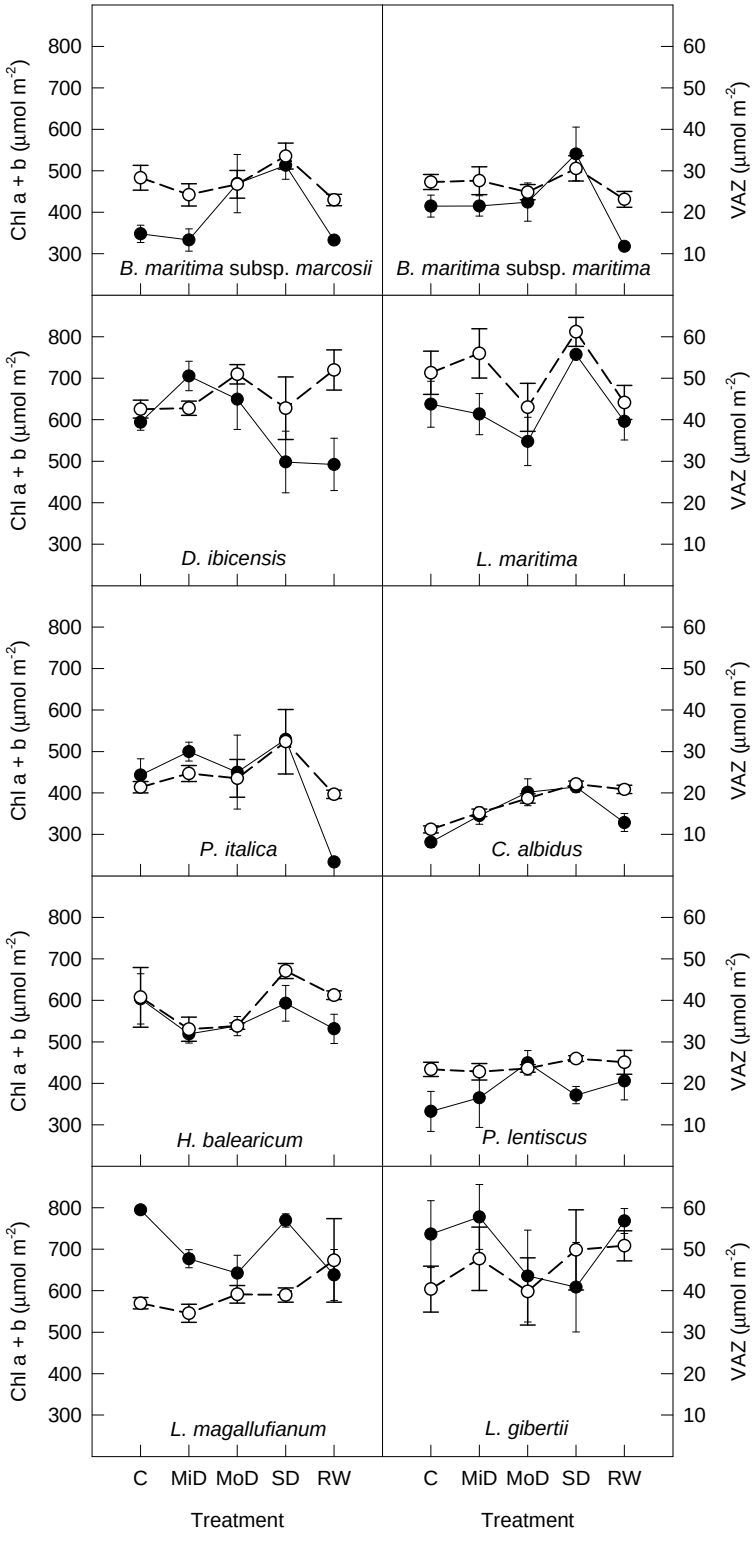
8.4.1. Pigment composition under drought and recovery

Seasonal changes in the amount of leaf pigments, particularly chlorophylls and xanthophylls, have been described in Mediterranean plants (Kyparissis *et al*., 1995; 2000; García-Plazaola *et al*., 1997; Faria *et al*., 1998; Munné-Bosch & Alegre 2000a, b; Munné-Bosch *et al*., 2003). However, the response to short-term drought treatments, as those applied in the present study, has received less attention (Munné-Bosch & Peñuelas, 2004; Peñuelas *et al*., 2004).

Decreased chlorophyll content in summer leaves has been described in some Mediterranean species as a regulatory mechanism to decrease leaf absorptance, which confers photoprotection under drought (Kyparissis *et al*., 1995, 2000; Balaguer *et al*., 2002). In the present study, leaf chlorophyll content (Chl) was generally unaffected by drought, with a few exceptions (Fig.8.1). In some species (the two *Beta*, *L. maritima* and *L. magallufianum*) Chl was increased while in other species (*D. ibicensis* and *L. gibertii*) it was decreased at moderate to severe drought. None of these changes were of great magnitude, which suggests that adjusting chlorophyll content may not be a major photoprotective response to short-term drought. In contrast, Chl decreases in many species after re-watering were considerable (Fig. 8.1). This may reflect a process of degradation and repair of drought-damaged or drought-modified components of PSII (see next section).

Figure 8.1

Total chlorophyll (filled symbols) and the sum of violaxanthin, anteraxanthin and zeaxanthin (empty symbols) concentrations, expressed in μmol m^{-2}, at midday under different irrigation treatments: control (C), mild drought (MiD), moderate drought (MoD), severe drought (SD) and re-watering (RW). Values are means ± standard error of four replicates per species and treatment.

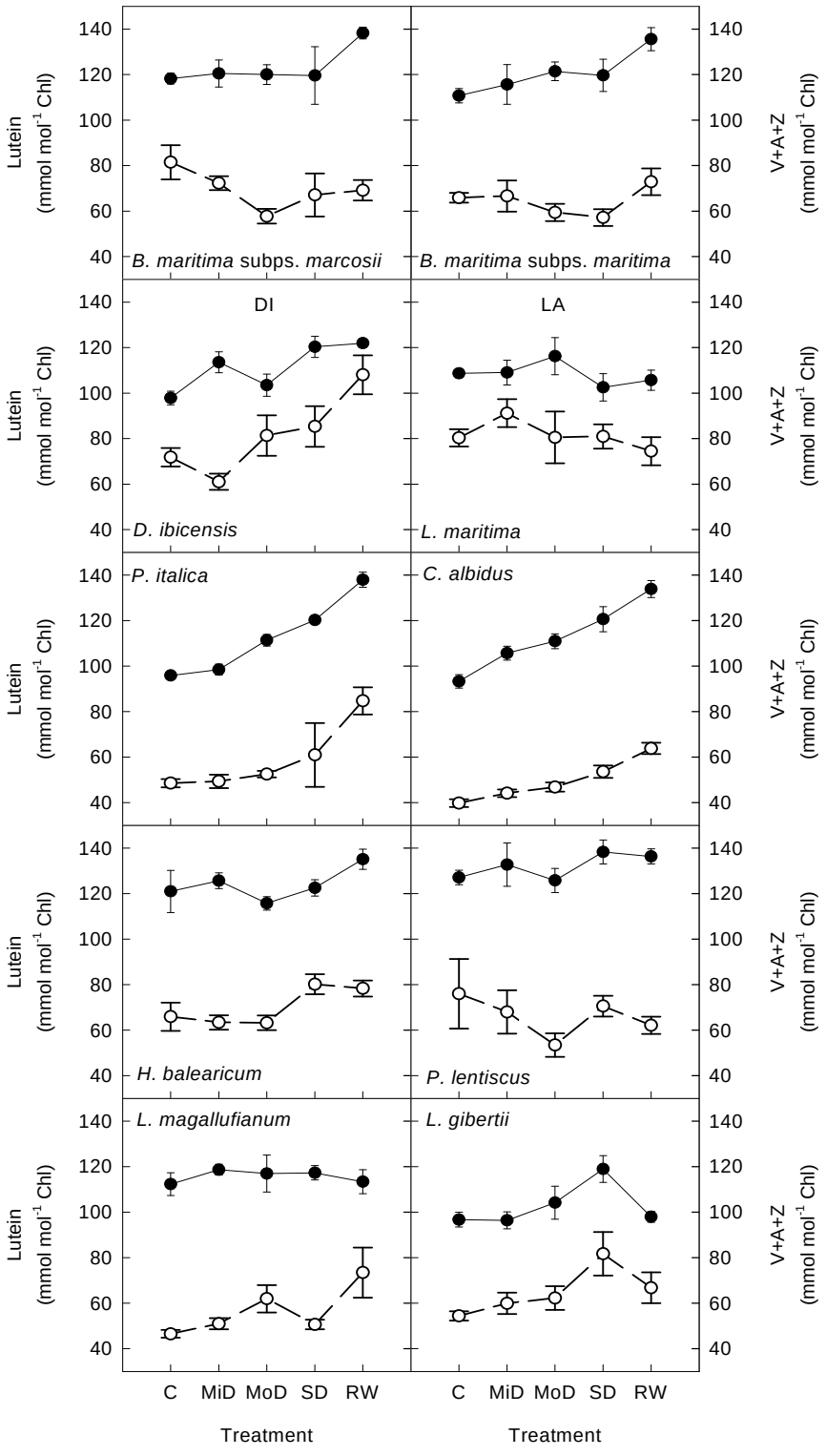


The total amount of the xanthophylls pool (VAZ) per unit leaf area has been shown to either increase (García-Plazaola *et al*., 1997), be kept constant (Kyparissis *et al*., 1995; Munné-Bosch *et al*., 2003) or decrease (Balaguer *et al*., 2002; Munné-Bosch & Peñuelas, 2003) in different Mediterranean species from spring to late summer. In the present study, VAZ per unit leaf area was not significantly affected by drought in any of the species analyzed (Fig. 8.1), except in *C. albidus*, in which it increased significantly ($P < 0.05$). Therefore, as with Chl, it may be concluded that adjusting VAZ content per leaf area may also not be a major photoprotective response to short-term drought in these species.

Increasing the VAZ pool per Chl may be another mechanism to increase the photoprotection capacity in leaves, and it has been shown to generally occur during summer both in evergreen sclerophylls and in semi-deciduous shrubs (Kyparissis *et al*., 1995, 2000; Faria *et al*., 1998). In short-term drought experiments, the VAZ/Chl ratio has been shown to increase in the evergreen sclerophylls *Arbutus unedo* (Munné-Bosch & Peñuelas, 2004) and *Phillyrea angustifolia* (Munné-Bosch & Peñuelas, 2003; Peñuelas *et al*., 2004). In the present study, none of the two evergreen sclerophyll analyzed (*P. lentiscus* and *H. balearicum*) presented such an increase (Fig. 8.2), suggesting that this may not be a specific feature of evergreen sclerophyll species. Two semi-deciduous shrubs (*C. albidus* and *P. italica*) and an evergreen semi-shrub (*L. gibertii*) were the only species showing an increased VAZ/Chl ratio in response to drought in the present study (Fig. 8.2). This was not a specific increase of VAZ, but rather it was also accompanied by increases in the lutein content (Fig. 8.2). The presence of the lutein expoxy cycle (García-Plazaola *et al*., 2002, 2003, 2004; Llorens *et al*., 2002) was not analyzed in the present study, so it cannot be discussed whether it could be operating in these species. Other carotenoids such as taraxanthine or neoxanthine did not show any specific trend of response to drought (data not shown).

Figure 8.2

Lutein (filled symbols) and the sum of violaxanthin, anteraxanthin and zeaxanthin (V+A+Z, empty symbols) concentrations at midday, expressed in mmol mol^{-1} Chl, at midday under different irrigation treatments: control (C), mild drought (MiD), moderate drought (MoD), severe drought (SD) and re-watering (RW). Values are means ± standard error of four replicates per species and treatment.

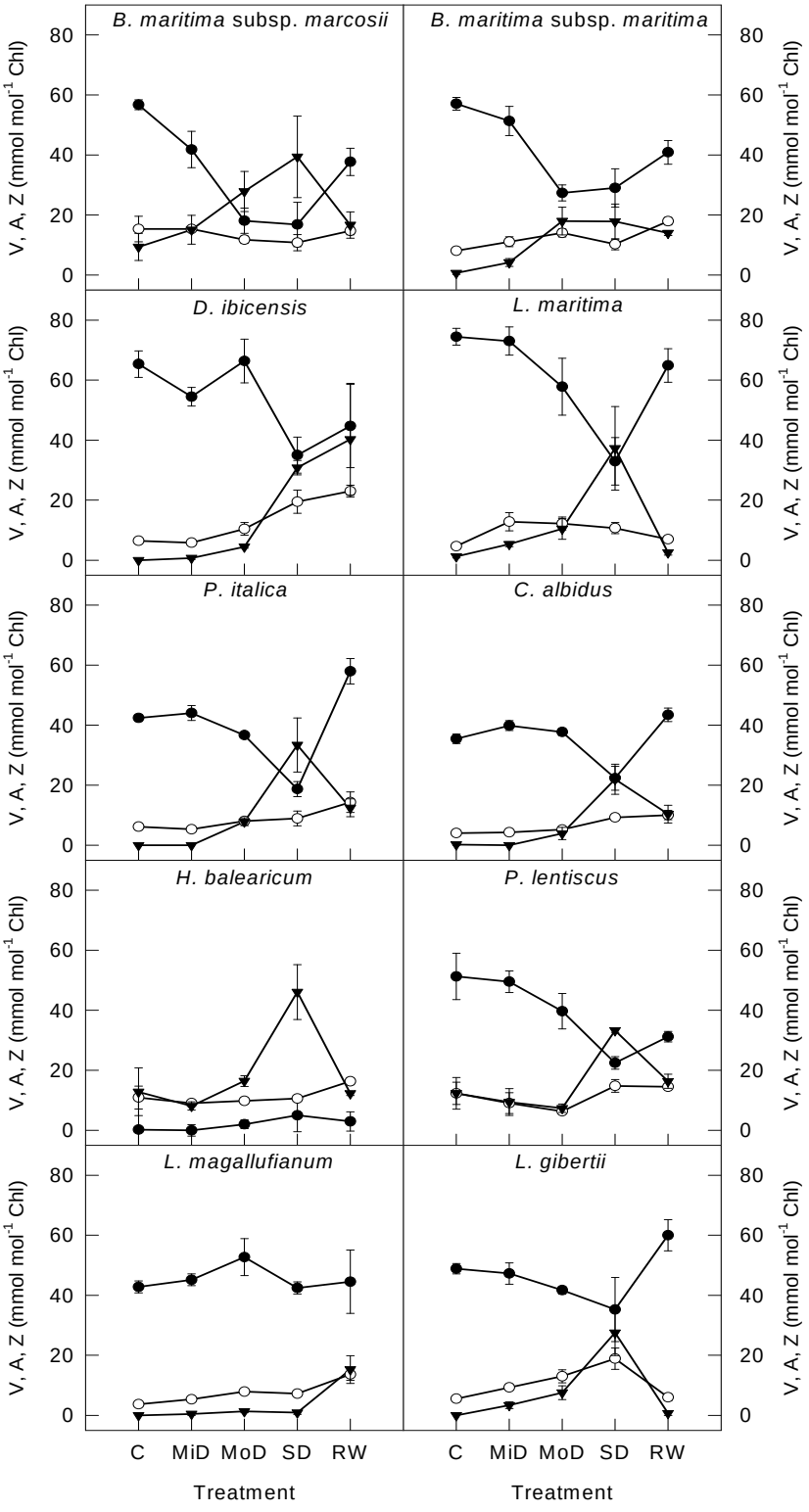


Therefore, nor Chl nor total VAZ pool adjustments seemed to be important responses to drought in the short term in the species analyzed. However, increased de-epoxidation of the xanthophylls cycle occurred in all the species (Fig. 8.3). In general, zeaxanthin increased from mild to severe drought, mostly at the expense of decreased violaxanthin, while anteraxanthin was kept at more or less constant concentration through the entire experiment (Fig. 8.3). However, the maximum de-epoxidation state reached at midday (DPS_{MD}) under severe drought strongly differed among species, ranging from only 0.10 in *L. magallufianum* to more than 0.60 in *B. maritima* subsp. *marcosii*, *P. italica* and *H. balearicum*. At pre-dawn (DPS_{PD}), the maximum de-epoxidation state under severe drought ranged between 0.15 in *P. italica* to 0.30 in *B. maritima* subsp. *maritima*. After re-watering, the extent of recovery of DPS_{MD} strongly differed among species, from none (*D. ibicensis*) to total (*L. gibertii*). In *L. magallufianum* DPS_{MD} increased from 0.10 to 0.32 after re-watering.

Therefore, the present results largely confirm that Mediterranean plants, regardless of their growth form, increase the de-epoxidation of the xanthophyll cycle in response to drought (Faria *et al*., 1996, 1998; García-Plazaola *et al*., 1997; Munné-Bosch & Alegre, 2000a, b, c; Balaguer *et al*., 2002; Gulías *et al*., 2002; Munné-Bosch *et al*., 2003; Peñuelas *et al*., 2004). However, the maximum DPS achieved under drought as well as the extent of DPS recovery differs strongly among species. The objective of the next sections is to discuss how these differences may affect photoprotection and photoinhibition in the different species.

Figure 8.3

Violaxanthin (V, filled circles), anteraxanthin (A, empty circles) and zeaxanthin (Z, filled triangles) concentrations at midday at midday under different irrigation treatments: control (C), mild drought (MiD), moderate drought (MoD), severe drought (SD) and re-watering (RW). Values are means ± standard error of four replicates per species and treatment.

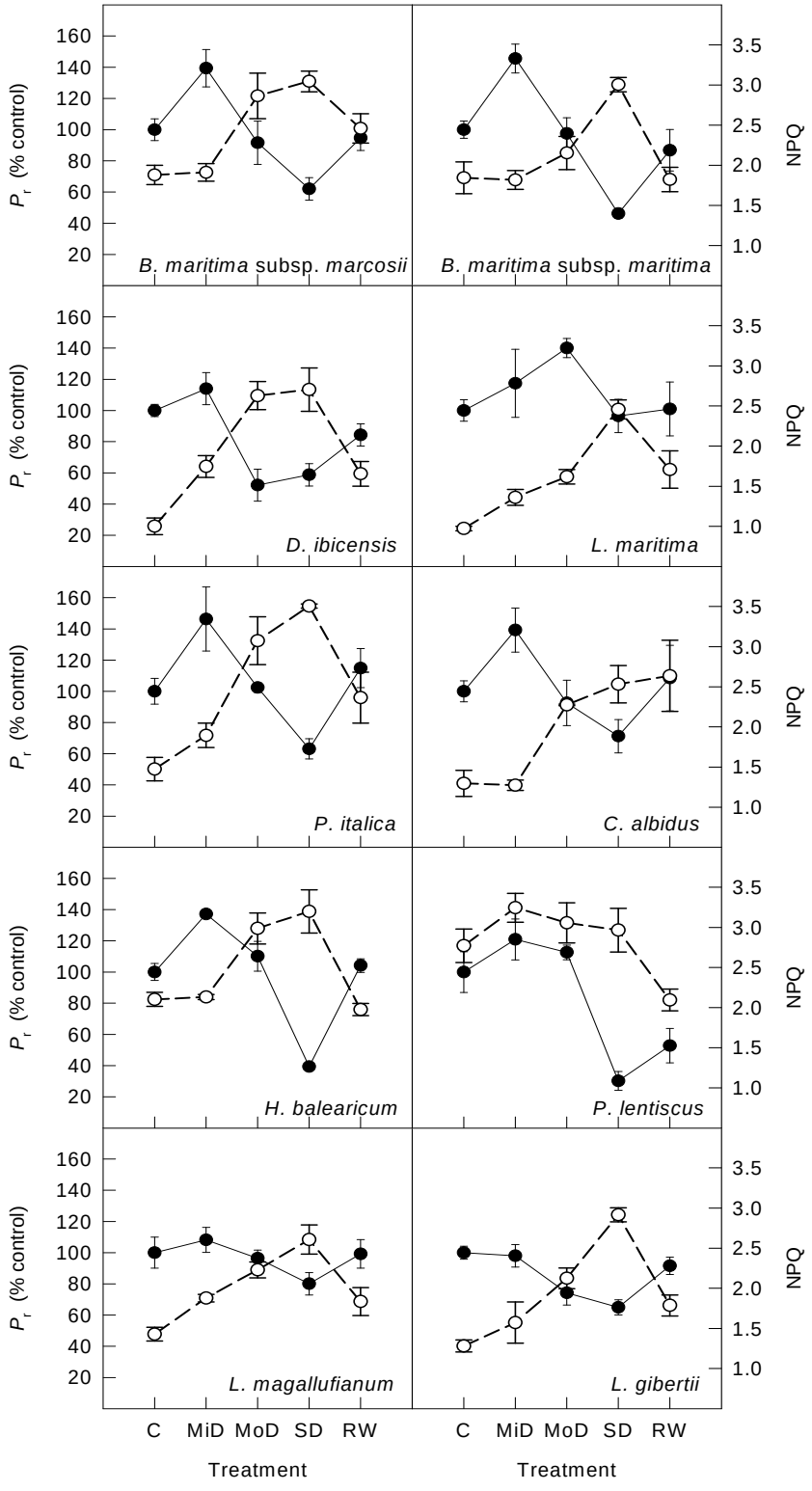


8.4.2. Photoprotection and photoinhibition under drought and recovery

When photosynthesis progressively declines with drought, photorespiration and thermal energy dissipation are regarded as the most important photoprotective mechanisms leading to dissipation of excess absorbed light (Lawlor & Fock, 1975; Powles & Osmond, 1978; Demmig *et al*., 1988; Faria *et al*., 1998; Wingler *et al*., 1999; Martínez-Ferri *et al*., 2000; Flexas & Medrano, 2002c). In the present study, as drought intensified photorespiration was kept at control values or increased, depending of the species (Fig. 8.4). Only at moderate to severe drought did photorespiration decline. In all the species analyzed, non-photochemical quenching of chlorophyll fluorescence (NPQ), an indicator of thermal energy dissipation in the pigment bed (Björkman & Demmig-Adams, 1994), increased progressively as drought intensified, particularly at moderate to severe drought (Fig. 8.4), i.e., once photorespiration started declining. The maximum NPQ values reached under stress were similar in all species (i.e. between 2.5 and 3.5). After re-watering, all the species except *Cistus albidus* showed some recovery of NPQ, but only in *Beta maritima* subsp. *marcosii*, *Hypericum balearicum* and *Pistacia lentiscus* the recovery was complete (Fig. 8.4). These results demonstrate that, as already shown for some Mediterranean evergreen sclerophylls (García-Plazaola *et al*., 1997; Faria *et al*., 1998; Martínez-Ferri *et al*., 2000; Gulías *et al*., 2002), maintaining photorespiration and increasing NPQ are common responses to water stress in other Mediterranean species, including herbs and semi-deciduous shrubs.

Figure 8.4

Photorespiration rates (P_r, expressed as % in respect to control treatment values, filled symbols) and mid-morning non-photochemical quenching (NPQ, empty symbols) under different irrigation treatments: control (C), mild drought (MiD), moderate drought (MoD), severe drought (SD) and re-watering (RW). Values are means ± standard error of four replicates per species and treatment.



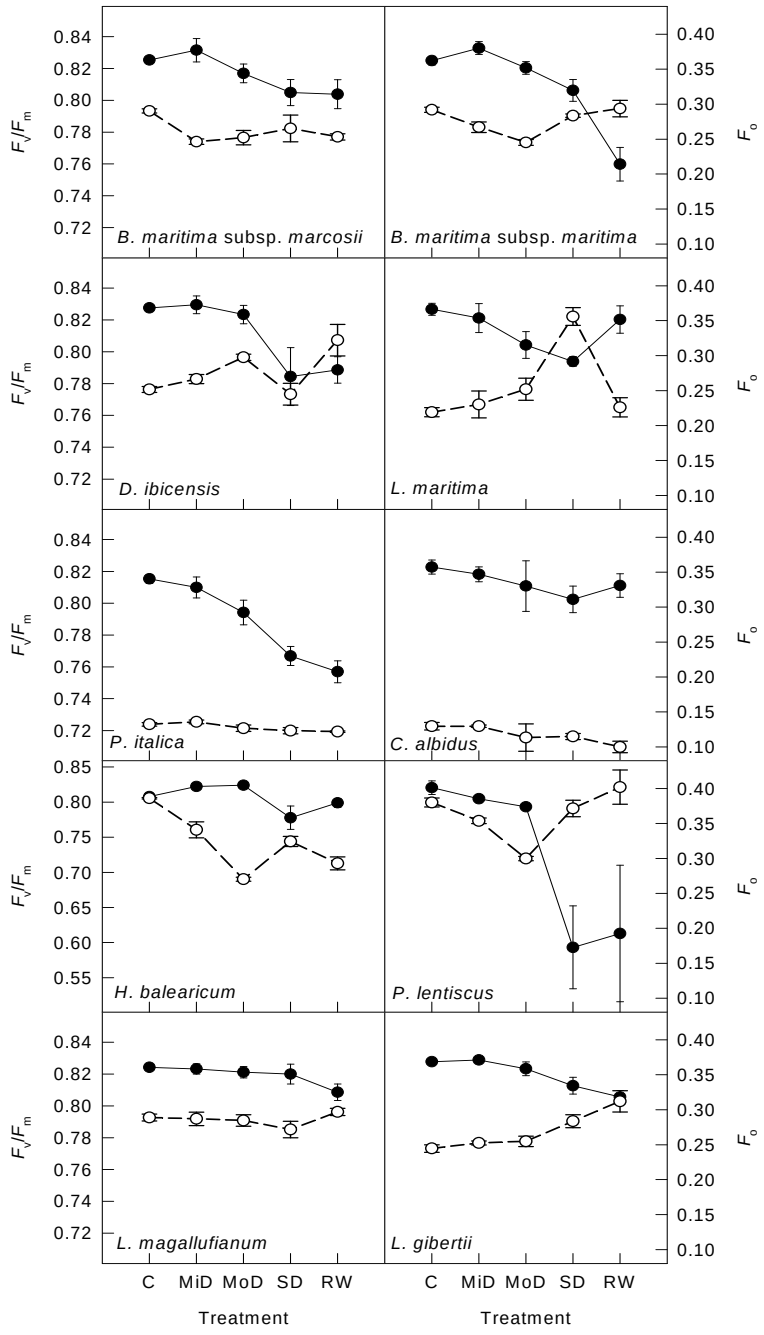
By contrast, the maximum photochemical efficiency of PSII (F_v/F_m), measured at pre-dawn, followed a pattern that differed among species (Fig. 8.5), although minimum values were generally higher than 0.75, indicating only a minor photoinhibitory effect of drought, as usually described in Mediterranean (Damesin & Rambal, 1995; Kyparissis *et al*., 1995; Méthy *et al*., 1996; Faria *et al*., 1998; Werner *et al*., 1999; Martínez-Ferri *et al*., 2000; Morales *et al*., 2002; Llorens *et al*., 2003; Ogayà & Peñuelas, 2003; Valladares *et al*., 2005) as well as in non-Mediterranean species (Flexas & Medrano, 2002c; Flexas *et al*., 2004a). Only in *P. lentiscus* F_v/F_m decreased to lower values (0.60), indicative of substantial photoinhibition. Therefore, the maximum extent of photoinhibition did not differ among growth forms. However, there was a certain effect of growth form in the pattern of F_v/F_m response to drought. Particularly, in semi-deciduous species (*L. maritima*, *P. italica* and *C. albidus*), F_v/F_m declined progressively from early stages of stress, although in *C. albidus* the decline was much more attenuated than in the other two species and resulted non-significant. This drought-induced decline in F_v/F_m was not accompanied by decreased chlorophyll content (Fig. 8.1), for which it may not be associated to the photoprotective mechanism consisting of decreasing light absorption described for *Phlomis fruticosa* or *Stipa tenacissima* (Kyparissis *et al*., 1995; Balaguer *et al*., 2002). According to Osmond (Osmond, 1994; Osmond *et al*., 1999), a decline of F_v/F_m can be indicative of either photoinactivation of PSII reaction centers or sustained photoprotection. In the first case, it may be accompanied by increased basal fluorescence (F_o), while in the second it may be accompanied by decreased F_o. Using this criterion, it becomes clear that two different mechanisms operate to decline F_v/F_m, depending on the species. In *L. maritima*, decreasing F_v/F_m was paralleled by a progressive increase of F_o (Fig. 8.5), therefore suggesting progressive photoinactivation of PSII centers since early drought stages. By contrast, F_o progressively declined in *P. italica* and *C. albidus*, suggesting that decreased F_v/F_m was reflecting sustained photoprotection in these two species.

In the other seven species, F_v/F_m declined only slightly from mild to moderate drought, and to some extent at severe drought (Fig. 8.5). The only exception was *L. magallufianum*, which maintained high values through the entire experiment. In these species, F_o progressively declined from mild to moderate drought, indicating some increase of sustained photoprotection, but then increased at severe drought in coincidence with the highest drop in F_v/F_m, which suggests that photoinactivation of PSII occurred (Fig. 8.5). Only *D. ibicensis* differed from the above patterns, showing

first an increase of F_o followed by a decrease at severe drought (Fig. 8.5). However, in this species the behavior of F_o seems to follow that of chlorophyll content (Fig. 8.1), for which it may not be indicative of either photoinactivation or photoprotection.

Figure 8.5

Maximum quantum yield of PSII measured at predawn (F_v/F_m, filled symbols) and the background fluorescence signal (F_o, empty symbols) under different irrigation treatments: control (C), mild drought (MiD), moderate drought (MoD), severe drought (SD) and re-watering (RW). Values are means ± standard error of four replicates per species and treatment.



Also a large variability was observed in the recovery of F_v/F_m after re-watering (Fig. 8.5), from total (*L. maritima*) or near total (*H. balearicum*, *C. albidus*) to almost no recovery (*D. ibicensis*, *P. lentiscus*, *B. maritima* subsp. *marcosii*). As with maximum photoinhibition, the extent of recovery was not dependent on the plant growth form. For instance, among the evergreens, recovery was null in *P. lentiscus* while almost complete in *H. balearicum*. Also, the extent of recovery did not depend on the maximum extent of photoinhibition achieved during drought. For instance, recovery was complete in *L. maritima* and null in *D. ibicensis*, while both species had achieved a similar degree of photoinhibition. In four species (*B. maritima* subsp. *maritima*, the two *Limonium* and *P. italica*) there was some decline of F_v/F_m after re-watering, which in three of them was accompanied by decreased chlorophyll but not xanthophylls content (Fig. 8.1). This has been already observed after water stress in *Vigna unguiculata* (De Souza *et al*., 2004) and after photoinhibitory experiments in different species (Flexas *et al*., unpublished), and it may be due to the fact that recovery of PSII after photoinhibition requires degradation and *de novo* synthesis of damaged components, particularly the D1 protein (Aro *et al*., 1994).

Despite the observed differences between species in photoprotection and photoinhibition response to drought, they all respond to a general pattern described for C_3 plants when stomatal conductance (g_s) is used as a reference for drought intensity (Flexas & Medrano, 2002a; Flexas *et al*., 2004a). This pattern is characterized by two phases of response to drought, a first phase corresponding to g_s declining from a maximum to about 0.15-0.20 mol H_2O m^{-2} s^{-1}, and a second phase corresponding to further decreases in g_s (Fig. 8.6). During the first phase, photorespiration acts as a major photoprotective mechanism, been kept at control values or even increased (Fig. 8.6A), while NPQ increases only slightly (Fig. 8.6B) and F_v/F_m is maintained above 0.8 (Fig. 8.6C), indicating little or no photoinhibition. During the second phase, photorespiration decreases (Fig. 8.6A) and NPQ largely increases (Fig. 8.6B), suggesting that thermal dissipation becomes the major photoprotective mechanism during this phase. In this phase, decreases of F_v/F_m eventually occur, to an extent that largely differs among species (Fig. 8.6C). The decline of F_v/F_m under severe drought may be understood as a consequence of rather than a cause for drought-induced photosynthesis decline, since electron transport rate and net photosynthesis start to decline far before F_v/F_m and achieve much larger reductions that this (see Chapter 7). Another indication that drought-induced variations in F_v/F_m did not limit photosynthesis comes from the fact

that despite F_v/F_m was further reduced after re-watering in four species, electron transport rate and net photosynthesis simultaneously recovered by about 50% in these species, as well as in those showing no F_v/F_m recovery except *P. lentiscus* (see Chapter 7). These results are consistent with the fact that leaves are doted with a much larger PSII concentration than needed for photosynthesis, so that up to 50% of PSII units can be damaged before any effect was detectable in photosynthesis (Lee *et al.*, 1999).

8.4.3. The relationship between photoprotection, photoinhibition and pigment composition

With the exception of *P. lentiscus* and, to some extent, good correlations in *B. maritima* subsp. *maritima*, significant correlations were found between NPQ and DPS_{MD}, particularly when data from the recovery treatment were excluded (Fig. 8.7). This may be viewed as an indication that thermal energy dissipation mostly depends on DPS_{MD} in all the species studied. Because all the species presented similar VAZ/Chl ratios (average between 55 and 80 mmol mol^{-1} Chl), it would be expected that a similar DPS_{MD} would be required to achieve a similar NPQ in all species. However, the slope of the relationship strongly differed among species, and in some of them there was a saturation of NPQ at a certain DPS_{MD} threshold. Remarkably, in some species the maximum NPQ (2.5-3) was achieved with a DPS_{MD} of only 0.1-0.2, suggesting that further de-epoxidation of the VAZ pool may have a function different than contributing to NPQ. In general, DPS_{MD} higher than 0.3 were achieved only when some degree of photoinactivation of PSII – as discussed in the previous section – have occurred. This happened only from moderate (*B. maritima* subsp. *marcosii* and *L. maritima*) to severe drought (*B. maritima* subsp. *maritima, D. ibicensis*, *H. balearicum, P. lentiscus* and *L. gibertii*). *P. italica* and *C. albidus* showed an increase of DPS_{MD} at severe drought despite the fact that reduced F_v/F_m was related to sustained photoprotection rather than photoinhibition in these species. The only species showing no photoinhibition at all during drought (*L. magallufianum*) did not increase DPS_{MD} above 0.1 during the stress treatment, although it has a much larger de-epoxidation capacity as revealed by the re-watering treatment.

In most species, significant negative relationships were observed between F_v/F_m and DPS_{PD} (Fig. 8.8). However, a closer inspection reveals that, with few exceptions, DPS_{PD} only differed significantly at severe drought, i.e. when, in most species,

decreased F_v/F_m was no longer reflecting sustained photoprotection but rather photoinactivation of PSII centers.

As discussed in previous sections, a large variability was found for recovery of DPS_{PD}, DPS_{MD}, F_v/F_m and NPQ. Remarkably, DPS_{MD} and NPQ were completely uncoupled during recovery in most species, as were DPS_{PD} and F_v/F_m (Figs. 8.7 and 8.8).

Figure 8.6

Relationship between stomatal conductance (g_s) and (A) photorespiration rates (P_r, expressed as % in respect to control treatment values), (B) mid-morning non-photochemical quenching (NPQ), and (C) maximum quantum yield of PSII measured at predawn (F_v/F_m). Values are means ± standard error of four replicates per species and treatment. Re-watering values were not included. Symbols and species as follows: ● *D. ibicensis*, ○ *B. maritima* subsp. *marcosii*, ■ *B. maritima* subsp. *maritima*, □ *L. magallufianum*, ▲ *L. gibertii*, Δ *P. italica*, ▼ *L. maritima*, ∇ *C. albidus*, ♦ *P. lentiscus*, ◊ *H. balearicum*.

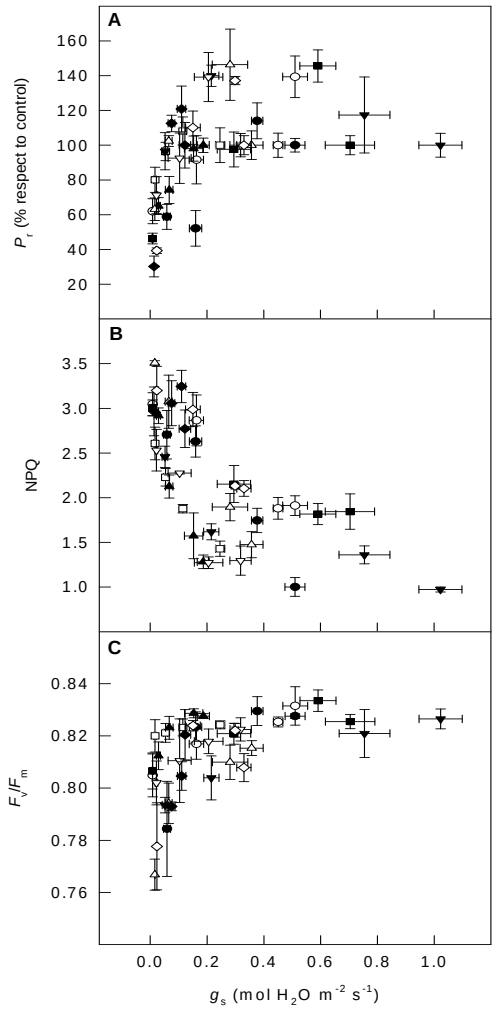


Figure 8.7

Relationship between the mid-morning non-photochemical quenching (NPQ) and the mid-morning DPS (DPS_{MD}) for the five treatments studied. Regression coefficients are shown for each one of the species. Measurements corresponding to re-watering treatment are indicated by empty symbols and were not considered for the regression adjustment. Values are means ± standard error of four replicates per species and treatment.

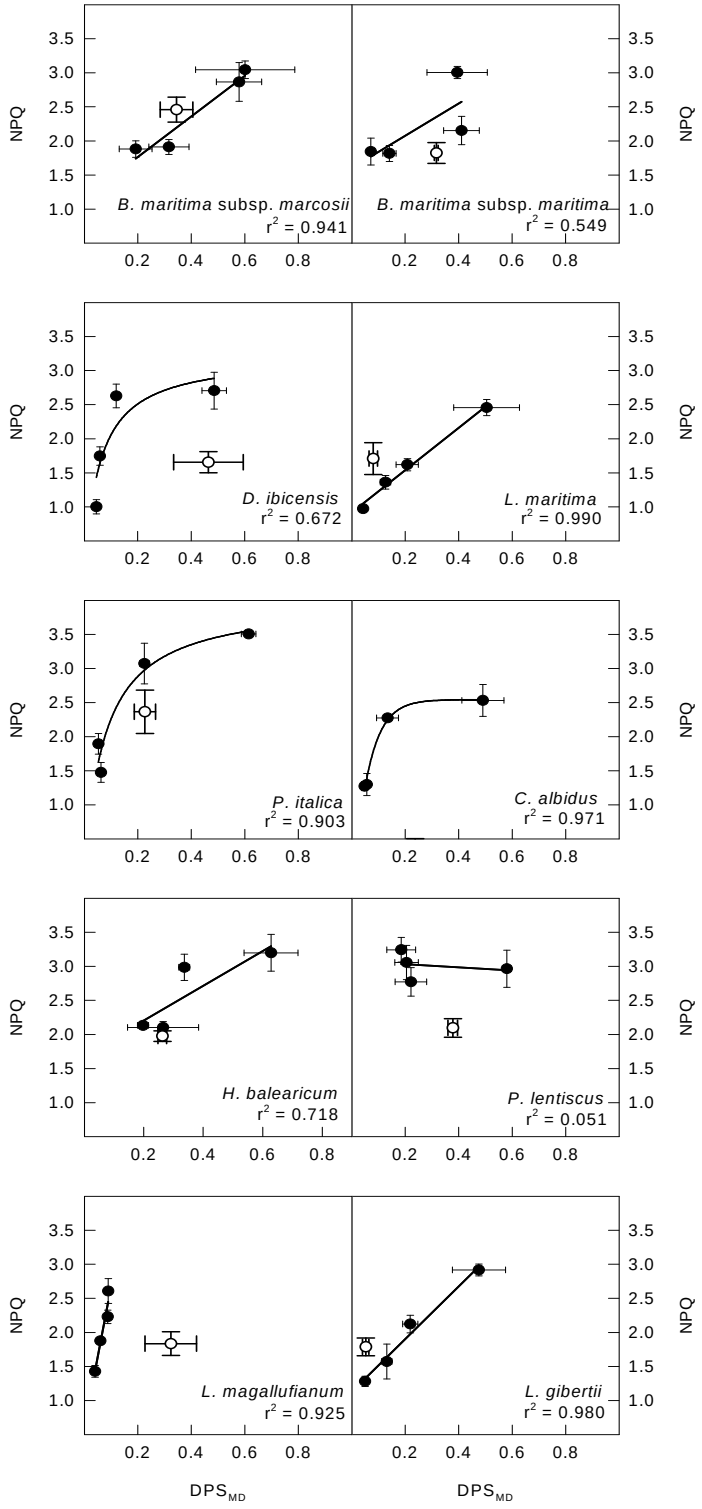
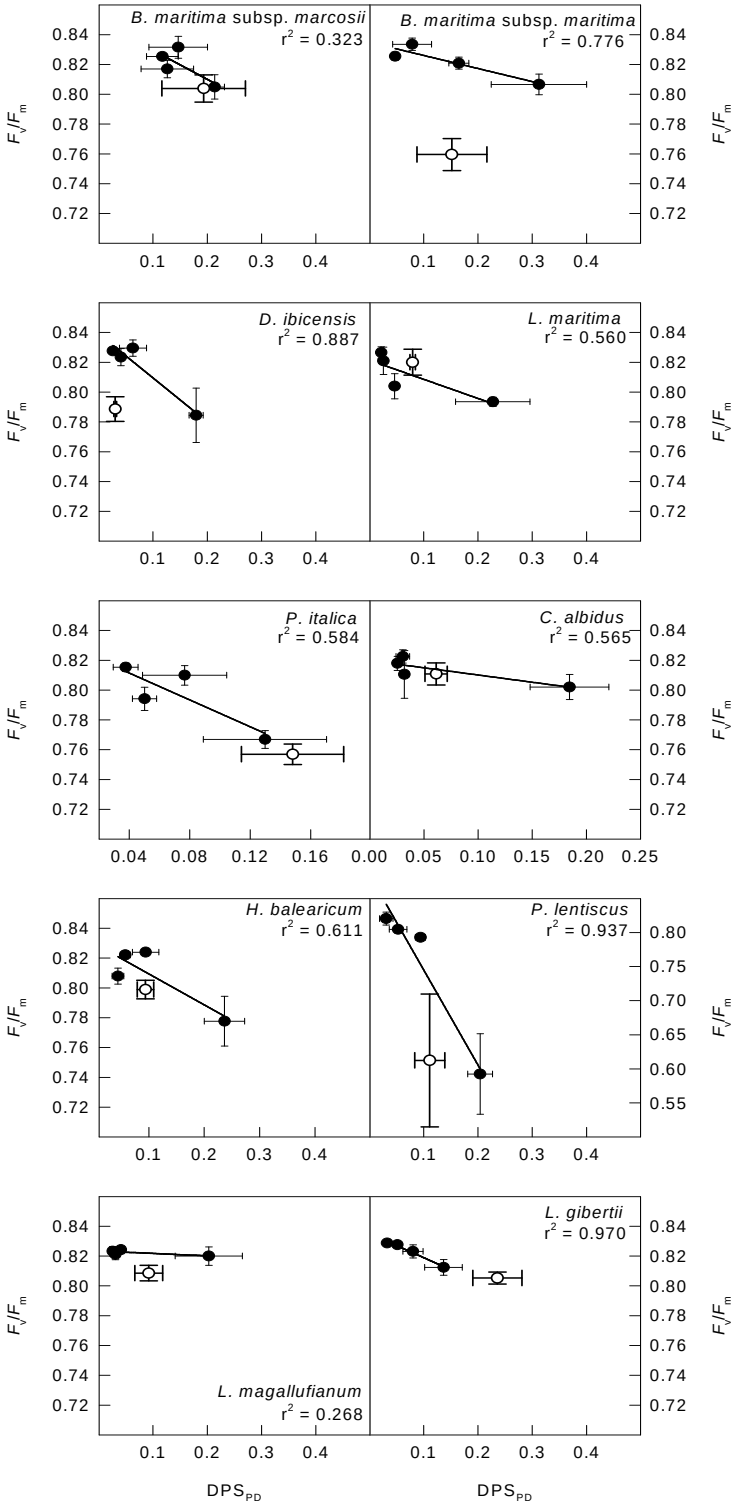


Figure 8.8

Relationship between the maximum quantum yield of PSII measured at predawn (F_v/F_m) and predawn de-epoxidation state of the xanthophylls cycle (DPS_{PD}), for the five treatments studied. Regression coefficients are show for each one of the species. Measurements corresponding to re-watering treatment are indicated by empty symbols and were not considered for the regression adjustment. Values are means ± standard error of four replicates per species and treatment.



All these observations (differences in the NPQ-DPS_{MD} slope, increased DPS_{PD} mostly when photoinactivation occurred, and recovery data outlying the general relationships), along with the fact that, in general, the species with highest DPS were also those showing lowest F_v/F_m during severe drought, strongly suggest that increased DPS during drought may be involved in processes other than contributing to thermal energy dissipation in the pigment bed. The involvement of zeaxanthin in membranes stabilization and decreased lipid peroxidation during conditions of high light stress has been demonstrated (Havaux, 1998; Havaux *et al*., 2000). We believe that this could likely be the function of increased DPS under drought and its maintenance during re-watering, since oxidative stress is likely to occur under severe drought, and increased antioxidant activities have been shown to be important in Mediterranean plants both under severe drought and re-watering (Flexas *et al*., 2006).

8.4.4. Concluding remarks

The present study shows that Mediterranean plants, regardless of their growth form, are substantially resistant to drought-induced photoinhibition. However, although all these species achieve photoprotection by a combination of photochemical (photorespiration) and non-photochemical (thermal dissipation) mechanisms, the mechanisms and/or pigments involved in the latter may differ among species, in a manner that is independent of the plant growth form. Similarly, the velocity of PSII recovery from photoinhibition also differs among species. In general, the studied species can be classified in the following groups according to their photoprotection and photoinhibition responses to drought:

1. Species showing very small drought-induced decreases of F_v/F_m, suggesting a very effective photoprotection. This group included only *Limonium magallufianum*. In this species the correlation between NPQ and DPS_{MD} was very high ($r^2 > 0.9$), and the maximum DPS_{MD} achieved during stress was the lowest among all species (0.1).

2. Species showing significant drought-induced decreases of F_v/F_m only under severe drought, suggesting a slightly less effective photoprotection. These include *P. lentiscus*, *H. balearicum*, *D. ibicensis*, *L. gibertii* and the two *Beta*. In all these species DPS_{PD} increased particularly at severe drought, but the decline in F_v/F_m was accompanied by increased F_o, suggesting photoinactivation of PSII rather than sustained photoprotection. In all except *P. lentiscus*, the relationship between NPQ and DPS_{MD}

was significant but less strong than in the previous group, although the type and slopes of the relationships differed strongly among them. In all species, DPS_{PD} increased at severe drought when photoinactivation occurred. This fact, together with the fact that little DPS_{MD} was sufficient to achieve maximum NPQ in some species, leads us to suggest that perhaps zeaxanthin, in addition to thermal dissipation, is involved in the antioxidant response of leaves once photoinhibition occurs (Havaux, 1998; Havaux *et al*., 2000).

3. Species showing a progressive drought-induced decrease of F_v/F_m. These are *L. maritima*, *P. italica* and, to some extent, *C. albidus*. However, the mechanism leading to decreased F_v/F_m differed among species. In *L. maritima* decreased F_v/F_m was paralleled by increased F_o, suggesting photoinactivation, while in *P. italica* and *C. albidus* it was paralleled by decreased F_o, suggesting increased sustained photoprotection. Remarkably, *L. maritima* was the species with the fastest recovery of F_v/F_m after re-watering.

These features may reflect adaptations to particular environments. For instance, a very effective photoprotection under drought may be of adaptive value for *Limonium*, *C. albidus* or *P. italica*, since they all inhabit sun-exposed areas, while being shallow-rooted species that retain green leaves in summer. Therefore, they may have the capacity to respond to frequent short episodes of drought in addition to the long summer drought period. An alternative adaptation for a species inhabiting similar areas, such as *L. maritima*, may be to possess a high plasticity, which includes a high capacity for rapid recovery. By contrast, the species belonging to the second group may have to endure less frequent periods of combined drought and high light intensity, the herbs because they do not retain leaves during summer and the large woody perennials because they use to live under the shade of adult plants when young, and be deep-rooted when adult.

Chapter 9

LEAF RESPIRATION AND CARBON BALANCE

ECOPHYSIOLOGICAL RESPONSES TO WATER STRESS AND RECOVERY IN MEDITERRANEAN PLANTS WITH DIFFERENT GROWTH FORMS. IV. LEAF RESPIRATION AND CARBON BALANCE

9.1. SUMMARY

The aim of the present study was to undertake a comparative analysis of the effects of water stress on leaf respiration rates and carbon balance, including a wide variety of Mediterranean species with different growth forms and evolutionary history. Plant water relations, photosynthetic and respiration rates, and specific leaf area were measured in plants grown in pots and subjected to four different levels of water stress, the most severe followed by re-watering. A high range of variation was observed in maximum leaf respiration rates, largely related to differences among plant growth forms. Leaf respiration rates decreased under drought in most but not all species. Such decline also resulted growth form-dependent, with herbs showing both the highest proportional decrease and the highest recovery after re-watering, and evergreens the lowest, reflecting differences in respiration requirements due to different life span and growth carbon costs between growth forms. The decline in respiration in response to drought occurred at a stomatal conductance threshold which is coincident with that observed to induce down-regulation of the activity of many other metabolic components, suggesting that decreased respiration is part of a systemic metabolic response, which occurs under conditions where drought severely restricts CO_2 availability inside leaves.

9.2. INTRODUCTION

Water stress is considered one of the most important factors limiting plant performance and yield worldwide (Boyer, 1982). Particularly in Mediterranean type ecosystems, summer drought is considered the main environmental constraint for plant growth and survival. Moreover, the climate in the western Mediterranean Basin tends to become warmer and drier (Piñol *et al*., 1998), as a result of global change (Annon, 2001).

The limitation to plant growth imposed by low water availability is mainly due to reductions of plant carbon balance, which is dependent on the balance between photosynthesis and respiration (Lambers *et al*., 1998). The regulation of photosynthesis by drought has been extensively studied and debated (Hsiao, 1973; Boyer, 1976; Chaves, 1991; Lawlor 1995; Cornic & Massacci 1996; Flexas & Medrano 2002a; Lawlor & Cornic 2002; Flexas *et al*., 2004a). Respiration rates are often an order of magnitude lower than photosynthesis rates. However, since photosynthesis is limited

temporally (i.e. to daytime hours) and spatially (i.e., to green biomass), while respiration occurs continuously in every plant organ, the latter may be an equally important factor controlling productivity, particularly when photosynthesis is largely depressed, such as under drought conditions (Lawlor & Cornic, 2002; Flexas *et al*., 2005). Nevertheless, and in spite of its well-recognized importance, the regulation of respiration by drought at the plant physiological level is largely unknown, partly because only a limited number of studies are available and partly because of the apparent contradictions among these studies (Flexas *et al*., 2005). Certainly, the available experimental evidences do not support a clear pattern of respiration in response to drought, different studies showing either increased, unaffected or decreased rates of respiration (Hsiao, 1973; Amthor, 1989). The lack of knowledge on plant respiration responses to drought is even greater when referring to Mediterranean species, since to the best of our knowledge there has been none attempt to make a comparative survey of the effects of water stress on respiration in Mediterranean species.

In Mediterranean environments, natural vegetation has developed an array of adaptations to drought, resulting in a high diversity of growth forms. The resulting vegetation consists mostly of deep rooted evergreen sclerophyll trees and shrubs, which maintain green leaves during the summer drought period, semi-deciduous shrubs, which lose part of their leaves during summer, and geophytes and winter annual and perennial herbs, which escape drought by finishing their annual cycle before summer (Ehleringer & Mooney, 1983). In addition to this diversity in morpho-phenological forms, we have observed in Mediterranean plants a strong diversity in ecophysiological traits that are likely of adaptive value, such as the specificity factor of Rubisco (Galmés *et al*., 2005a), and the response to water stress of relative growth rate and its components (Galmés *et al*., 2005b), leaf water relations, stomatal control, photosynthetic limitations, photoprotection and energy dissipation (see Chapters 6, 7 and 8).

Moreover, islands biotas differ from those of continents for being generally species-poor and disharmonic, yet rich in species found nowhere else (Whittaker, 1998). The present study was performed in plants from the Balearic Islands, located within the Mediterranean Basin, which represent an example of such biotas, with up to 1700 taxons described and with a richness of endemic flora of about 7-8% (Cardona & Contandriopoulos, 1979).

Several works have indented to elucidate the underlying ecophysiological causes that force endemic species to a limited distribution. Recently, in a study including 73 West Mediterranean species occurring in the Balearic Islands, in which 22 were endemics, Gulías *et al*. (2003) suggested photosynthetic capacity to be an important factor contributing to the limited distribution of the endemic species. Endemic species presented, on average, significantly lower net photosynthetic rates than non-endemics and crops, and this was particularly marked for species with high specific leaf area (SLA). Gulías *et al.* (2003) suggested that one of the possible factors for the lower photosynthetic capacity of endemics could be higher respiration rates in the endemic species. In fact, Gulías *et al*. (2002) had shown that the endemic *Rhamnus ludovici-salvatoris* presented respiration rates more than twice that of the non-endemic *Rhamnus alaternus*. In Hawaii, another insular system with a high percentage of endemicity, invasive species assimilate more CO_2 at a lower respiratory cost than native species, and this fact has been related to the competitive and invasive ability of these species (Pattison *et al*., 1998).

The aim of the present study was to undertake a comparative analysis of the effects of water stress on leaf respiration rates and their relation to photosynthetic variations, including a wide variety of Mediterranean species with different growth forms and evolutionary history. Some specific questions were addressed: (1) which are the ranges in leaf respiration rates within Mediterranean species?; (2) are leaf respiration rates affected by a decrease in water availability?; (3) if so, what determines the extent and intensity of change in the respiration processes?; (4) do these characteristics differ between species belonging to different growth forms?; and (5) are there any differences between endemic and non-endemic species of the Balearic Islands that would help explaining the regressive and limited distribution of the endemic species?

9.3. MATERIAL AND METHODS

9.3.1. Plant material

Eleven Mediterranean species naturally occurring in the Balearic Islands, six of them endemic to these islands, were selected for this study (Table 9.1). Special care was taken in the selection of the species, in order to include taxons representative of different growth forms: four evergreen sclerophyll shrubs (*Pistacia lentiscus*, *Hypericum balearicum*, *Limonium gibertii* and *L. magallufianum*), three summer semi-

deciduous shrubs (*Lavatera maritima*, *Phlomis italica* and *Cistus albidus*) and four herbs (*Diplotaxis ibicensis*, *Beta maritima* subsp. *maritima*, *Beta maritima* subsp. *marcosii* and *Lysimachia minoricensis*). Seeds of each species were collected in the field from natural populations and taken from several parent plants to obtain a representative sample of the genetic diversity in their natural populations. Seeds were germinated on filter paper moistened with deionized water in a controlled environment (germination chamber, at 18ºC in darkness). After germination and emergence of one true leaf, ten seedlings were transplanted into pots (25 L volume, 40 cm height) containing a 40:40:20 mixture of clay-calcareous soil, horticultural substrate and pearlite (granulometry A13). Plants were grown outdoors at the University of the Balearic Islands (Mallorca, Spain). The experiment was performed in five rounds, each one with one couple of species at the same time. Four weeks before starting the experiment, plants were placed in a controlled growth chamber with a 12h photoperiod (26ºC day/20ºC night) and a photon flux density at the top of the leaves of about 600 μmol m^{-2} s^{-1}.

Plants were daily fertitirrigated with 100% Hoagland's solution. Measurements corresponding to control treatments were made during the first day of the experiment, when all the plants were well watered. Thereafter, irrigation was stopped in five plants for each species. Pots were weighted every day to determine the amount of water loss and consequently available for plants respect to control. To obtain different degrees of drought, measurements were made on days 4, 8 and 13-17 after the last irrigation, when plants were subjected to mild, moderate and severe drought intensities. Each drought experiment was stopped when g_s was close to zero, 13-17 days after water withholding, depending on species. Once achieved such g_s values, pots were again irrigated at field capacity, and considered for the re-watering treatment measurement on the next day. Control plants were watered daily during all the experiment and eventually measured to ensure that they maintained constant values of each parameter during the experiment.

Table 9.1.

List of species considered for study with their family and a brief description. The number of plants used was 10 per species, and the age differed because of the different phenology of the species selected. Plants of *P. lentiscus*, *H. balearicum*, *C. albidus*, *P. italica* and *L. maritima* were three years old, plants of *L. minoricensis*, *L. magallufianum* and *L. gibertii* were a year and half old and plants of *D. ibicensis*, *B. maritima* subsp. *marcosii* and *B. maritima* subsp. *maritima* were six months old at the onset of the experiments.

Species	Family	Description
Diplotaxis ibicensis Pau	Brassicaceae	Annual herb, endemic of the Balearic Islands and inhabiting a few coastal locations.
Beta maritima L. subsp. *marcosii* A. Juan & M. B. Crespo	Chenopodiaceae	Perennial herb. Endemic of the Balearic Islands, inhabiting a few small islets subjected to strong saline spray.
Beta maritima L. subsp. *maritima*	Chenopodiaceae	Perennial herb inhabiting coastal ecosystems. Widespread in Mediterranean and temperate climates.
Lysimachia minoricensis J. J. Rodr.	Primulaceae	Biannual herb endemic to the island of Menorca, considered to be now extinct in the wild, although some specimens are still conserved in botanical and private gardens. Its natural habitat was close to water streams.
Lavatera maritima Gouan	Malvaceae	Semi-deciduous shrub up to 2 m, densely covered by hairs. Inhabits in coastal locations.
Phlomis italica L.	Labiatae	Semi-deciduous shrub up to 1 m, densely covered by hairs. Endemic of the Balearic Islands. The biggest populations are found 500 m above the sea level, where they co-exist with *Cistus albidus*.
Cistus albidus L.	Cistaceae	Semi-deciduous shrub up to 1 m. Commonly found in the Mediterranean garrigue. Its leaves are densely covered by hairs.
Hypericum balearicum L.	Guttiferae	Woody evergreen shrub up to 2 m, endemic of the Balearic Islands. The biggest populations are found in the garrigue 500 m above the sea level, where competes with PL.
Pistacia lentiscus L.	Anacardiaceae	Woody evergreen shrub up to 5 m, commonly found in the Mediterranean garrigue.
Limonium magallufianum L. Llorens	Plumbaginaceae	Woody evergreen shrub, in cushion-like rosettes. Endemic of the Balearic Islands, inhabiting just in one coastal marsh located in Magalluf, Mallorca.
Limonium gibertii (Sennen) Sennen	Plumbaginaceae	Woody evergreen shrub, in cushion-like rosettes. Occurring in West Mediterranean rocky and sandy coastal areas.

9.3.2. Plant water status

The relative water content at pre-dawn (RWC_{PD}) and midday (RWC_{MD}) were determined as follows: RWC = (Fresh weight - Dry weight) / (Turgid weight – Dry weight) × 100. To determine the turgid weight of the samples, these were kept in

distilled water in darkness at 4°C to minimise respiration losses, until they reached a constant weight (full turgor, typically after 12 h). Their dry weight was obtained after 48 h at 70ºC in an oven. Four replicates per species and treatment were obtained.

9.3.3. Specific leaf area

Specific leaf area (SLA) was calculated in four leaves per species under the five drought treatment, as the ratio of leaf area to leaf dry mass. First, the leaf area was determined with an AM-100 Area Meter (Analytical Development Company, Herts, UK). Then, the dry mass of these leaves was determined after oven drying for 48 h at 60ºC.

9.3.4. Gas exchange measurements

Instantaneous determinations of net CO_2 assimilation rate (A_N) and stomatal conductance (g_s) at saturating light (1500 μmol photon m^{-2} s^{-1}), 25ºC and 400 μmol mol^{-1} CO_2 were performed at mid-morning, using a Li-6400 (Li-Cor Inc., Nebraska, USA) in one leaf of four different plants per treatment and species. Relative humidity was kept at 50 ± 5% during measurements. The IRGA was calibrated every day, according to manufacturer's recommendations.

For dark respiration measurements, four leaf samples per treatment and species were collected during the light period and stored 20 min in the dark in 0.2 mM $CaCl_2$ for membrane stabilization. O_2 uptake rates were measured in the dark, using a liquid-phase oxygen electrode (Hansatech Instruments Ltd., England) in ambient air-equilibrated 10 mM Mes buffer (pH 5.7), as previously described (Delieu & Walker, 1981; Azcón-Bieto *et al*., 1994). Leaf 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, except at low O_2 concentrations. To avoid oxygen-limiting conditions inside the cuvette, all measurements were determined with O_2 concentration above 60% of saturation. Respiration measurements were performed with the oxygen electrode technique to avoid the gasket-related leak with the CO_2 gas exchange measurements (Long & Bernacchi, 2003; Hurry *et al*., 2005). It is well known that the precision of the oxygen electrode techniques for respiration measurements are much higher than techniques based on CO_2 gas-exchange measurements (Hurry *et al*., 2005).

9.3.5. Statistical analysis

Regressions coefficients were calculated with the 8.0 Sigma Plot software package (SPSS). Differences between means were revealed by Duncan analyses ($P < 0.05$) performed with the SPSS 12.0 software package (SPSS, Chicago, USA).

9.4. RESULTS AND DISCUSSION

9.4.1. Range of variation of respiration rates among Mediterranean species: influence of growth form and evolutionary history

Under well-watered conditions, dark respiration rates largely differed among species and varied in an 8-fold range. The lowest R_{Dm} values were found for *P. lentiscus*, with 12.6 μmol O_2 kg^{-1} s^{-1}, while the highest were found for *D. ibicensis*, with 100.6 μmol O_2 kg^{-1} s^{-1} (Table 9.2). Averaged per growth forms, herbaceous species presented the highest respiration rates (90.6 ± 6.4 μmol O_2 kg^{-1} s^{-1}), which resulted significantly different ($P < 0.05$) from those of evergreen shrubs (31.2 ± 6.8 μmol O_2 kg^{-1} s^{-1}). Semi-deciduous shrubs showed somewhat intermediate values, with 39.7 ± 12.6 μmol O_2 kg^{-1} s^{-1}), but they were not significantly different ($P > 0.05$) from evergreen shrubs (Table 9.3).

Table 9.2.

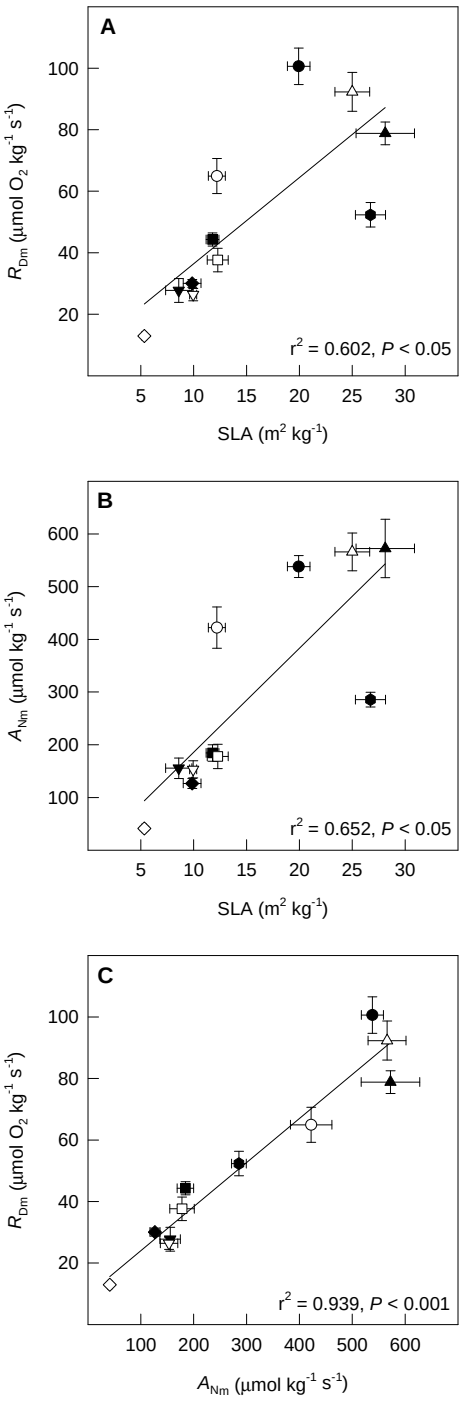
Specific leaf area (SLA), dark respiration rates (R_{Dm}), net CO_2 assimilation rates (A_{Nm}), and the ratio of net CO_2 assimilation to dark respiration rates (A_{Nm}/R_{Dm}) for the eleven selected species. Values are means ± standard error of four replicates per species and treatment.

Species	SLA (m^2 kg^{-1})	R_{Dm} (μmol O_2 kg^{-1} s^{-1})	A_{Nm} (μmol CO_2 kg^{-1} s^{-1})	A_{Nm}/R_{Dm} (μmol CO_2 μmol^{-1} O_2)
B. maritima subps.*marcosii*	28.1 ± 2.8	78.8 ± 3.7	572.4 ± 55.3	7.3 ± 0.7
B. maritima subps. *maritima*	25.0 ± 1.6	92.4 ± 6.3	565.9 ± 36.0	6.1 ± 0.3
D. ibicensis	19.9 ± 1.1	100.6 ± 5.9	538.1 ± 20.8	5.3 ± 0.4
L. minoricensis	26.7 ± 1.4	52.3 ± 4.0	285.6 ± 14.0	5.5 ± 0.4
L. maritima	12.2 ± 0.8	64.9 ± 5.7	422.3 ± 39.2	6.5 ± 0.3
P. italica	8.6 ± 1.2	27.7 ± 3.9	155.5 ± 19.4	5.6 ± 0.8
C. albidus	10.0 ± 0.3	26.4 ± 2.0	153.4 ± 16.3	5.8 ± 0.6
H. balearicum	9.9 ± 0.8	30.0 ± 1.3	126.7 ± 9.6	4.2 ± 0.5
P. lentiscus	5.3 ± 0.2	12.9 ± 0.4	41.2 ± 4.2	3.2 ± 0.2
L. magallufianum	11.8 ± 0.6	44.3 ± 2.1	184.4 ± 15.7	4.2 ± 0.3
L. gibertii	12.3 ± 1.0	37.6 ± 3.8	177.9 ± 23.0	4.7 ± 0.2

These variations were closely and positively related to leaf structure (approached by specific leaf area, SLA) and photosynthesis per mass (A_{Nm}, Fig. 9.1). Hence, evergreen shrubs had the lowest SLA, A_{Nm} and R_{Dm}, while herbaceous species had the highest values for all parameters (Table 9.3). Both R_{Dm} (Fig. 9.1A) and A_{Nm} (Fig. 9.1B) scaled with SLA, which resulted in a highly significant correlation between R_{Dm} and A_{Nm} (Fig. 9.1C). Therefore, the species included in the present study followed the well-known, worldwide pattern of leaf economics spectrum (Reich *et al*., 1997; Wright *et al*., 2004). It is remarkable that these relationships did not differ between species non-endemic and endemic to the Balearic Islands (Fig. 9.1), contrary to what Gulías *et al*. (2003) showed for the relationship between A_{Nm} and SLA when pooling up to 73 species. Also, the differences in R_{Dm} between endemic and non-endemic species of the Balearic Islands were not significantly different ($P > 0.05$, 55.6 ± 11.7 and 46.8 ± 14.2 μmol O_2 kg^{-1} s^{-1}, respectively), nor were the differences in A_{Nm} and SLA. In the earlier study (Gulías *et al*., 2003), differences between endemics and non-endemics were particularly marked for species with high SLA. However, with the species considered in the present survey, non-significant differences were found in the regression line between endemics and non-endemic species. Thus, regarding to the hypothesis that endemics species with high SLA would have a lower photosynthetic capacity than non-endemics (Gulías *et al*., 2003), the present results show that it cannot be generalized to all endemic species.

Figure 9.1.

(A) Relationship between dark respiration rate on a mass basis (R_{Dm}) and specific leaf area (SLA) under control conditions. (B) Relationship between net CO_2 assimilation rate on a mass basis (A_{Nm}) and specific leaf area (SLA) under control conditions. (C) Relationship between dark respiration rate on a mass basis (R_{Dm}) and net CO_2 assimilation rate on a mass basis (A_{Nm}) under control conditions. Values are means ± standard error of four replicates per species and treatment. Symbols and species as follows: ● *D. ibicensis*, ○ *L. maritima*, ■ *L. magallufianum*, □ *L. gibertii*, ▲ *B. maritima* subsp. *marcosii*, Δ *B. maritima* subsp. *maritima*, ▼ *P. italica*, ∇ *C. albidus*, ♦ *H. balearicum*, ◊ *P. lentiscus*, ⬣ *L. minoricensis*.



The balance between photosynthesis and respiration determines the leaf (and plant, when including respiration of heterotrophic organs) carbon balance, and hence the capacity of plants to produce new biomass for growing and developing reproductive structures (Poorter *et al*., 1992). We used the ratio of net carbon assimilation to dark respiration rates (A_{Nm}/R_{Dm}), i.e. mols of CO_2 incorporated per mols of O_2 consumed, as a simple approach to leaf carbon balance. It may be considered only as a preliminary approach, since both photosynthesis and respiration may be affected by light intensity (Kowallik, 1982; Azcón-Bieto & Osmond, 1983; Kromer, 1995; Ribas-Carbó *et al*., 2000; Pinelli & Loreto, 2003), and respiration but not photosynthesis continues in the dark. Nevertheless, this simple ratio may allow a qualitative comparison between species (Pattison *et al*., 1998). The ratio A_{Nm}/R_{Dm} varied between species, the highest value (7.3 μmol CO_2 μmol^{-1} O_2) corresponding to *B. maritima* subsp. *marcosii*, and the lowest (4.2 μmol CO_2 μmol^{-1} O_2) to *P. lentiscus* (Table 9.2). Although the herbaceous species showed the highest respiration rates, they presented the lowest respiration costs with respect to the assimilation capacity, with 6.2 ± 0.6 μmol CO_2 μmol^{-1} O_2, which resulted significantly different to those of evergreen shrubs (4.1 ± 0.3 μmol CO_2 μmol^{-1} O_2). The semi-deciduous shrubs (6.0 ± 0.3 μmol CO_2 μmol^{-1} O_2) did not differ significantly from herbs (Table 9.3). It is worth noting that herbs and semi-deciduous shrubs presented a higher carbon balance than evergreens, which is consistent with the fact that their growth capacities are higher (Galmés *et al*., 2005b).

Table 9.3.

Specific leaf area (SLA), dark respiration rates (R_{Dm}), net CO_2 assimilation rates (A_{Nm}), and the ratio of net CO_2 assimilation to dark respiration rates (A_{Nm}/R_{Dm}) for the three growth forms. Values are means ± standard error of four replicates per species and treatment.

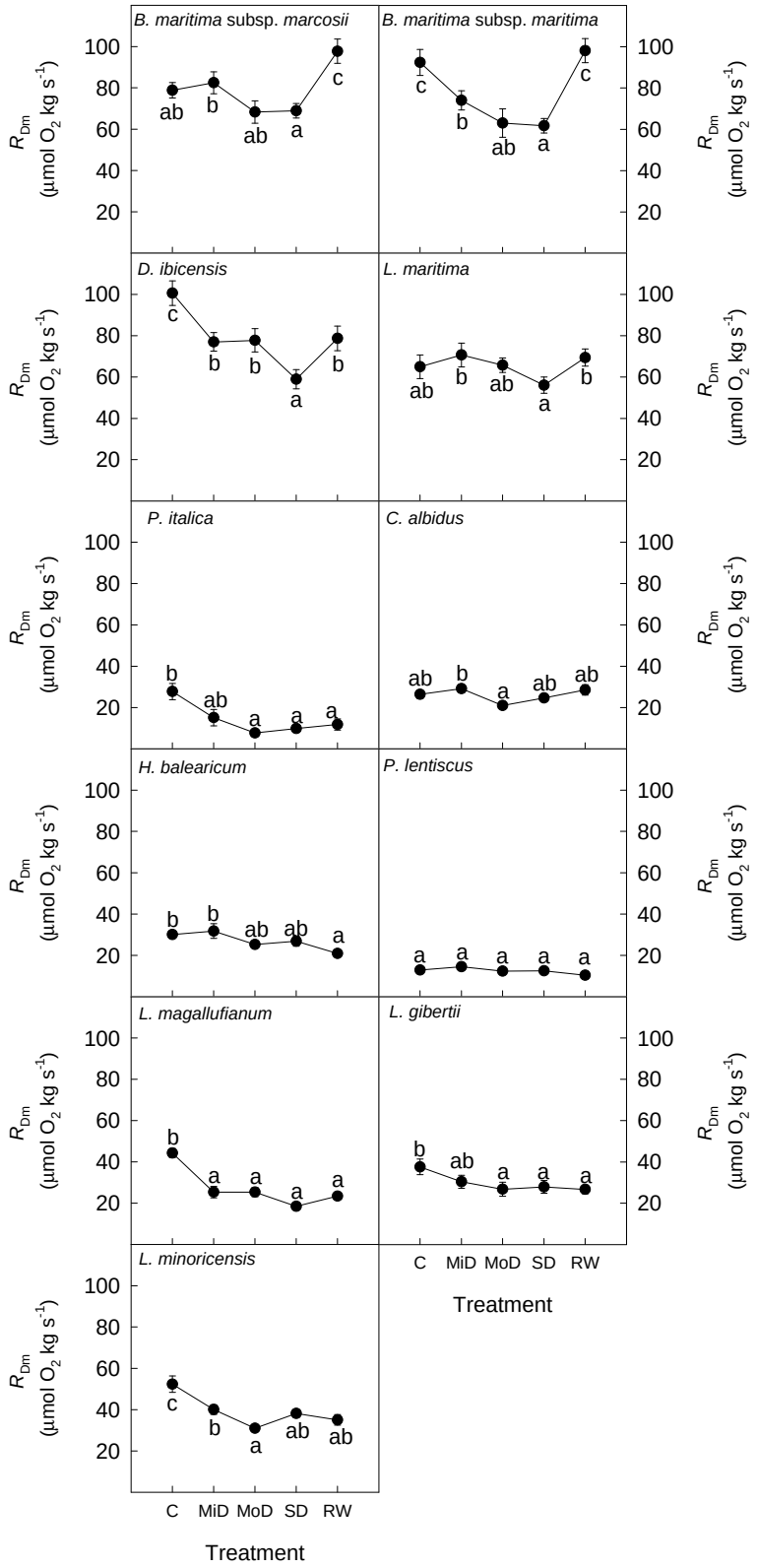
Growth forms	SLA (m^2 kg^{-1})	R_{Dm} (μmol O_2 kg^{-1} s^{-1})	A_{Nm} (μmol CO_2 kg^{-1} s^{-1})	A_{Nm}/R_{Dm} (μmol CO_2 μmol^{-1} O_2)
Herbaceous plants	24.9 ± 1.8	81.0 ± 11.9	490.5 ± 68.7	6.2 ± 0.6
Semi-deciduous shrubs	10.2 ± 1.1	39.7 ± 12.6	243.7 ± 89.3	6.0 ± 0.3
Evergreen shrubs	9.8 ± 1.6	31.2 ± 6.8	132.6 ± 33.1	4.1 ± 0.3

9.4.2. The effects of water stress on respiration rates

The response of dark respiration (R_{Dm}) to gradual drought was determined in eleven different Mediterranean species representative of different growth forms (Fig. 9.2). The evolution of dark respiration rates under water stress largely depended on the species. *B. maritima* subsp. *maritima*, *D. ibicensis*, *P. italica*, *L. magallufianum* and *L. gibertii* showed significant decreases in R_{Dm} due to drought imposition in respect to control values, while in *B. maritima* subsp. *marcosii*, *L. maritima*, *C. albidus*, *H. balearicum* and *P. lentiscus* dark respiration resulted non-affected by water stress (Fig. 9.2). In addition to this diversity in the species response to water limitation, a high variability in the intensity and the *timing* of the R_{Dm} decrease due to drought stress was also found. *B. maritima* subsp. *maritima*, *D. ibicensis*, *P. italica* and *L. magallufianum* decreases of R_{Dm} were significant at earlier stages of water limitation, under mild drought treatment (Fig. 9.2). The effect of re-watering on R_{Dm} also resulted to be species-dependent, from total recovery in both *Beta* species to no recovery in the four evergreen shrubs, and partial recovery in the other species (Fig. 9.2).

Figure 9.2.

Dark respiration rate on a mass basis (R_{Dm}) under different irrigation treatments: control (C), mild drought (MiD), moderate drought (MoD), severe drought (SD) and re-watering (RW), for the eleven species selected to study. Values are means ± standard error of four replicates per species and treatment.



Regarding plant growth forms, herbs showed the most marked and progressive decreases of R_{Dm} as drought intensified, as well as the most complete recovery after re-watering (Fig. 9.3A). In semi-deciduous and evergreen shrubs, R_{Dm} showed only a slight declining tendency under water stress. These variations were roughly parallel to those of A_{Nm} (Fig. 9.3B), but the latter did not fully recover after re-watering. Therefore, the ratio of A_{Nm}/R_{Dm} declined progressively with drought in all growth forms (Fig. 9.3C), although in herbs and semi-deciduous species this ratio was maintained higher than in evergreens except under severe drought. Also, the ratio showed higher recovery after re-watering in herbs and semi-deciduous than in evergreens.

This large variability in plant respiration responses to drought is consistent with the variable responses found among studies in literature (Hsiao, 1973; Hanson & Hitz, 1982; Amthor, 1989; Amthor & McCree, 1990). While several studies described a water stress-induced decreased respiration rate (Brix, 1962; Brown & Thomas, 1980; Palta & Nobel, 1989; González-Meler *et al*., 1997; Escalona *et al*., 1999; Ghashghaire *et al*., 2001; Haupt-Herting *et al*., 2001), others have shown unaffected rates (Lawlor, 1976; Loboda, 1993), or even an increased respiration rate under drought stress (Zagdanska, 1995). Flexas *et al*. (2005) attributed this controversy to three possible causes: (i) the use of different species, organs and techniques for respiration studies; (ii) the presence of complex interactions of respiration rates with other environmental factors; and (iii) the presence of a threshold of water stress intensity in which a change in the response of respiration to water stress occurs. The first two possible causes were constrained by growing all plants under identical conditions, as studying the effects of water stress on a single plant tissue (i.e., leaves). The present results confirm that the response of respiration to water stress is largely species dependent, and suggest that a large part of the observed differences can be attributed to the effects of growth form. Leaves of herbs and semi-deciduous plants have a shorter life-span than those of evergreens. Therefore, they need to optimize their carbon balance over shorter time periods (Mooney & Ehleringer, 1997), which helps explaining why they maintain higher respiration rates but also higher A_{Nm}/R_{Dm} during drought than evergreens, as well as why they show a faster recovery after re-watering. By contrast, leaf productivity in evergreens relies on larger time periods, and they usually have deeper root systems, therefore experiencing a slower rate of water stress imposition and requiring slower responses (Lloret *et al*., 1999; Gratani & Varone, 2004).

Figure 9.3.

(A) Dark respiration rate on a mass basis (R_{Dm}), (B) net CO_2 assimilation rate on a mass basis (A_{Nm}), and (C) the ratio of net CO_2 assimilation to dark respiration rates (A_{Nm}/R_{Dm}) for the different growth forms under different irrigation treatments: control (C), mild drought (MiD), moderate drought (MoD), severe drought (SD) and re-watering (RW). Values are means ± standard error of four replicates per species and treatment. Symbols and growth forms as follows: ● herbaceous species, ○ semi-deciduous shrubs, ▼ evergreen shrubs.

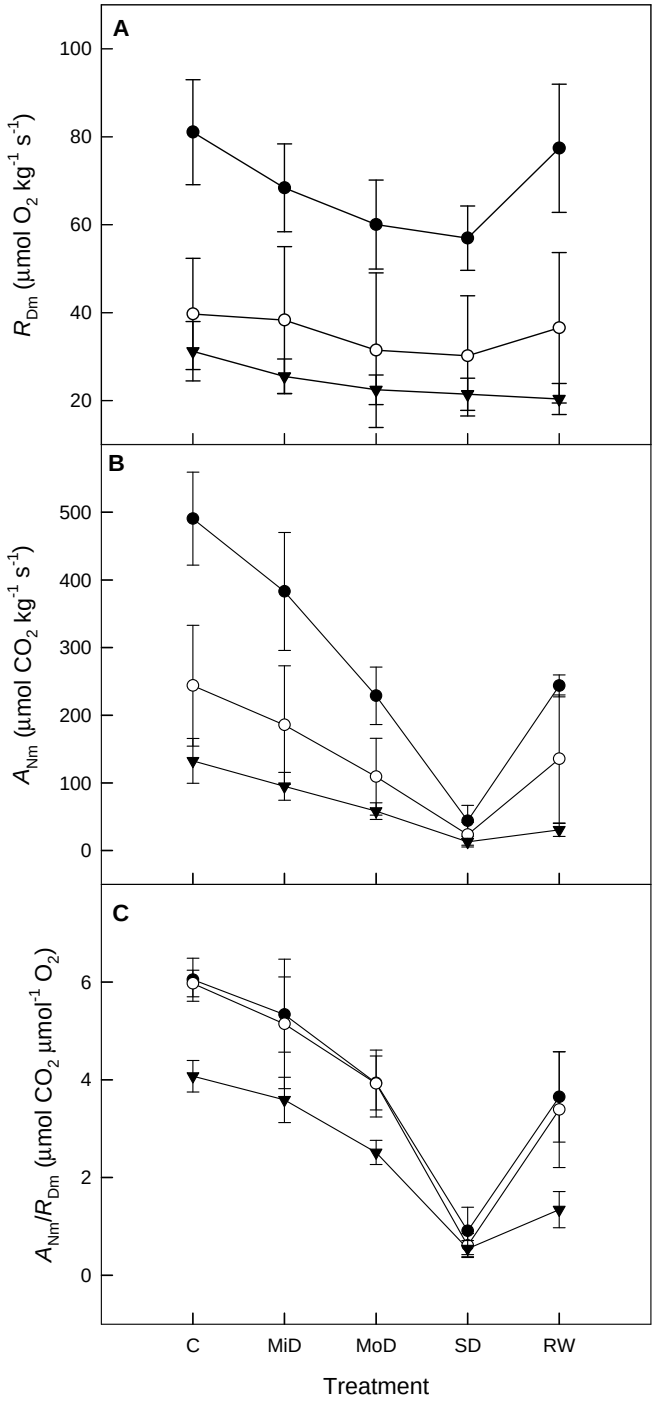
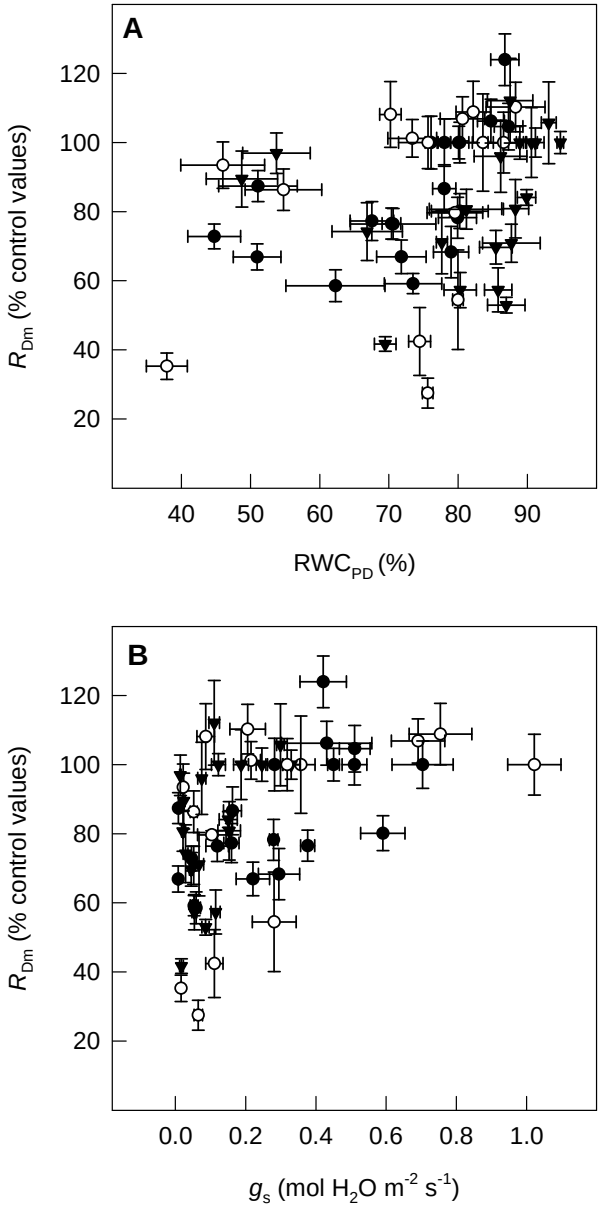


Figure 9.4.

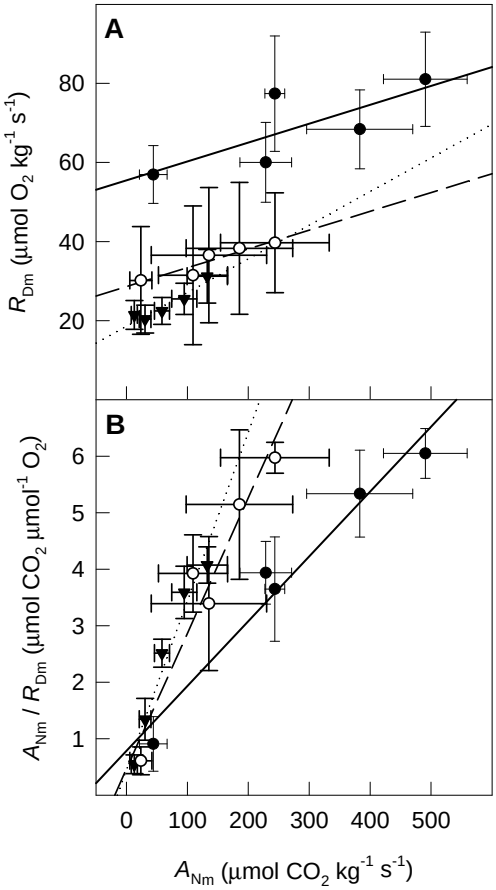
(A) Relationship between the dark respiration rate on a mass basis (R_{Dm}, expressed as percentage of control values) and predawn leaf relative water content (RWC_{PD}). (B) Relationship between the dark respiration rate on a mass basis (R_{Dm}, expressed as percentage of control values) and the stomatal conductance (g_s). Values are means ± standard error of four replicates per species and treatment. Symbols and growth forms as follows: ● herbaceous species, ○ semi-deciduous shrubs, ▼ evergreen shrubs.



The mechanisms allowing different growth forms to show different extents and velocities of respiration response to drought are presently unknown. One possible explanation would be the existence of a threshold of water stress intensity triggering the induction of the respiration response. In this respect, Flexas *et al*. (2005) suggested a biphasic response on the relationship between respiration and relative water content (RWC). However, the present results do not support the existence of such biphasic response of respiration to drought imposition (Fig. 9.4A). When respiration rates, either expressed in area or mass basis, are plotted against the RWC_{PD} (Fig. 9.4A) or RWC_{MD} (data not shown) no clear relationship was found. By contrast, although with some scattering, plotting the response of R_{Dm} to daily maximum stomatal conductance (g_s) resulted in a recognizable pattern, consisting in R_{Dm} declining only when g_s dropped below ca. 0.2 μmol m^{-2} s^{-1} (Fig. 9.4B). This g_s threshold largely coincides with that inducing down-regulation of the activity of other metabolic components, such as photosynthetic enzymes, nitrate reductase or sucrose phosphate synthase (Flexas *et al*., 2004a, 2004b), as well as the induction of cell antioxidant responses (Flexas *et al*., 2006) and an increase in electron transport partitioning towards the alternative respiration pathway at the expense of cytochromic pathway (Ribas-Carbó *et al*., 2006). Therefore, the decline in respiration in response to drought seems to be part of a systemic metabolic response, which occurs under conditions where drought severely restricts CO_2 availability inside leaf cells, therefore creating the risk of a secondary oxidative stress (Flexas *et al*., 2004a, 2004b, 2006). Nevertheless, some of the species showing the most plastic responses (i.e. *D. ibicensis*, *B. maritima* subsp. *maritima* and *P. italica*) behaved somewhat as outliers of this general relationship (outlying points in Fig. 9.4B), suggesting that additional regulatory mechanisms may act in those species. Alternatively, the decline in respiration under stress could be related to a decreased availability of photosynthates, and in fact a close relationship between photosynthesis and respiration was maintained during drought and recovery (Fig. 9.5A), although this relationship strongly differed among growth forms. At any given A_{Nm}, R_{Dm} was lower in evergreens than in herbs, and so their A_{Nm}/R_{Dm} ratio was higher (Fig. 9.5B). Semi-deciduous plants showed an intermediate relationship, but more close to evergreens. The different slopes of the A_{Nm}/R_{Dm} *vs*. A_{Nm} among growth forms likely reflects the well known differences in carbon costs of growth (Poorter & De Jong, 1999; Bouma, 2005, and references therein).

Figure 9.5.

(A) Relationship between dark respiration rate on a mass basis (R_{Dm}) and net CO_2 assimilation rate on a mass basis (A_{Nm}). (B) Relationship between the ratio of net CO_2 assimilation to dark respiration rates (A_{Nm} /R_{Dm}) and net CO_2 assimilation rate on a mass basis (A_{Nm}). Symbols and growth forms as follows: ● and solid line, herbaceous species; ○ and medium dash line, semi-deciduous shrubs; ▼ and dotted line, evergreen shrubs.



9.4.3. Concluding remarks

The aim of the present study was to undertake a comparative analysis of the effects of water stress on leaf respiration rates and carbon balance, including a wide variety of Mediterranean species with different growth forms and evolutionary history. A ten-fold variation in maximum leaf respiration rates was observed, from about 10 to 100 µmol O_2 kg^{-1} s^{-1}. This range was largely determined by differences among growth forms, and respiration values were strongly correlated with photosynthesis and specific leaf area, thus confirming global trends (Wright *et al*., 2004). Endemic species did not

differ from non-endemics in the present study, showing that the trend described by Gulías *et al*. (2003) cannot be generalized.

Leaf respiration rates were decreased under drought in most but not all species, but the decline was always smaller than that of photosynthesis, therefore resulting in a decreased photosynthesis to respiration ratio (a rough indicative of leaf carbon balance). The response of leaf respiration to drought was growth form-dependent, with herbs showing both the highest proportional decrease and the highest recovery after re-watering, and evergreens the lowest. These differences likely reflect differences in respiration requirements due to different life span and growth carbon costs between growth forms (Bloom *et al*., 1985; Lambers & Poorter, 1992; Baruch & Goldstein, 1999; Navas *et al*., 2003). The decline in respiration in response to drought occurs at a stomatal conductance threshold of 0.2 μmol m^{-2} s^{-1}. This g_s threshold value is coincident with that observed in the induction of down-regulation of the activity of many other metabolic components (Flexas *et al*., 2004a, 2004b, 2006; Ribas-Carbó *et al*., 2006), suggesting that it forms part of a systemic metabolic response, which occurs under conditions where drought severely restricts CO_2 availability inside leaves.

Chapter 10

BIOCHEMISTRY OF RUBISCO

ADAPTATION AND ACCLIMATION OF RUBISCO SPECIFICITY

10.1. SUMMARY

Drought conditions strongly influence photosynthetic metabolism in a way that might be extremely important to determine a positive carbon balance in highly stressed environments, such as the Mediterranean area. However, plants may have evolved towards more efficient photosynthetic mechanisms, possibly including acclimation and adaptation of photosynthetic enzymatic traits. Because of its central role in photosynthesis, Rubisco is one of these potential traits to be selected under stressful conditions. Plants respond to low water availability by decreasing leaf diffusive conductances, which, however, leads to an increase of the barriers to diffusion of CO_2 to the primary site of carboxylation. We hypothesized that arid environments leading to water stress and, thus, decreased CO_2 availability for photosynthesis, may impose

increased selection pressure on Rubisco for improving its specificity factor (τ), a measure of the relative affinity of the enzyme for CO_2 and O_2.

To test this hypothesis, τ was measured on purified Rubiscos from 24 Mediterranean species having a variety of ecological, phylogenetic and morphological traits. A high variability in Rubisco τ was found among plants, which was related to environmental pressure factors and not to phylogeny. Rubisco τ was significantly higher in species inhabiting the most arid areas, and the Rubisco of a xeric species, *Limonium gibertii*, presented the highest τ value hitherto reported among higher plants. This was sequenced and some interesting residues were found to be different to other higher plant Rubiscos but identical to *Galdieria*. Finally, to check whether plants can also acclimate Rubisco kinetic properties to drought, τ was measured in tobacco leaves developed under different drought intensities. The results showed that Rubisco τ does not acclimate to water stress in the short term.

10.2. INTRODUCTION

The most important characteristic of plants is their ability to harness energy from the sun to fix atmospheric carbon dioxide into a range of more complex organic molecules. This process of photosynthesis provides the major input of free energy into the biosphere; it is the basis for most terrestrial food chains, the ultimate source of the fuel energy in oil and coal, and is responsible for the origin and maintenance of oxygen in the atmosphere.

Photosynthesis is known to be very sensitive to environmental stresses, such as salt, temperature, drought and excessive light. Among these, drought is considered the most important constraint for plant production world-wide (Boyer, 1982). Moreover drought frequently takes place together with other stresses like high temperature and excess of light, particularly under Mediterranean summer conditions (Flexas & Medrano, 2002c). Mediterranean climate is mainly characterized by a dry season in which plants undergo water deficit conditions. The severity and duration of this season depends on the location. In the Balearic Islands, where this study was performed, most of the precipitation typically falls between September and May, leading to a severe dry period of at least four months (Flexas *et al.*, 2003).

Despite the importance of photosynthesis for plant growth and survival, the study of adaptation of plant traits to Mediterranean conditions has focused mainly on

morphological features (Ehleringer & Mooney, 1983; Joffre *et al.*, 1999). Some physiological traits have also been identified as adaptation and acclimation responses, e.g. tolerance to low tissue water potential by osmotic adjustments (Morgan, 1984), more rigid cell walls, or smaller cells (Wilson *et al*., 1980). Within the short-term plant acclimation responses to drought, leaves that survive drought often show higher rates of photosynthesis (Ludlow & Ng, 1974; Stewart *et al*., 1994) and chloroplastic electron transport (Panković *et al*., 1999; Kitao *et al*., 2003), and larger Rubisco content per unit area (David *et al*., 1998), after drought is released than leaves of similar age that have not been endured previous drought. However, acclimation and adaptation of photosynthetic features have been much less studied than morphology, with the exception of photosynthetic types (C_3 versus C_4 and CAM) (Winter & Smith, 1996; Hatch, 1999; Keeley & Rundel, 2003).

It is hypothesized that some photosynthetic traits of C_3 plants may have diverged under Mediterranean conditions, allowing for the appearance of some characteristics conferring a higher photosynthetic efficiency under drought. In particular, the present study is focused to analyze whether Rubisco, the central enzyme of the photosynthetic process, can acclimate and/or has adapted to drought in Mediterranean plants.

10.2.1. Rubisco's key role in photosynthesis: the entry point of life

The biochemistry supporting life on earth depends, in terms of gain of energy, on oxidative reactions. The final product of metabolic pathways based on carbon is carbon dioxide, which is released into the atmosphere. To close the carbon cycle it is necessary to feed the carbon dioxide back to the food chain. The only enzyme capable of this task is a protein located at the chloroplast stroma, Rubisco (Ribulose-1,5-bisphosphate carboxylase/oxygenase, EC 4.1.1.39) (Andrews & Lorimer, 1987). Rubisco then comprises the starting point of any food chain.

Rubisco plays its role in a cyclic, autocalatytic process, called the Calvin cycle, reductive pentose phosphate pathway or photosynthetic carbon reduction cycle. Rubisco is one of the 11 enzymes involved in this carboxylation cycle, and its importance resides in catalysing the first step, the reaction of CO_2 with an acceptor molecule, ribulose bisphosphate (RuBP), producing 3-phosphoglycerate (3PGA). Five-sixths of the 3PGA thus formed is used for regeneration of the acceptor, in reactions consuming NADPH and ATP. NADPH and ATP are produced as a consequence of the light-driven

photochemical reactions in the thylakoids. The remaining one-sixth is exported from the chloroplasts as triose phosphate for the synthesis of sucrose and other products in the cytosolic compartment or metabolized to form starch within the chloroplasts.

Assimilation of CO_2 can be summarised as:

$$3\ CO_2 + 9\ ATP + 6\ NADPH + 5\ H^+ \rightarrow C_3H_5O_3P + 9\ ADP + 8\ P_i + 6\ NADP^+ + 3\ H_2O + 468\ kJ\ mol^{-1}$$

10.2.2. Rubisco structure

There are two major structural forms of Rubisco (Tabita, 1988):

- Form I Rubisco, always found in higher plants and in most autotrophic and chemoautotrophic bacteria. It consists of eight ca. 53 kDa large (L) subunits and eight ca. 14 kDa small (S) subunits. According to phylogeny, two subdivisions can be made (Horken & Tabita, 1999): (i) a "green-type" division composed of enzymes from higher plants, green algae, cyanobacteria, and proteobacteria sharing recent ancestry with cyanobacteria; and (ii) a "red-type" division composed of enzymes from α and β purple proteobacteria and from red and brown plastids.
- Form II Rubisco, found in some dinoflagellates and certain autotrophic bacteria, including the obligate anaerobe *Rhodospirillium rubrum*, lacks S subunits and contains only L subunits (Morse *et al*., 1995; Whitney & Andrews, 1998; Tabita, 1999).

Rubisco from archaebacteria comprises a variation of these two major structural forms, comprising five L subunit dimers (Maeda *et al*., 1999).

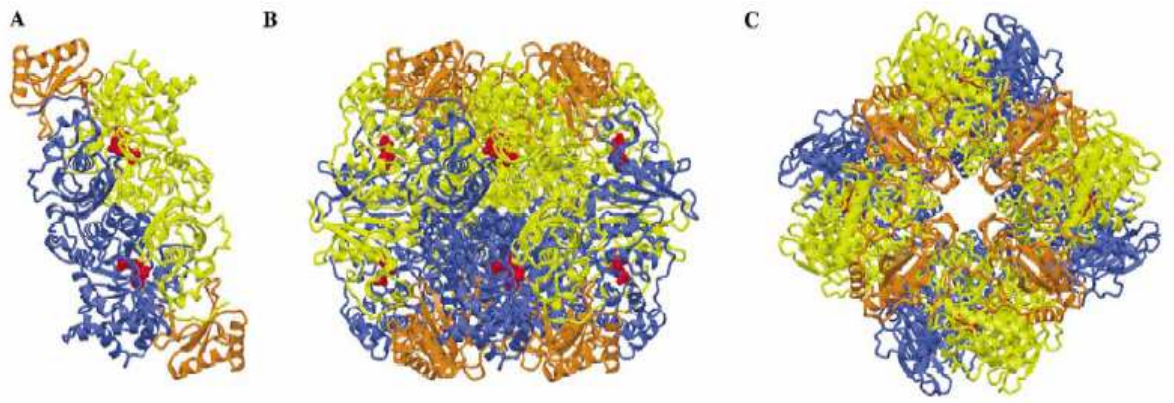
In green algae and higher plants, Rubisco occurs in the chloroplast and the L subunit is encoded by a single *rbc*L gene in the chloroplast genome (Eilenberg *et al*., 1998). In contrast, an *rbc*S gene family having 2-12 nuclear genes encodes the S subunit, which is synthesized as a precursor polypeptide on the cytosolic ribosomes and imported into the chloroplast in an ATP-dependent reaction (reviewed in Dean *et al*., 1989; Spreitzer, 1993; Hartman & Harpel, 1994). Once in the chloroplast, S subunits are added to a core of chaperone-assembled L subunits (reviewed in Spreitzer, 1999; Roy & Andrews, 2000).

In order to understand the function and structure of Rubisco, much attention has been given to X-ray crystallographic studies of the enzyme. Three dimensional high

resolution-structures of Rubisco are now known for a number of species, allowing insights of the enzyme with bound substrate, product and transition-state analogues (Knight *et al*., 1990; Curmi *et al*., 1992; Andersson, 1996; Taylor *et al*., 1996; Taylor & Andersson, 1996; Taylor & Andersson, 1997a; Taylor & Andersson, 1997b; Taylor *et al*., 2001; Mizohata *et al*., 2002). Moreover, based on these and other studies, it is now believed that Rubisco from higher plants is a hexadecamer described as four dimers of L subunits surrounded by two tetramers of S subunits (Fig. 10.1A). The L subunit has two separate domains, an N-terminal domain encompassing amino acid residues 1 to 150 and a C-terminal built up of residues 151 to 475. The C-terminal domain contains eight-stranded parallel α/β barrel. Two active sites are found at the interface of the L subunits in each L_2 dimmer (Fig. 10.1B), i. e. eight catalytic sites per complete complex (holoenzyme) for the higher plant enzyme. Most of the active site residues are contributed by the loops connecting the β-strands to the α-helices at the C-terminal end of the barrel. In addition two loop regions in the N-terminal domain of the second L subunit in the dimmer supply the remainder of the residues in the active site (Andersson, 1996). The S subunit is arranged in a four-stranded anti-parallel β-sheet flanked by two helices on one side (Knight *et al*., 1989).

Figure 10.1

Quaternary structure of type I Rubisco from spinach. (A) L_2S_2 unit viewed down the twofold symmetry axis. (B and C) Entire L_8S_8 hexadecamer viewed down the twofold and fourfold axes. From Andersson & Taylor (2003).



10.2.3. Rubisco's tendency to mistake

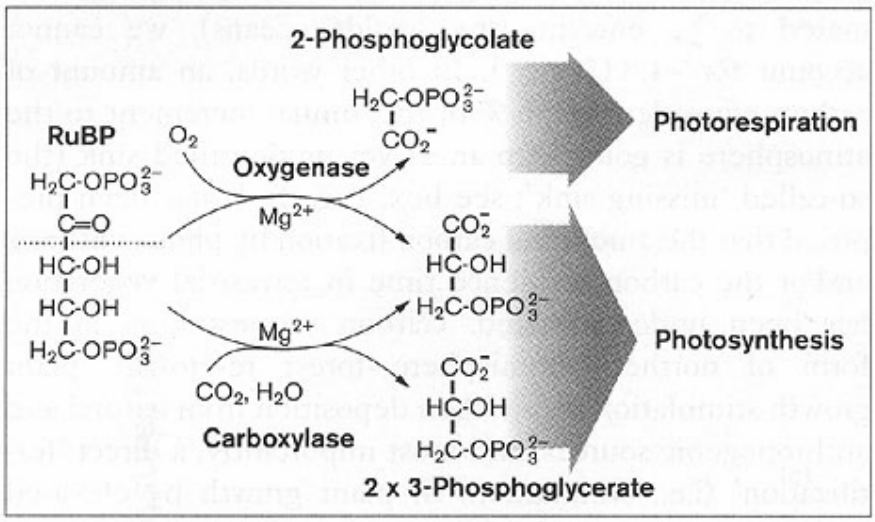
Besides its unique role in incorporating carbon from atmospheric CO_2 into the organic substances of the biosphere, Rubisco has a peculiar catalyst inefficiency: its carboxylase activity is reduced by a major competing reaction with another atmospheric gas, O_2, involving the same active site as the natural substrate, CO_2 (Fig. 10.2). The opposing oxygenase activity of Rubisco catalyses the first reaction in the photorespiratory pathway (Ögren & Bowes, 1971; Laing *et al*., 1974), causing in C_3 plants the loss of up to 50% of carbon fixed by Rubisco and greatly decreasing the efficiency with which light energy is used (Zelitch, 1973). The balance between the two competitive reactions is determined by the kinetic properties of Rubisco and the CO_2 and O_2 concentrations at the site of the enzyme (Laing *et al*., 1974):

$$\frac{v_c}{v_o} = \left(\frac{V_c \cdot K_o}{V_o \cdot K_c} \right) \cdot \frac{C}{O}$$

where v_c and v_o are the velocities of carboxylation and oxygenation, respectively, V_c and V_o are the maximal velocities of the two reactions, K_c and K_o the Michaelis constants for CO_2 and O_2, and C and O are the CO_2 and O_2 molar concentration at the site of Rubisco. The substrate specificity factor, τ (V_cK_o/V_oK_c), determines the relative rates of the two reactions at any given CO_2 and O_2 concentrations.

Figure 10.2

The oxygenation and carboxylation of RuBP catalyzed by Rubisco.



Oxygenation of RuBP leads to the production of one molecule of 3PGA and one molecule of glycolate-2-P, a two carbon compound which is salvaged in the photorespiratory pathway or C_2 cycle. In the course of this pathway, two molecules of glycolate-2-P are metabolised to form one molecule of PGA and one molecule of CO_2. The PGA is returned to the photosynthetic carbon reduction cycle. This recycling of carbon from glycolate-2-P to RuBP is not only a very costly reaction, consuming one third or more of the total energy requirement of CO_2 fixation, it also requires a complex metabolic machinery, consisting of more than 15 enzymes and translocators, distributed over three different organelles, i.e. the chloroplast, peroxisome and mitochondrion (reviewed in Roy & Andrews, 2000).

However, photorespiratory metabolism uses much energy to convert 75% of the carbon diverted out of the photosynthetic carbon reduction cycle back to the photosynthesis (Lorimer & Andrews, 1981), and the only control step is at the level of competition between O_2 and CO_2 for reacting with RuBP (reviewed in Douce & Heldt, 2000). This use of energy at high irradiance helps to prevent the formation of the excited triplet state of chlorophyll and consequently reactive O_2 species (superoxide radicals and singlet oxygen), which would be damaging to the chloroplast membranes (Osmond & Grace, 1995). In other words, photorespiration, in concert with other reactions (Mehler-ascorbate peroxidase reaction, carotenoid pigments cycle), potentially mitigates chronic photoinhibition, which is especially present under conditions where intracellular CO_2 levels are low but light levels are high (Oliver, 1998), a situation very common in water stressed environments such as those found during the Mediterranean summer (Flexas *et al*., 2003).

10.2.4. Is Rubisco τ an immutable parameter?

Nature has been unable to avoid the lack of specificity of Rubisco (Andrews & Lorimer, 1978). The reason is probably that early evolution of Rubisco occurred at a time when there was little oxygen in the atmosphere. When, more than a billion years later, due mainly to photosynthesis, oxygen appeared in the atmosphere in higher concentrations, the complexity of Rubisco protein probably precluded rapid adaptation of the catalytic centre to eliminate oxygenase activity. Rubisco inevitably initiates inefficiencies of carbon assimilation (Osmond & Grace, 1995). The comparison of τ values, which vary over a 20-fold range, from divergent photosynthetic organisms

supports this hypothesis (Jordan & Ogren, 1981; Tortell, 2000) (Table 10.1). Effectively, τ increases from the lower photosynthetic forms to higher plant forms (Fig. 10.3). Form II Rubiscos, which lack small subunits, have the lowest τ values (10-15). Form I Rubiscos have values ranging from a low of 25-45 in proteobacterias "green-type" up to a high in excess of 225 in some thermopilic rhodophytes. Higher plants cluster between 70 and 100.

Table 10.1

List of τ values (rounded) found in literature, with their structural form of Rubisco. Form I Rubiscos are further classified according to whether they fall in the "green-type" or "red-type" phylogenies. τ values are given with errors when provided by the authors. All data procede from *in vitro* τ measurements. The diversity of enzymatic determination procedures is likely responsible for some differences in τ within the same species. Indeed, and specially in some procedures, the huge dependency of τ measurement on pH, temperature and gas concentrations makes it difficult to compare results from different literature sources, even if the same methodology was used.

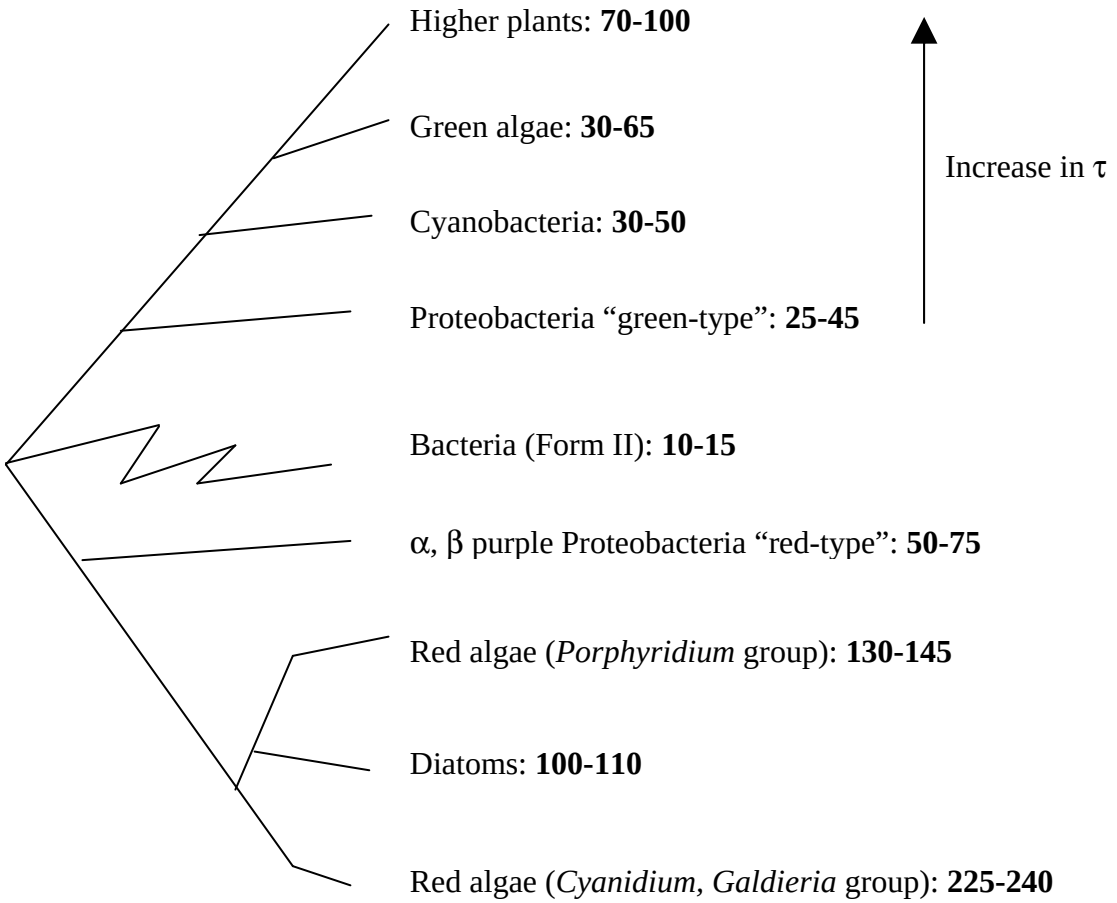
	Source	**Form**	**τ**	**Reference**
C_3 plants				
	Agropyron intermedium	I (green)	99	Kent & Tomany (1995)
	Agrostis scabra	I (green)	107	Kent & Tomany (1995)
	Avena sativa	I (green)	79	Kent & Tomany (1995)
	Bromus arenarius	I (green)	94	Kent & Tomany (1995)
	Bromus pseudodanthoniae	I (green)	102	Kent & Tomany (1995)
	Carex crinita	I (green)	101±5	Kent & Tomany (1995)
	Ceratonia siliqua	I (green)	98	Delgado *et al.* (1995)
	Chelidonium majus	I (green)	101	Kent & Tomany (1995)
	Chrysanthemum coronarium	I (green)	107	Delgado *et al.* (1995)
	Elodea sp.	I (green)	111	Kent & Tomany (1995)
	Equisetum sp.	I (green)	90	Kent & Tomany (1995)
	Glycine max	I (green)	82±5	Jordan & Ogren (1981)
	Helianthus annuus	I (green)	109±4	Kent *et al.* (1992)
	Helianthus annuus cv Sungro	I (green)	104±3	Parry *et al.* (1989)
	Helianthus annuus SH 229	I (green)	105±4	Parry *et al.* (1989)
	Helianthus maximus	I (green)	77	Cited in Keys (1986)
	Helleborus lividus subsp. *corsicus*	I (green)	108	Delgado *et al.* (1995)
	Hippocrepis balearica	I (green)	94	Delgado *et al.* (1995)
	Holcus lanatus	I (green)	110	Kent & Tomany (1995)
	Hordeum bulbosum	I (green)	101	Kent & Tomany (1995)
	Hordeum marinum	I (green)	100	Kent & Tomany (1995)
	Hordeum vulgare	I (green)	87±1	Kane *et al.* (1994)
	Laportea canadensis	I (green)	89±3	Kent & Tomany (1995)
	Leersia hexandra	I (green)	111	Kent & Tomany (1995)
	Lolium perenne	I (green)	80±1	Jordan & Ogren (1981)
	Lonicera sp.	I (green)	112	Kent & Tomany (1995)
	Lotus creticus subsp. *cytisoides*	I (green)	107	Delgado *et al.* (1995)
	Lupinus polyphyllus	I (green)	92±4	Kent & Tomany (1995)

	Source	Form	τ	Reference
	Lycopersicon esculetum	I (green)	82	Cited in Keys (1986)
	Medicago arborea ssp. *citrina*	I (green)	96	Delgado *et al.* (1995)
	Medicago sativa	I (green)	77	Cited in Keys (1986)
	Mertensia virginica	I (green)	107	Kent & Tomany (1995)
	Nicotiana tabacum	I (green)	77±1	Jordan & Ogren (1981)
	Nicotiana tabacum	I (green)	82±1	Kane *et al.* (1994)
	Nicotiana tabacum	I (green)	90	Gutteridge *et al.* (1986)
	Nicotiana tabacum cv. Bright yellow	I (green)	89±4	Parry *et al.* (1989)
	Oryza punctata	I (green)	85±1	Kane *et al.* (1994)
	Pastinaca lucida	I (green)	85	Delgado *et al.* (1995)
	Petroselinum crispum	I (green)	77	Cited in Keys (1986)
	Plantago lanceolata	I (green)	105±3	Kent & Tomany (1995)
	Quercus ilex	I (green)	93±5	Balaguer *et al.* (1996)
	Quercus robur	I (green)	102±2	Balaguer *et al.* (1996)
	Ranunculus acris	I (green)	99±3	Kent & Tomany (1995)
	Smilacina racemosa	I (green)	107±5	Kent & Tomany (1995)
	Spinacea oleracea	I (green)	80±1	Jordan & Ogren (1981)
	Spinacea oleracea	I (green)	83±4	Jordan & Ogren (1984)
	Spinacea oleracea	I (green)	82±1	Kane *et al.* (1994)
	Spinacea oleracea	I (green)	94±1	Uemura *et al.* (1997)
	Spinacea oleracea	I (green)	74	Kent & Tomany (1984)
	Spinacea oleracea	I (green)	100±4	Kent & Tomany (1995)
	Syringa vulgaris	I (green)	88±2	Kent *et al.* (1992)
	Syringa vulgaris	I (green)	90	Kent & Tomany (1995)
	Tetragonium expansa	I (green)	81±1	Jordan & Ogren (1981)
	Trifolium subterraneun	I (green)	97	Delgado *et al.* (1995)
	Triticum aestivum	I (green)	90±1	Kane *et al.* (1994)
	Triticum aestivum	I (green)	100	Gutteridge *et al.* (1986)
	Triticum aestivum	I (green)	100	Delgado *et al.* (1995)
	Triticum aestivum cv. Maris Mardler	I (green)	107±3	Parry *et al.* (1989)
	Veratrum viride	I (green)	109	Kent & Tomany (1995)
	Vitis vinifera cv. Manto Negro	I (green)	100±5	Bota *et al.* (2002)
	Vitis vinifera cv. Tempranillo	I (green)	101±3	Bota *et al.* (2002)
C_4 plants				
	Amaranthus hybridus	I (green)	82±4	Jordan & Ogren (1981)
	Anoxopus compressus	I (green)	95	Kent & Tomany (1995)
	Chloris gayana	I (green)	79	Kent & Tomany (1995)
	Echinochloa crus-galli	I (green)	85±3	Kent & Tomany (1995)
	Echinochloa crus-pavonis	I (green)	83	Kent & Tomany (1995)
	Panicum bergi	I (green)	97	Kent & Tomany (1995)
	Panicum maximum	I (green)	100	Kent & Tomany (1995)
	Portulaca oleracea	I (green)	78	Cited in Keys (1986)
	Setaria italica	I (green)	58	Jordan & Ogren (1983)
	Setaria italica	I (green)	80±4	Kent & Tomany (1995)
	Sorghum bicolor	I (green)	20	Cited in Keys (1986)
	Zea mays	I (green)	78±3	Jordan & Ogren (1981)
	Zea mays	I (green)	79±1	Kane *et al.* (1994)
	Zea mays	I (green)	95	Kent & Tomany (1995)
Green algae				
	Chlamydomonas reinhardtii	I (green)	61±5	Jordan & Ogren (1981)
	Chlamydomonas reinhardtii	I (green)	60	Cited in Horken & Tabita (1999)

	Source	Form	τ	Reference
	Chlorella pyrenoidosa	I (green)	31	Kent & Tomany (1984)
	Coccomyxa sp.	I (green)	83	Palmqvist *et al.* (1995)
	Euglena gracilis	I (green)	54±2	Jordan & Ogren (1981)
	Scenedesmus obliquus	I (green)	63±2	Jordan & Ogren (1981)
Non-green eukaryotic algae				
	Cyanidium calderium	I (red)	225±5	Uemura et al. (1997)
	Cylindrotheca sp. strain N1	I (red)	105	Cited in Tabita (1995)
	Cylindrotheca fusiformis	I (red)	110	Cited in Tabita (1995)
	Galdieria partita	I (red)	238±9	Uemura *et al.* (1997)
	Galdieria partita	I (red)	240	Cited in Horken & Tabita (1999)
	Olisthodiscus luteus	I (red)	100	Cited in Tabita (1995)
	Porphyridium cruentum	I (red)	130	Cited in Tabita (1995)
	Porphyridium purpureum	I (red)	144±6	Uemura *et al.* (1997)
Cyanobacteria				
	Anabaena caldarium PCC7120	I (green)	35	Igarashi & Kodama (1996)
	Aphanizomemon flos-aquae	I (green)	48±2	Jordan & Ogren (1983)
	Aphanocaspa alpicola	I (green)	48	Cited in Keys (1986)
	Coccochloris peniocystis	I (green)	47±2	Jordan & Ogren (1981)
	Plectonema boryanum	I (green)	54	Cited in Keys (1986)
	Plenctonema boryanum	I (green)	32	Kent & Tomany (1984)
	Synechococcus PCC6301	I (green)	43	Kane *et al.* (1994)
	Synechococcus 6301	I (green)	39±3	Cited in Horken & Tabita (1999)
Bacteria				
	Alcaligenes eutrophus	I (red)	75	Cited in Tabita (1995)
	Bradyrhizobium japonicum	I (red)	75±3	Cited in Horken & Tabita (1999)
	Chromatium vinosum	I (green)	40	Jordan & Chollet (1985)
	Chromatium vinosum	I (green)	44±1	Uemura *et al.* (1997)
	Hydrogenovibrio marinus	I (green)	25	Cited in Horken & Tabita (1999)
	Ralstonia eutrophus	I (red)	75	Cited in Horken & Tabita (1999)
	Rhodobacter sphaeroides	I (red)	62	Jordan & Ogren (1981)
	Rhodobacter sphaeroides	I (red)	62±4	Jordan & Ogren (1981)
	Rhodobacter sphaeroides FI	I (red)	56±3	Cited in Horken & Tabita (1999)
	Rhodobacter sphaeroides FI (L341M)	I (red)	51±2	Cited in Horken & Tabita (1999)
	Rhodobacter sphaeroides	I (red)	60	Cited in Horken & Tabita (1999)
	Rhodobacter sphaeroides	II	10	Cited in Horken & Tabita (1999)
	Rhodobacter sphaeroides	II	9±1	Jordan & Ogren (1981)
	Rhodospirillum rubrum	II	15±1	Jordan & Ogren (1981)
	Rhodospirillum rubrum	II	15	Cited in Horken & Tabita (1999)
	Rhodospirillum rubrum	II	12	Kane *et al.* (1994)
	Rhodospirillum rubrum	II	8	Kent & Tomany (1984)
	Rhodospirillum rubrum	II	9±1	Parry *et al.* (1989)
	Thiobacillus denitrificans	II	10	Cited in Horken & Tabita (1999)
	Thiobacillus denitrificans	II	10	Cited in Tabita (1995)
	Thiobacillus denitrificans	I (green)	46	Cited in Horken & Tabita (1999)
	Xanthobacter flavus	I (red)	44±4	Cited in Horken & Tabita (1999)

Figure 10.3

Rubisco τ depending on the evolutionary grouping of Rubisco structures. τ ranges were obtained from grouping organism from Table 10.1. The phylogenetic tree was made according the evolution of the gene for the L subunits of Rubisco (Ueda & Shibata, 1992; Zetsche & Valentin, 1994; Yokota, 1999).



Although differences in τ among distant phylogenetic groups (i.e., photosynthetic bacteria, cyanobacteria, higher plants, etc.) were generally accepted (Jordan & Ogren, 1984; Andrews & Lorimer, 1987), in the early 80's there was a general consensus that non-significant differences would be found among C_3 plants. However, as the errors of the methods used were becoming lower, studies reporting differences among higher plants appeared (references listed in Table 10.1). Nowadays, more than 20 Rubisco X-ray crystal structures exist within the Protein Data Bank, and more than 2200 *rbc*L and 350 *rbc*S amino acid sequences reside within the GenBank, allowing an insight into the existing differences in Rubisco from higher plants.

10.2.4.1. Long-term environmental effects: adaptation of τ

Despite this strong evident phylogeny-dependence of τ, it has been hypothesised that the enzyme could evolve according to the organisms' specific need for CO_2 assimilation depending on environment conditions in which the organism evolved (Horken & Tabita, 1999). More specifically, Delgado *et al*. (1995) and Kent & Tomany (1995) hypothesised that hot environments associated with water stress may impose increased selection pressure on Rubisco for improved specificity. Such postulation may be based on the following aspects:

- Temperature dependence of τ. Rubisco τ decreases with increasing temperature (Chen & Spreitzer, 1991).
- Under water stress conditions, the leaf conductances to gas diffusion decrease and, as a consequence, the CO_2 concentration at the site of Rubisco (C_c) is decreased (Flexas *et al*., 2004a). Rubisco is estimated to work at a C_c just one-quarter of its effective K_c under optimal conditions (Sharkey, 1998). When water limitation and therefore leaf resistances increase, C_c may be even more far from its effective K_c, increasing the ratio of photorespiration to photosynthesis (Flexas & Medrano, 2002a).

Although it is well known that Mediterranean species are well adapted to drought, the Mediterranean basin is characterised by a long dry, hot summer, which severely stresses plants and influences their distribution. Therefore, such conditions of simultaneously high temperatures and water deficit are well represented. Within the Balearic Islands there is a steep gradient of both precipitation (from 300 to 1500 mm) and temperature, which may impose different degrees of such environmental stresses. Therefore, such a climatically-variable area appears ideal to search for species-specific differences in τ.

On the other hand, Gulías *et al*. (2003) showed that species endemic to the Balearic Islands have significantly lower photosynthetic capacities than widespread species and proposed that one factor could be a lower τ in the endemic species to explain the observed differences. Geographical isolation could have favoured selection of distinctive properties for Rubisco.

Finally, it has also been proposed that there is a strong correlation between plant physiological parameters and ecological plant traits (Reich *et al*., 1997; Wright *et al*.,

2004). Therefore, a possible relationship between τ and leaf traits, such as leaf life span and leaf sclerophylly, is likely.

10.2.4.2. Short-term environmental effects: acclimation of τ

Current models of leaf photosynthesis assume constant τ values among C_3 plants (Long & Bernacchi, 2003) or derive them indirectly from the CO_2 compensation point in the absence of mitochondrial respiration (Γ*) after gas exchange measurements (Bernacchi *et al*., 2001). However, τ may not be constant under varying environmental conditions, such as light quality and quantity or water stress, although there has been no attempt to assess this possibility. The molecular basis and/or structural features determining Rubisco τ are still poorly understood (Spreitzer & Salvucci, 2002; Andersson & Taylor, 2003; Spreitzer, 2003). The Rubisco large subunit is encoded by multiple identical copies of *rbc*L in the chloroplast genome (Eilenberg *et al*., 1998), whereas an *rbc*S gene family having 2 to 12 nuclear genes encodes small subunit peptides (Dean *et al*., 1989; Spreitzer, 2003). Thus, while the copies of the large subunit would likely be the same, the differential expression of *rbc*S genes may depend on the environment. For instance, transcription of specific *rbc*S genes appears to be dependent on light quality in the fern *Pteris vittata* (Eilenberg *et al*., 1998). Although large subunits have the main influence on catalytic properties, the Rubisco small subunits have also been hypothesised to affect key kinetic characteristics like τ (Roy & Andrews, 2000; Parry *et al*., 2003). Hence, in principle, any environmental condition capable of modulating *rbc*S gene expression could induce changes in Rubisco τ.

Water stress, in particular, induces stomatal closure and a decrease in leaf internal CO_2 concentration, which results in increased oxygenase over carboxylase activity, thus increasing the ratio of photorespiration to photosynthesis (Flexas & Medrano, 2002c).

When applying photosynthesis models to study the effects of water stress on photosynthetic limitations and/or on mesophyll conductance to CO_2 (g_i), Rubisco τ (or Γ*) is usually assumed to be constant (Wilson *et al*., 2000; Xu & Baldocchi, 2003; Peña-Rojas *et al*., 2004; Warren *et al*., 2004). However, Bota *et al*. (2002) and Warren *et al*. (2004) have reported an apparent increase of Γ* under water stress. This fact merits some attention, since it may change substantially the interpretations of water stress on photosynthesis coming from studies in which Γ* was assumed as constant.

Fluorescence estimates of g_i, in particular, are very sensitive to Γ* (Harley *et al*., 1992). However, estimations of Γ* under water stress include a number of assumptions that may not be correct and, as noted by Warren *et al*. (2004), it would be preferably to determine Rubisco τ (or Γ*) independently, using an alternative method.

10.2.5. Temperature-dependence of Rubisco τ

The above explained phylogenetical and environmental factors possibly affect the characteristic value of τ for a given species. However, it is well established that τ can vary in a more dynamic way. Particularly, the oxygenase efficiency constant (V_o/K_o) results more rapidly increased with increasing temperature than the carboxylase efficiency constant (V_c/K_c) (Jordan & Ogren, 1984). Nevertheless, the temperature response of τ has been studied in very few species, although differences in this response have been demonstrated between bacterial, red algae and higher plant enzyme (Lorimer *et al*., 1993; Uemura *et al*., 1997). Nevertheless, other authors suggested a general response pattern (Jordan & Ogren, 1984; Brooks & Farquhar, 1985).

The balance between the carboxylase and the oxygenase reactions of Rubisco can be illustrated as:

$$\frac{v_c}{v_o} = \tau \frac{C}{O} = \frac{k_c [E]_0 C}{k_o [E]_0 O} \qquad (1)$$

where k_c and k_o are the rate constants for the partial reactions and $[E]_0$ is the concentration of the enzyme-enol-RuBP complex. By cancelling terms in Eq. (1), it becomes clear that τ must also be equal to the ratio of the rate constants (k_c/k_o) for these two partial reactions (Pierce *et al*., 1986). Based on transition-state theory (Fersht, 1985), a rate constant (k_i) can be defined as:

$$k_i = \frac{kT}{h} e^{-\Delta G^{\ddagger}/RT} \qquad (2)$$

where k is the Boltzmann constant, *h* is the Planck's constant, *R* is the gas constant, *T* is the absolute temperature, and $\Delta G^{\ddagger}$ is the free energy of activation. k_c/k_o is related to the

carboxylation and oxygenation free energies of activation ($\Delta G_c^{\ddagger}$ and $\Delta G_o^{\ddagger}$) by the following equation (Chen & Spreitzer, 1991):

$$\ln \frac{k_c}{k_o} = \frac{\Delta G_o^{\ddagger} - \Delta G_c^{\ddagger}}{RT} \qquad (3)$$

It is apparent from this equation that k_c/k_o can be influenced by temperature (Chen & Spreitzer, 1991), or by changes in $\Delta G_o^{\ddagger} - \Delta G_c^{\ddagger}$.

10.2.6. Catalytic Mechanism

Much is known about the Rubisco catalytic mechanism from chemical modification, directed mutagenesis and structural studies (reviewed in Spreitzer, 1993; Hartman & Harpel, 1994; Cleland *et al*., 1998).

Rubisco must be reversibly activated before catalysis can occur. Demonstrations that pre-incubation with CO_2/HCO_3^- and Mg^{2+} before assay *in vitro* increased activity (Pon *et al*., 1963; Andrews *et a*l., 1975), that CO_2/HCO_3^- became bound to the protein (Akoyunoglou & Calvin, 1963; Miziorko & Mildvan, 1974), and that activity and catalytic properties changed rapidly following extraction from chloroplasts and leaves (Badger & Andrews, 1974; Bahr & Jensen, 1974), led to the discovery that sequential binding of CO_2 and a divalent metal ion (physiologically Mg^{2+}) is a prerequisite for catalysis (Laing & Christeller, 1976; Lorimer *et al*., 1976) (Fig. 10.4). The oxygenase activity also requires this activation (Badger & Lorimer, 1976). It is important to note that the CO_2 molecule which binds in a reversible and rate limiting process before subsequent rapid binding of Mg^{2+} (Lorimer *et al*., 1976), is distinct from the substrate CO_2 molecule fixed during carboxylation (Lorimer, 1979).

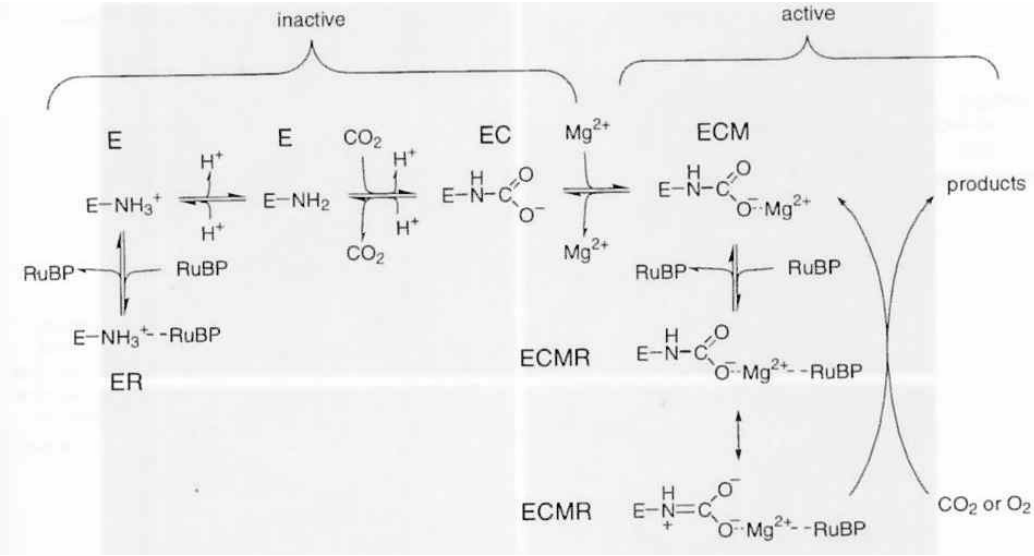
The activator CO_2 molecule becomes covalently attached as a carbamate to the amino group of a specific lysyl residue, lysine-201, within the active site (Lorimer, 1981). This carbamylation of lysine-201 occurs spontaneously at slightly alkaline pH (Lorimer & Miziorko, 1980) and converts the side chain of lysine-201 from a positive to a negative charge. This process creates a binding site (one of the resulting carbamino oxygen atoms) for Mg^{2+} that stabilises the carbamate and completes the active site of the enzyme (Andersson, 1996). Within the active site, carboxyl oxygen atoms of

aspartic acid-203 and glutamic acid-204 provide other ligands to Mg^{2+} (reviewed in Roy & Andrews, 2000).

Once activated, the reaction is ordered with RuBP binding before addition of the gaseous substrates, CO_2 or O_2 (Pierce *et al.*, 1986). The catalytic processes of carboxylation and oxygenation start with the preliminary enolization of RuBP. CO_2 or O_2 then compete for reaction with the resulting enediol. This evidence seems convincing that neither gas binds in the absence of RuBP, therefore, it generally has inferred that neither CO_2 nor O_2 forms a Michaelis complex with the enzyme (Roy & Andrews, 2000). Finally, a sequence of analogous intermediates, except for a final protonation that is lacking in the case of oxygenation, ends both catalytic processes (reviewed in Roy & Andrews, 2000).

Figure 10.4

The activation of Rubisco. Activation of Rubisco (E) by carbamylation of K201 (producing EC) followed by binding of Mg^{2+} (producing ECM). While RuBP may bind to either the carbamylated form (producing ECMR) or the non-carbamylated form (producing ER), only the carbamylated form is able to carry out catalysis. From Roy & Andrews (2000).



Carbamylation causes only minor changes in the conformation of the Rubisco L subunit (Schreuder *et al.*, 1993; Taylor & Andersson, 1996). In contrast, binding of RuBP and other phosphorylated ligands induces several major structural changes in and around the active site (Newman & Gutteridge, 1993; Schreuder *et al.*, 1993; Taylor & Andersson, 1996; Taylor *et al.*, 1996). Most obvious is a 12-Å shift of α/β barrel loop 6

from retracted (open) to an extended (closed) position (Schreuder *et al.*, 1993; Taylor & Andersson, 1996). A closed, desolvated active site would be necessary to protect the enediol from incorrect protonation (Edmondson *et al.*, 1990). The closed active site reopens for product release, however, conversion of Rubisco from the closed to the open conformation is extremely slow (Spreitzer & Salvucci, 2002). To facilitate the process, plants contain Rubisco activase, an ATP-dependent enzyme that releases tight-binding sugar phosphates from the Rubisco active site (reviewed in Portis, 1995; Salvucci & Ogren, 1996). The interaction almost certainly involves changing the conformation of Rubisco in a way that promotes opening of the closed configuration. Once activase opens a closed site, the sugar phosphate can dissociate and free the site for activation via spontaneous carbamylation and metal binding to start the next catalytic cycle (Werneke *et al.*, 1988; Wang & Portis, 1992).

10.2.7. Kinetic insights: Catalytic efficiency

An enzyme perfectly adapted for speed and specificity would convert its substrate as fast as it diffused into its active site and never mistake nonsubstrate for substrate. There are many examples of enzymes that approach this standard (Fersht, 1985). However, Rubisco, even in higher plants, has several catalytic disadvantages as an enzyme (Yokota, 1999):

- As discussed extensively above, Rubisco τ is very far from an infinite specificity for CO_2 relative to O_2.
- The reaction turnover rate (k_c^{cat}) is 2 to 5 s^{-1} per site, i.e. each site can catalyse three reactions per second (Horken & Tabita, 1999). It is 1/100 to 1/1000 the turnover number of enzymes found in nature. Because its slowness and since carbon reduction is the major sink for energy captured in photosynthetic electron transport (Sharkey, 1998), plants invest in large quantities of Rubisco, requiring a huge proportion of cellular nitrogen for Rubisco biosynthesis (Andrews & Lorimer, 1987). In fact, approximately 50% of soluble leaf protein is Rubisco (Roy & Andrews, 2000).
- The affinity of the enzyme for CO_2 (K_c) is 10 to 15 μM (Yokota, 1999), roughly equal to the concentration of CO_2 in aqueous solutions in equilibrium with air. However, the competitive inhibition by O_2 nearly doubles the effective K_c. Moreover, the leaf diffusion resistances lower the CO_2 level in the chloroplast

stroma by a half of that in the atmosphere. So, instead of working near the K_c, Rubisco is working at just one-quarter of its effective K_c, i.e. just a quarter of the enzyme can participate in photosynthesis (Sharkey, 1998). Therefore, Rubisco cannot exert its maximum ability to fix CO_2, since the concentration of CO_2 in the stroma is smaller than the Michaelis constant for CO_2 (Mizohata *et al*., 2002).

10.2.8. The temptation to engineer Rubisco

Genetic engineering provides powerful tools to enhance the modification of plants to the potential benefit of society. In this way, manipulating Rubisco to decrease the inhibitory effect of oxygen and its competitive involvement in reaction with RuBP, as opposed to reaction with CO_2, is a tempting target to increase the productivity of plants (Parry *et al*., 2003; Zhu *et al*., 2004). In particular, one major objective for Rubisco manipulation has been to alter the discrimination between the gaseous substrate gases, therefore, to alter τ. One problem with this manipulation of Rubisco in higher plants is derived from the complexity of the hexadecameric holoenzyme. This explains why many protein engireering projects have been conducted using cyanobacterial, algal and bacterial Rubiscos, for which assembly into the holoenzyme is less problematic (Parry *et al*., 2003). Logically, the extreme achievement may be the transference of sequences coding for the non-green algae forms of Rubisco, with the highest τ (Fig. 10.3), to the higher plant plastome. Unfortunately, although providing valuable information on the relationship between structure and function, this has not succeeded in providing a functional Rubisco (Whitney *et al*., 2001) because of problems in either folding or assembly of these quite evolutionary diverse forms. However, successful folding and assembly of Rubisco transferred from other higher plants has been achieved (Kanevski *et al*., 1999; Whitney & Andrews, 2001). These facts make reasonable, at least until enough is known on assembly and folding of both enzyme subunits, to concentrate efforts on transferring Rubiscos within higher plants. Once statistically meaningful variation in Rubisco τ among C_3 species has been confirmed (Gutteridge *et al*., 1986; Parry *et al*., 1989; Kent *et al*., 1992; Kane *et al*., 1994; Delgado *et al*., 1995; Kent & Tomany, 1995), the identification of higher plants with high Rubisco τ has become potentially an important step towards the improvement of the enzyme (Andrews & Lorimer, 1987). However, the exploration of natural variation in the kinetic properties of this enzyme among C_3 plants is not extensive and less than 100 species

have been analysed (most of them listed in Table 10.1) from a plant population in excess of 300 000 species. This limited sampling of C_3 plants may be a significant limitation to crop improvement, since Rubiscos from many major crops already have some of the highest specificity factors described up to now. Therefore, the first step must be to deeply explore the natural range among higher plants to identify species with high τ. Since it is evident that no survey is able to analyse Rubisco from all species, the sampling strategy becomes a crucial step. It is particularly important to study plants from an environment where there would be a particular advantage to the plant a Rubisco with a high τ (Delgado *et al*., 1995). It has been already mentioned that the hot and dry Mediterranean summer match some of the conditions hypothesised to increase the selection pressure on Rubisco for improved specificity.

Another way to increase the natural range of τ variation has been mutagenic techniques, which enabled potential substitutions in DNA encoding both large and small subunits to be evaluated *in vitro*. However, so far, efforts to increase Rubisco-limited photosynthetic rate by increasing τ via directed mutagenesis have had little success (Chène *et al*., 1992; Romanova *et al*., 1997; Madgwick *et al*., 1998; Ramage *et al*., 1998). Most mutants exhibit a lower τ than the wild-type from which they were obtained (Bainbridge *et al*., 1995; Parry *et al*., 2003).

Once a species with optimum kinetic characteristics has been identified, sequencing of both the large and the small subunits has to be performed. Then, from the comparison of amino acid sequences between this species and crops, potentially relevant differences which may confer key catalytic properties of the enzyme, such as τ, have to be analysed by 3-D structures modelling, which might ratify the biochemical differences. Finally, either the *Agrobacterium* (Horsch *et al*., 1985) or the ballistic (Svab & Maliga, 1993) methods have been proved to provide enough tools to allow chloroplastic and plastome transformation of Rubisco.

Nevertheless, when the kinetic properties of different Rubiscos are modelled into the situation of the higher-plant chloroplast, it becomes obvious that more efficient catalytic performance under physiological conditions is not a simple function of any single kinetic parameter (Whitney *et al*., 2001). In this way, a "perfect" Rubisco would have: (1) a k_c^{cat}/K_c quotient of at least 10^8 M^{-1} s^{-1}, (2) a K_c lower than the concentration in the chloroplast stroma and (3) infinitely large τ (Andrews & Whitney, 2003). After analysing the effect of these variables on the dependency of the photosynthetic CO_2

assimilation rate on the chloroplast stromal CO_2 partial pressure modelled according to Farquhar *et al*. (1980), it has been concluded that the ability to discriminate between CO_2 and O_2, i.e. τ, can, nevertheless, confer better performance when the CO_2 concentration is very low (Whitney *et al*., 2001). These are, in fact, the concentrations of CO_2 typically found under the dry Mediterranean summer and many other regions. Moreover, water scarcity is likely to increase world-wide and therefore water availability for agricultural purposes will become one of the greatest limitations to increasing productivity (Araus, 2004). In addition, species with improved Rubisco kinetics would result in increased water-use efficiency, currently a priority for the United Nations policy, what is called the 'Blue Revolution' and summarized as 'more crop per drop' (Annan, 2000).

In conclusion, drought conditions strongly influence photosynthetic metabolism and in a way that might be extremely important to determine positive carbon balance in highly stressed environments, such as most of the Mediterranean habitats. Therefore, it is reasonable to hypothesise that, as has been demonstrated for other morphological and physiological traits, plants could have evolved a better, more efficient enzymatic machinery. Because of its central role in photosynthesis, Rubisco is one of the potential traits to be selected in such stressing conditions. In consequence, the main objectives addressed in the present chapter are:

1. To explore more deeply the natural variability of τ, increasing the number of taxa for which its value is known.
2. To elucidate the dependency of τ on ecological (growth form and habitat xericity), phylogenetic and evolutionary (endemicity) factors.
3. To explore the temperature dependency of the catalytic activities of Rubiscos from different sources.
4. To discern the differences in the tertiary protein structure that may account for the differences in τ between species with a high τ and other plants.
5. If an adaptation (i.e. in the long term scale) of τ to environmental stresses such as drought and temperature is found, to check whether plants can also acclimate (i.e. in the short term) their Rubisco to these stresses.

10.3. MATERIAL AND METHODS

The present chapter consisted in three experiments, related to the objectives mentioned before.

- Experiment 1: τ was measured on purified Rubiscos from 24 C_3 Mediterranean species having a variety of ecological, phylogenetic and morphological traits.

- Experiment 2: consisted in sequencing the *rbc*L and *rbc*S of *Limonium gibertii*.

- Experiment 3: τ was determined in leaves of *Nicotiana tabacum* developed under different water stress conditions.

10.3.1. Experiment 1

10.3.1.1. Plant material

Twenty-four dicotyledonous species (Table 10.2) from different communities in the Balearic Islands were selected for study. The criteria used to select these species were based on their evolutionary history, ecological characters and phylogenetic relationships. For evolutionary history, species were classified into: endemic species, those only occurring in the Balearic Islands, and non-endemic species, species that are not restricted to the Balearic Islands. Two different criteria were used to classify the species with respect to their ecology. Firstly, species were classified depending on their growth form into: herbaceous annuals, herbaceous evergreens, woody semi-deciduous and woody evergreen species. Herbaceous annual species comprised all non-woody species that complete their life cycle in one year. Herbaceous evergreen species comprised all non-woody species that maintain functional leaves during the whole year. Woody semi-deciduous species comprised all woody species that lose a certain amount of their leaves during the unfavourable season, depending on its length and severity. Woody evergreen species comprised all woody species that maintain their leaves during the whole year. The second ecological classification was made on the basis of habitat xericity. Group 1 comprised the species inhabiting the coastal, driest and hottest areas with annual precipitation typically below 400 L m^{-2}. Species typical of Mediterranean macchia with annual precipitation typically between 400 and 800 L m^{-2} were classified in group 2 together with some ruderal species. Group 3 comprised species inhabiting the wettest and coolest mountain areas with annual precipitation above 800 L m^{-2}, species growing only near open water sources, and species maintaining their leaves only during the wet season. Species were finally plotted in a phylogenetic tree from the specific

categories of genera and families to the more general categories of orders and subclasses.

Table 10.2

List of the species analysed, with their evolutionary history, growth form and xericity.

Species	Evolutionary history	Growth form	Xericity index
Diplotaxis ibicensis Pau	Endemic	Herb annual	1
Urtica atrovirens subsp. *bianorii* (Knoche) Paira	Endemic	Herb annual	3
Pimpinella bicknelli Briq.	Endemic	Herb annual	3
Paeonia cambessedesii Willk.	Endemic	Herb annual	3
Beta maritima L. subsp. *marcosii* A. Juan & M. B. Crespo	Endemic	Herb evergreen	1
Crepis triasii (Camb.) Nyman	Endemic	Herb evergreen	2
Lysimachia minoricensis J. J. Rodr.	Endemic	Herb evergreen	3
Digitalis minor L. *var. palaui* (G. Font) Hinz & Rosselló	Endemic	Herb evergreen	3
Digitalis minor L. *var. minor*	Endemic	Herb evergreen	3
Phlomis italica L.	Endemic	Woody semi-deciduous	2
Limonium magallufianum L. Llorens	Endemic	Woody evergreen	1
Rhamnus ludovici-salvatoris R. Chodat	Endemic	Woody evergreen	2
Hypericum balearicum L.	Endemic	Woody evergreen	2
Urtica membranacea Poiret	Non-endemic	Herb annual	2
Kundmannia sicula (L.) D. C.	Non-endemic	Herb annual	2
Helleborus foetidus L.	Non-endemic	Herb annual	3
Beta maritima L. subsp. maritima	Non-endemic	Herb evergreen	1
Mentha aquatica L.	Non-endemic	Herb evergreen	3
Lavatera maritima Gouan	Non-endemic	Woody semi-deciduous	1
Cistus albidus L.	Non-endemic	Woody semi-deciduous	2
Limonium gibertii (Sennen) Sennen	Non-endemic	Woody evergreen	1
Limonium virgatum (Willd.) Fourr.	Non-endemic	Woody evergreen	1
Rhamnus alaternus L.	Non-endemic	Woody evergreen	2
Pistacia lentiscus L.	Non-endemic	Woody evergreen	2

Most of the plant species were grown from seed. Specimens of *M. aquatica*, *D. minor* var. *minor*, *D. minor* var. *palaui* and *C. triasii* were collected in the field and propagated asexually. The resulting plants were grown in a glasshouse at Rothamsted Research, UK, with supplementary lighting to give a photoperiod of 16 h. The temperature was 25ºC during the light period and 18ºC during the dark period. Growth was in soil-based compost supplemented with slow release fertilizer. Water was added sparingly by hand. For *L. virgatum*, *R. alaternus*, *R. ludovici-salvatoris*, *P. bicknelli*, *P. cambessedesii* and *H. foetidus*, young mature leaves were collected directly in the field and transported to England packed in dry ice.

10.3.1.2. Extraction and purification of Rubisco

Young but mature leaves (30-50 g) from each species were immediately frozen in liquid nitrogen. The leaf material was ground to a powder in a mortar, buffer was added and grinding continued from time to time as the mixture thawed. After extensive preliminary tests, the most appropriate protein extraction media for Rubisco were found to be: (A) 0.1 M bicine, 50 mM β-mercaptoethanol, 11 mM Na-DIECA, 6% (w/v) PEG 4000, 1 mM benzamidine, 1 mM ε-amino-n-caproic acid and 1 mM PMSF, at pH 8, and (B) containing 0.1 M HEPES, 3% (w/v) PVP 25, 6% (w/v) PEG 4000, 50 mM β-mercaptoethanol, 2 mM DTT, 10% glycerol, 5 mM $MgCl_2$, 5 mM EGTA and 2 mM PMSF, at pH 8.0. Buffer A was used to extract Rubisco from most of the species. Buffer B was used with *M. aquatica*, *P. lentiscus* and *C. albidus*. For *H. balearicum*, buffer A was used with the following modifications: the concentration of bicine was increased to 0.2 M and the volume used was doubled. For *P. lentiscus*, the following modifications were made to buffer B: the concentration of HEPES was increased to 0.25 M and the volume used was doubled.

All the purification steps were carried out at 0 to 4 ºC. Fully thawed but still cold homogenates were filtered through butter muslin and then centrifuged at 22000 x g for 20 min. The supernatant liquid was decanted through 50 μm mesh nylon and PEG 4000 was added as a 60% (w/v) aqueous solution to the supernatant liquid to produce a final concentration of 20% (w/v). Also, 1 M $MgCl_2$ was added to a final concentration of 20 mM followed by gentle mixing. After standing for 10 min the mixture was centrifuged again at 22000 x g for 20 min. The pellet was re-suspended in 40 mL of column buffer (10 mM Tris pH 8.0 with 10 mM $MgCl_2$, 10 mM $NaHCO_3$, 1 mM EDTA and 1 mM KH_2PO_4) containing 1mM each of PMSF, benzamidine and ε-amino-n-caproic acid. The suspension was centrifuged to remove insoluble material. The supernatant liquid was applied to an 88 x 1.6 cm column of Q Sepharose Fast Flow anion exchanger (Pharmacia) previously equilibrated with column buffer and operated at 1 ml min^{-1}. The effluent was monitored for absorbance at 280 nm. The proteins were eluted using a linear gradient from 0 to 0.75 M NaCl in column buffer in 16 h and fractions were collected at 10 min intervals. Those fractions with high Rubisco activity were combined and de-salted using a Sephadex G25 (Pharmacia) column 44 x 5 cm operated at 200 ml h^{-1} with fractions collected at 3 min intervals. Finally, fractions containing high amounts of protein were pooled together, the concentration of Rubisco was estimated as A_{280} x

0.61 mg ml^{-1} (Paulsen & Lane, 1966), and the solution dispensed into vials and freeze dried.

10.3.1.3. Rubisco activity measurements

Rubisco activity was measured at different stages of the purifications by adding 10 or 25 µl of solution containing protein to 0.2 ml of a solution containing 1 ml 0.1 M $NaH^{14}CO_3$, 0.5 μC_i per µmol, 5 mL 0.2 M bicine containing 40 mM $MgCl_2$ pH 8.2 and 4 ml H_2O. After 3 min 10 µl 20 mM RuBP was added and after a further 1 min the reaction was stopped by adding 0.1 ml of 10 M formic acid. To activate the slowly activating form of Rubisco present in solution after desalting, reaction mixtures, less the RuBP, were heated at 37°C for 40 min and then cooled to room temperature before adding the RuBP and completing the assay. The acidified reaction mixes were dried down in an oven placed in a fume hood. After cooling, 0.4 ml of H_2O and 3.5 ml of Ultima Gold scintillation cocktail (Packard, Canberra, Australia) were added. ^{14}C in PGA (D-3-phosphoglycerate) was measured using a Scintillation Spectrometer.

10.3.1.4. Specificity factor determinations

For Rubisco from each species, between 6 and 12 measurements of specificity factor were made as follows. For measurements at 25°C the freeze-dried Rubisco samples from the 24 species were dissolved and desalted by centrifugation through G25 Sephadex columns (Helmerhorst & Stokes, 1980) previously equilibrated with CO_2-free 0.1 M bicine pH 8.2 containing 20 mM $MgCl_2$. The desalted solutions were made 10 mM to $NaH^{14}CO_3$ and 0.4 mM to orthophosphate. These mixtures were incubated at 37°C for 40 min to activate the Rubisco. Reaction mixtures were prepared in an oxygen electrode (Model DW1, Hansatech, Kings Lynn., UK) by first adding 0.95 ml of a solution of 100 mM bicine pH 8.2, 10 mM $MgCl_2$ containing 1.5 mg (7000 W-A units) per 100 ml of carbonic anhydrase and equilibrated with CO_2-free air at 25ºC. After adding 0.02 ml of 0.1 M $NaH^{14}CO_3$ the plug was fitted to the oxygen electrode vessel. Enough activated Rubisco was then added in 20 µl for the reaction to be completed within 5 min. The reaction was started by the addition of 10 µl of 15 mM RuBP to give a total reaction volume of 1 cm^3. RuBP oxygenation was calculated from the oxygen consumption and carboxylation from the amount of ^{14}C incorporated into PGA when all the RuBP had been consumed (Parry *et al.*, 1989). A sequence of reaction mixtures

containing pure wheat Rubisco were interspersed with those containing Rubisco from the test species and the results normalised to the average value obtained from wheat Rubisco, 100.0 at 25ºC.

In addition, 14 species were selected for measurement of τ at 15ºC and 35ºC. The procedure followed was the same as at 25ºC except that the buffer was prepared and equilibrated with CO_2–free air at 15 or 35ºC as appropriate and the volume of 0.1 M $NaH^{14}CO_3$ used was 0.015 and 0.03 ml respectively. The total volume of reaction mixtures was again 1 cm^3. The results were normalised to the average values for wheat Rubisco, 139.6 at 15ºC and 77.1 at 35ºC. From the slopes of the regressions between ln τ and 1000/RT, the difference in the free energy of activation to the transition state intermediates for the oxygenase and the carboxylase reactions of Rubisco ($\Delta G_o^{\ddagger} - \Delta G_c^{\ddagger}$) was calculated according to Uemura *et al*. (1997).

10.3.1.5. Estimation of the CO_2 concentration at the active site of Rubisco

The CO_2 concentration at the active site of carboxylation (C_c) was estimated under different degrees of water availability in the following species: *C. albidus*, *P. italica*, *H. balearicum*, *P. lentiscus*, *D. ibicensis*, *L. maritima*, *B. maritima* subsp. *marcosii*, *B. maritima* subsp. *maritima*, *L. magallufianum* and *L. gibertii*. The experimental design, treatments and methods used to estimate C_c are shown in Chapter 7.

10.3.1.6. Statistical analysis

An analysis of variance (Manugistics, 1998) was made for τ with the following treatments: species at 15ºC, 25ºC and 35ºC, evolutionary history, growth form, xericity index and phylogeny (from genera to classes). Finally, an analysis of variance was made for $\Delta G_o^{\ddagger} - \Delta G_c^{\ddagger}$ in each species. Duncan tests at the 95% confidence limit were used to separate the means for τ and $\Delta G_o^{\ddagger} - \Delta G_c^{\ddagger}$ within each treatment.

10.3.2. Experiment 2

10.3.2.1. Plant material

Seeds of *Limonium gibertii* were collected in the field from natural populations and germinated on filter paper moistened with deionized water in a controlled environment (germination chamber, at 18ºC in darkness). After germination and

emergence of one true leaf, seedlings were transplanted to in large pots (20 cm height, 4.1 L volume) and grown in a glasshouse at Rothamsted Research, UK, with supplementary lighting to give a photoperiod of 16 h. The minimum temperature was 25ºC in the photoperiod and 18ºC in the dark. Growth was in soil-based compost supplemented with slow release fertilizer. Water was added sparingly by hand.

Young, mature leaves were collected and immediately submerged in *RNAlater* (Ambion) and stored at –70ºC until nucleic acids extraction.

10.3.2.2. cDNA of *rbc*S

Protocols described by Gehrig *et al*. (2000) and Doyle & Doyle (1987) were modified to increase the efficiency in the extraction of total RNA from plants, such as the Mediterranean species, with high amount of polyphenols and polysaccharides, which make difficult the isolation of RNA (Loomis, 1974).

Leaf samples were ground to a powder under liquid in a mortar, and the following extraction buffer was added: 0.5 M Tris (pH 8.0), 0.3 M LiCl, 10 mM DTT, 10 mM EDTA, 1 % (v/v) nonidet pyrrolidone 40000, 5 mM urea and 2 % (v/v) PEG 20000. Sample and buffer were mixed by vortex and, after incubating at 60ºC for 40 min, were centrifuged 13000 rpm for 10 min at 4ºC. The supernatant was then transferred to a clean tube and 1 ml TRizol Reagent was added. After mixing and incubating for 5 min at room temperature, chloroform was added and samples were mixed by inverting tubes. To precipitate the RNA, isopropanol and 1.2 M NaCl / 0.8 M Na-citrate were added to the supernatant and samples were incubated at –20ºC for at least 1 h, before centrifuging at 13,000 rpm for 25 min at 4ºC. Finally, the RNA pellet was washed with 70% ethanol, dissolved in diethylpyrocarbonate-treated water and quantified at 260:280 nm.

Reverse transcription was performed using a SuperScript^{TM} II RNase H^{-} Reverse Transcriptase (Invitrogen Co., Carlsbad, USA). For each reaction, first-strand cDNA was synthesized in 20 µl containing 2 µl Oligo $(dT)_{12\text{-}18}$ (about 500 µg/ml), 4 µg total RNA, 1 µl of 10 mM dNTP, 4 µl of 5× RT buffer, 2 µl 0.1 M DTT, 1 µl of SuperScriptRT, and 7 µl of diethylpyrocarbonate-treated water. To start the reaction, the mix was incubated at 42°C for 2 min. For the PCR reaction, 2 µl of cDNA from the RT reaction was added to *Taq* DNA polymerase (Invitrogen; 2.5 units *Taq* DNA polymerase), PCR buffer (20 mM Tris-HCl, pH 8.4 and 50 mM KCl), 1.5 mM $MgCl_2$,

0.2 mM dNTP, and specific primers (0.5 μM each primer: $(dT)_{20}$ and L5) in a total volume of 50 μl.

To design the specific primer, no *rbc*S from *Limonium* was found on the databases, so taxonomy was used to consider other closely related species. Thus, this primer was designed from known sequences of other *Caryophyllales* species (GenBank), in conservative regions, as follows:

L5: 5' -GGT GG(AC) A(AG)A GTC C(AG)(AG) TGC ATG CAG GT(AG) TGG CC-3'

The PCR program consisted of 2 min at 95°C, followed by 34 denaturation cycles of 15 s at 95°C, annealing at 54°C to 59°C for 15 s, and extension at 72°C for 1 min and 30 s. The final extension step was for 5 min at 72°C.

10.3.2.3. DNA of *rbc*L

The following adaptation of the protocol described by Doyle & Doyle (1987) was used to extract DNA.

Leaf samples were ground in a mortar with the following extraction buffer: 100 mM Tris-HCl (pH 8), 1.4 M NaCl, 20 mM EDTA and 2% CTAB. After incubating the homogenate at 60°C for 45 min, chloroform:isoamyl alcohol (24:1) was added and mixed by vortex. The mixture was centrifuged at 13000 rpm for 10 min. Two volumes of ethanol 96% were added to the upper aqueous layer and solution was mixed by inverting tubes. After incubation at -20°C for at least 20 min, DNA was precipitated with centrifugation at 13000 rpm for 15 min. DNA pellet was washed with cold ethanol 70% and centrifuged again at 13,000 rpm for 10 min, to finally be resuspended in destilled water. DNA quantity and quality were checked at 260:280.

For the PCR reaction, 10 μl DNA solution was added to *Taq* DNA polymerase (Invitrogen; 2.5 units *Taq* DNA polymerase), PCR buffer (10 mM Tris-HCl, pH 9, 50 mM KCl and 0.1% Triton X-100), 1.5 mM $MgCl_2$, 0.4 mM dNTP, and specific primers (0.4 μM each primer: ATP1 and ACC1; Fig. 3.10) in a total volume of 50 μl.

The PCR program consisted of 2 min at 95°C, followed by 9 denaturation cycles of 15 s at 95°C, annealing at 49°C for 15 s, and extension at 72°C for 3 min and 20 s. Then it was proceed to 34 cycles of denaturation at 95°C for 15 s, annealing at 56°C for 15 s, and extension at 72°C for 3 min and 20 s. The final extension step was for 5 min at 72°C.

Figure 3.11

Location of the designed primers in the *rbc*L and neighbouring genes *atp*B and *acc*D. Primers sequences were as follows:

L1: GGA ATT CAC CCT AGA TAC TGA TAT CTT GG

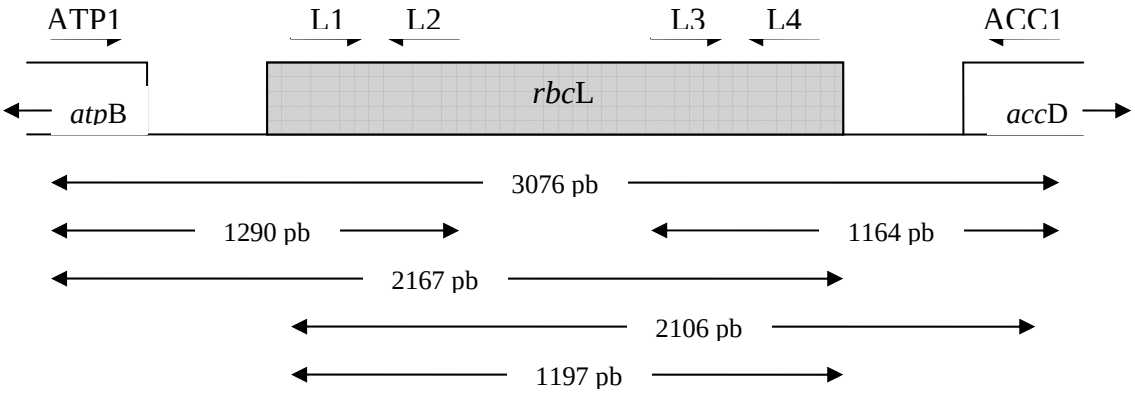
L2: ATC TTC CAA ACG TAG AGC ACC

L3: GTT TGT TGA TTT ACT ACG TGA TG

L4: GGA ATT CAA GTA CAC AGG CTT CTA GAG C

ATP1: GGA ATT CGG ATC CGC TAC (A/G)TC CAG TAC (C/T)GG ACC (A/G)AT (A/G)AT TTG

ACC1: GGA ATT CGG ATC CTC GAA TT(A/G) AAC CAC CAT TTT T(T/C)C ATA GAG C



10.3.2.4. PCR products purification

Both *rbc*L and *rbc*S DNA were purified from PCR using Wizard PCR Preps DNA Purification System Kit (Promega Co., Madison, USA) following manufacturer's instructions. However, *rbc*S DNA was purified from the agarose gel bands and *rbc*L DNA directly from PCR mixture.

10.3.2.5. Cloning and transformation

Cloning and transformation were performed with the TOPO TA Cloning Kit for Sequencing (Invitrogen) following manufacturer's instructions. Both genes were cloned with the pCR4-TOPO plasmid (Invitrogen) and transformed in *E. coli* Mach1^{TM}-T1^{R} (Invitrogen).

10.3.2.6. Plasmid purification

Plasmid purification from *E. coli* Mach1^{TM}-T1^{R} was performed with the Wizard Plus Minipreps DNA Purification System Kit (Promega) following manufacturer's

instructions. The analysis of positive transformants was performed with both PCR and enzymatic digestion with EcoRI.

10.3.2.7. Sequencing

Sequencing reactions were set up with approximately 300 ng of DNA, 3.2 pmole primer, 4 μl BigDye v3.1 Ready Reaction mix (Applied Biosystems, Foster City, USA) and 2 μl 5x reaction buffer. Mixtures were cycled on the following PCR program: 1 min at 96°C, followed by denaturation cycles of 10 s at 96°C, annealing at 50°C for 5 s, and extension at 60°C for 4 min. It was proceed to 25 cycles.

After PCR cycling, DNA was precipitated with NaOAc and ethanol, and pellets were sent to the Department of Biochemistry of the University of Oxford to be sequenced.

10.3.3. Experiment 3

10.3.3.1. Plant material and treatments

Thirty seeds of *Nicotiana tacabum* var. White Burley were germinated and grown individually in pots (20 cm height, 4.1 L volume) containing a mixture of clay-calcareous soil, horticultural substrate and perlite (40:40:20). The experiment was performed during spring 2004 inside a greenhouse located at the University of the Balearic Islands (Mallorca, Spain). Plants were randomly distributed in growing and spread out to avoid mutual shading and to give similar light and temperature.

All seedlings were well-watered until they had four fully expanded leaves and, presumably, an adequate root system to cope with water constraints. Irrigation treatments were started on 22-April-04. Ten such plants were randomly selected for each of the irrigation treatments: a) maintained at field capacity throughout the experiment (control treatment, C), b) maintained at 40% field capacity (moderate drought treatment, MoD), and c) maintained at 15% field capacity (severe drought treatment, SD). Desired moisture levels were attained by allowing the soil to dry until close to the selected moisture level, as determined gravimetrically weighting pots on alternate days and, from then on, compensating their daily water losses with the addition of an equal amount of 50% Hoagland's solution.

New leaves were allowed to develop and expand under the three irrigation treatments until 15-June-04. Then, leaves developed during irrigation treatments (i.e.,

acclimated to different water availability) were sampled to determine the specific leaf weight (SLW), the relative water content (RWC), τ, total leaf soluble protein, Rubisco activity, gas exchange and chlorophyll fluorescence.

10.3.3.2. Rubisco purification and specificity factor measurement

Leaves (30-50 g) of each treatment (C, MoD and SD) were collected and immediately frozen in liquid nitrogen. The leaf material was ground to a powder in a mortar, buffer was added and grinding continued from time to time as the mixture thawed. The protein extraction media used contained: 0.1 M bicine, 10 mM Na-DIECA, 6% PEG (polyethylene glycol) 4000, 3% (w/v) PVP (polyvinylpyrrolidon) 25000, 1 mM DTT (dithiothreitol), 1 mM benzamidine, 1 mM ε-amino-n-caproic acid and 1 mM PMSF (phenylmethylsulphonylfluoride), at pH 8.

All the purification steps were carried out at 0 to 4ºC. Fully thawed but still cold homogenates were filtered through butter muslin and centrifuged at 18,000 x g for 20 min. The supernatant liquid was decanted through 50 μm mesh nylon and PEG 4000 was added as a 60% aqueous solution to the supernatant liquid to produce a final concentration of 20% w/v. Also, 1 M $MgCl_2$ was added to a final concentration of 20 mM followed by gentle mixing. After standing for 10 min the mixture was centrifuged again at 18,000 x g for 20 min. The pellet was re-suspended in 6 ml of column buffer (10 mM Tris pH 8.0 with 10 mM $MgCl_2$, 10 mM $NaHCO_3$, 1 mM EDTA and 1 mM KH_2PO_4) containing 1 mM each of DTT, PMSF, benzamidine and ε-amino-n-caproic acid. The suspension was then centrifuged to remove insoluble material. The supernatant liquid was layered onto step gradients from 1.2 to 0.4 M in sucrose in column buffer. Gradients were centrifuged at 50 000 rpm for 120 min in a 70.1Ti rotor (Beckman, High Wycombe, UK). Fractions with high protein concentration were combined and applied to two 1 ml HiTrap Q HP columns (Amersham Biosciences) connected in series previously equilibrated with column buffer and operated at 1 ml min^{-1}. The proteins were eluted using a step gradient from 0 to 0.8 M NaCl in column buffer and fractions were collected in 1 ml intervals. Total soluble protein content in fractions was confirmed using Bradford assay (Bradford, 1976). Those fractions with high protein (Rubisco) concentration were combined and stored at –70ºC.

Rubisco-rich fractions were used to make 8 to 10 measurements of Rubisco specificity factor (τ) per treatment, which were made at 25ºC as in the Experiment 1,

with the following differences: the oxygen electrode was a Dual digital Model 20 (Rank Brothers Ltd., Cambridge, UK); 0.93 ml 100 mM bicine pH 8.2, 10 mM $MgCl_2$ containing 1.5 mg (7000 W-A units) per 100 ml of carbonic anhydrase and equilibrated with CO_2-free air at 25ºC were added; activated Rubisco was added in 40 µl; and the average τ value obtained from wheat Rubisco and used to normalize tobacco results was 102.5 at 25ºC.

10.3.3.3. Rubisco carboxylase activity and total soluble protein

For Rubisco carboxylase activity, 3 to 4 samples per treatment were ground to a fine powder in a mortar, previously chilled with liquid nitrogen and homogenised in 1 ml of an ice-cold extraction medium. The extraction medium was the same used for Rubisco purification for τ measurements. Extracts were clarified by centrifugation (12,000 rpm at 4ºC for 2 min) and the supernatant immediately assayed at 25ºC for Rubisco activity. The initial and total activities were determined according to Parry *et al*. (1997). Total soluble protein was determined according to the method of Bradford (1976).

10.3.3.4. Relative water content and specific leaf weight

RWC at mid morning was determined as follows: RWC = (Fresh weight − Dry weight) / (Turgid weight – Dry weight) × 100. To determine the turgid weight of the leaves, these were kept in distilled water in darkness at 4°C to minimise respiration losses, until they reached a constant weight (full turgor, typically after 24 h). Their dry weight was obtained after 48 h at 70ºC in an oven. Six replicates per treatment were obtained.

SLW was calculated, in six replicates per treatment, as the ratio of dry mass to leaf area. Leaf area was determined in fresh leaves using an AM-100 leaf area meter (ADC, Herts, UK).

10.3.3.5. Gas exchange and chlorophyll fluorescence measurements

Leaf gas exchange parameters were measured simultaneously with measurements of chlorophyll fluorescence using an open gas exchange system (Li-6400; Li-Cor, Inc., Nebraska, USA) with an integrated fluorescence chamber head (Li-6400-40 leaf chamber fluorometer; Li-Cor, Inc.).

In light adapted leaves, the actual photochemical efficiency of photosystem II ($\Delta F/F_m$') was determined by measuring steady-state fluorescence (F_s) and maximum fluorescence during a light-saturating pulse of ca. 10 000 μmol m^{-2} s^{-1} (F_m'), following the procedures of Genty *et al*. (1989):

$$\Delta F/F_m' = (F_m'-F_s)/F_m'$$

The electron transport rate (ETR) was then calculated as:

$$ETR = \Delta F/F_m' \cdot PPFD \cdot \alpha \cdot \beta$$

where PPFD is the photosynthetically active photon flux density and $\alpha \cdot \beta$ is a term which includes the product of leaf absorptance and the partitioning of absorbed quanta between photosystems I and II. $\alpha \cdot \beta$ was previously determined for each treatment as the slope of the relationship between $\Delta F/F_m$' and ϕ_{CO2} obtained by varying light intensity under non-photorespiratory conditions in an atmosphere containing less than 1% O_2 (Valentini *et al*., 1995). $\alpha \cdot \beta$ resulted 0.357, with no difference between treatments.

All measurements were started at 25ºC and at 1500 μmol m^{-2} s^{-1} to ensure light saturation, with 10% blue light. Cuvette CO_2 concentration (C_a) was set at 400 μmol CO_2 mol air^{-1} and the vapour pressure deficit was maintained between 1.0 and 1.5 kPa. After inducing photosynthesis under the above conditions and once steady-state was reached, photosynthesis response curves to varying sub-stomatal CO_2 concentration (C_i) were obtained. First, the C_a was lowered stepwise from 400 to 50 μmol mol^{-1} and then fixed again at 400 μmol mol^{-1} until reaching a steady-state value similar to that obtained at the beginning of the curve. Then, C_a was increased stepwise from 400 to 1500 μmol mol^{-1}. Gas exchange measurements were determined at each step after maintaining the leaf for at least 5 min at the new C_a. Measurements consisted in 12-13 measurements for each curve. From the linear relationship at low C_i between gross photosynthesis (i.e., the sum of A_N and R_L) and C_i, Γ* was calculated (Long & Bernacchi, 2003). Mitochondrial respiration (R_D) was measured at the same temperature and CO_2 concentration in the same leaves after keeping them for 30 min in darkness.

From combined gas-exchange and chlorophyll fluorescence measurements, the CO_2 concentration at the site of carboxylation (C_c) and the mesophyll conductance (g_i) were estimated in all three treatments as described by Epron *et al*. (1995). For such calculations, the *in vitro* Rubisco specificity factor was considered to be 99.3, which was the mean of the values obtained for the three treatments (see Results).

10.3.3.6. Statistical analysis

Differences between means were revealed by Duncan analyses ($P < 0.05$) performed with the SPSS 12.0 software package (SPSS, Chicago, USA).

10.4. RESULTS

10.4.1. Experiment 1

10.4.1.1. Rubisco extraction

Amounts and specific activities of purified Rubiscos from the different species of plant varied. The Rubisco from *D. minor* var. *minor* and *D. minor* var. *palaui* had particularly low specific activities. In general, Rubisco specific activity and amount decreased through the purification steps, but the amount of fresh leaf material initially used (about 40 g) always yielded sufficient Rubisco and activity for τ determination. The highest specific activity obtained at the end of the purification procedure was from *T. aestivum* (0.70 μmol CO_2 min^{-1} mg^{-1} protein), and the lowest from *D. minor* var. *minor* (0.05 μmol CO_2 min^{-1} mg^{-1} protein).

Recovery of Rubisco activity depended on the species. *L. maritima*, the two *Digitalis* and *P. bicknelli*, lost about 90% or so of their enzymatic activity from the crude extract to the G25 fractions. Nevertheless, other species maintained or even increased the Rubisco activity through the purification steps (*T. aestivum*, *R. ludovici-salvatoris*, *M. aquatica* and *L. minoricensis*). If the extreme values are omitted, most activity was lost on the Q Sepharose Fast Flow column (54.1 ± 5.1% of Rubisco activity was recovered). Losses of activity in the remaining purification steps was significantly lower (25.6 and 16.7%).

10.4.1.2. Species variation in specificity factor at 25ºC

Table 10.3 shows τ measured at 25ºC. The values ranged from 88.7 for *H. foetidus* to 110.5 for *L. gibertii*. Only Rubiscos from two *Limonium* species, *L. gibertii* and *L. magallufianum*, had significantly higher τ than that of wheat ($P < 0.05$). Rubiscos from *U. membranacea*, *P. italica*, *L. virgatum*, *P. lentiscus* and *M. aquatica* did not differ significantly from wheat τ ($P < 0.05$), while the remaining seventeen species had significantly lower values ($P < 0.05$).

Table 10.3

List of the species analysed, with their τ measured at 25 ºC. Different letters denote statistical differences at $P < 0.05$ by Duncan Analysis.

Species	**τ at 25ºC**
Diplotaxis ibicensis Pau	95.6 ^{efg}
Urtica atrovirens subsp. *bianorii* (Knoche) Paira	90.2 ^{abc}
Pimpinella bicknelli Briq.	92.2 ^{abcde}
Paeonia cambessedesii Willk.	94.1 ^{defg}
Beta maritima L. subsp. *marcosii* A. Juan & M. B. Crespo	96.3 ^{fg}
Crepis triasii (Camb.) Nyman	94.8 ^{defg}
Lysimachia minoricensis J. J. Rodr.	93.8 ^{cdefg}
Digitalis minor L. var. *palaui* (G. Font) Hinz & Rosselló	97.1 ^{g}
Digitalis minor L. var. *Minor*	91.0 ^{abcd}
Phlomis italica L.	100.8 ^{hi}
Limonium magallufianum L. Llorens	106.1 ^{j}
Rhamnus ludovici-salvatoris R. Chodat	94.4 ^{defg}
Hypericum balearicum L.	93.6 ^{cdefg}
Urtica membranacea Poiret	102.4 ^{i}
Kundmannia sicula (L.) D. C.	89.2 ^{ab}
Helleborus foetidus L.	88.7 ^{a}
Beta maritima L. subsp. *maritima*	92.9 ^{bcdef}
Mentha aquatica L.	97.2 ^{gh}
Lavatera maritima Gouan	92.5 ^{abcdef}
Cistus albidus L.	92.1 ^{abcde}
Limonium gibertii (Sennen) Sennen	110.5 ^{k}
Limonium virgatum (Willd.) Fourr.	100.7 ^{hi}
Rhamnus alaternus L.	94.7 ^{defg}
Pistacia lentiscus L.	97.2 ^{gh}

Significant differences in τ were found for Rubisco from species within the same genus. Within the *Limonium* genus, τ differed significantly ($P < 0.05$) between the three species analysed at 25ºC, *L. gibertii* (110.5), *L. magallufianum* (106.1) and *L. virgatum* (100.7). Even larger differences were observed for Rubiscos from the *Urtica* genus, with τ of 102.4 for *U. membranecea* and 90.2 for *U. atrovirens* subsp. *bianorii*. Statistically significant differences in τ were indeed found even between Rubiscos from two varieties of a single species, *D. minor*, 91.0 for *D. minor* var. *minor* and 97.1 for *D. minor* var. *palaui* ($P < 0.05$). By contrast, no significant differences were observed between the two species of *Beta* analysed.

10.4.1.3. Ecological, phylogenetical and evolutionary influences on specificity factor

Non-significant differences in τ were observed between endemic and non-endemic species of the Balearic Islands at any of the temperatures analysed (data not shown). Even if the values obtained by Delgado *et al*. (1995) for other Balearic species were included in the analysis, the differences still remained non-significant.

By contrast, significant differences were observed in τ for Rubiscos from plants of different growth forms. In woody evergreen species τ averaged 99.5, which was significantly higher ($P < 0.05$) than τ from both herb annual and herb evergreen species (93.5 and 95.5, respectively, Table 9.4). Woody semi-deciduous species presented intermediate values (96.6). Therefore, in general, woody species showed higher τ values than herbs.

Table 10.4

τ variation among growth form and xericity groups. Different letters denote statistical differences at $P < 0.05$ within each criterion by Duncan Analysis.

Growth form	**τ at 25ºC**
Herb annual	93.5 [a]
Herb evergreen	94.5 [ab]
Woody semi-deciduous	96.6 [bc]
Woody evergreen	99.5 [c]
Xericity index	
1	98.7 [c]
2	96.4 [b]
3	92.6 [a]

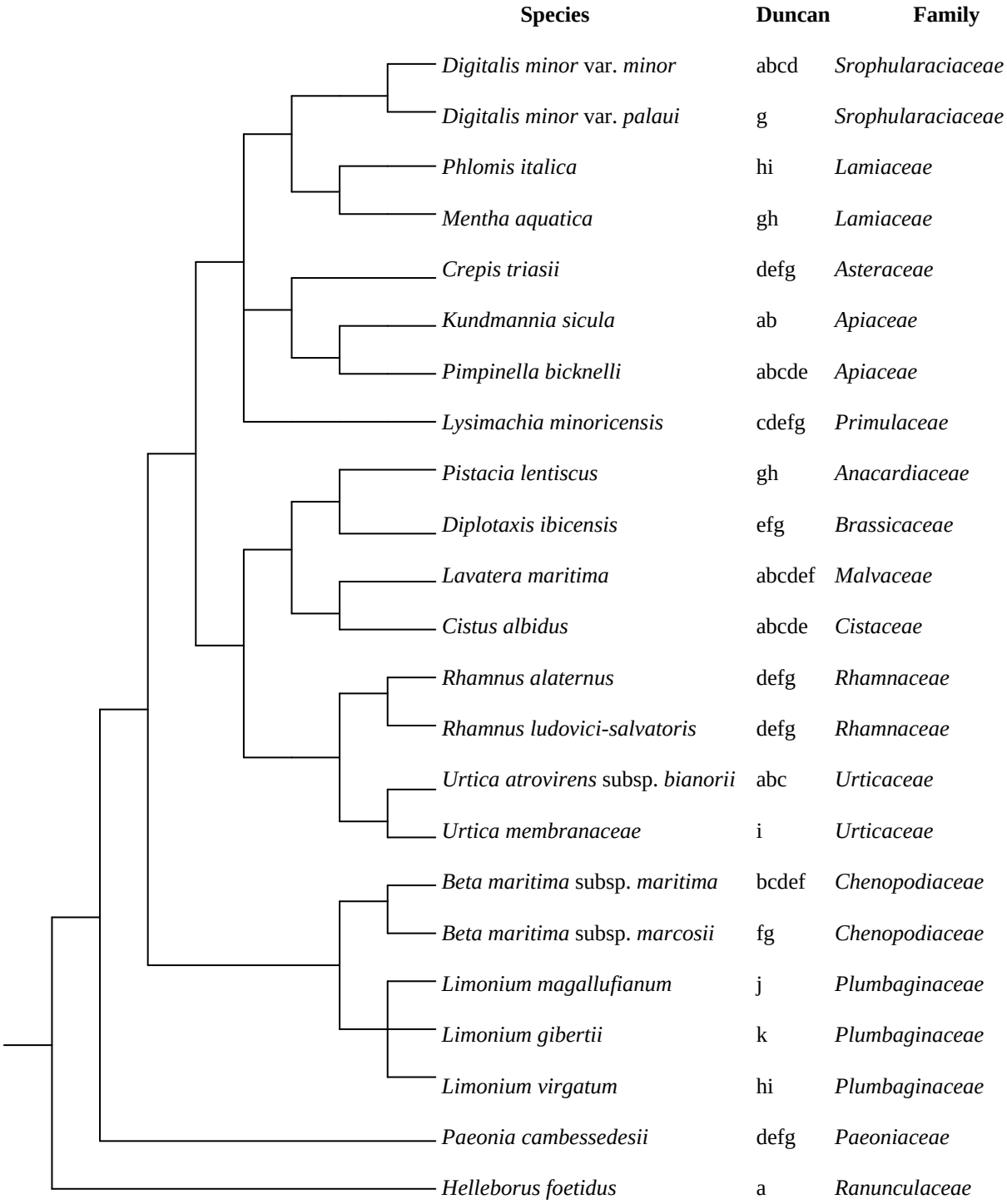
Significant differences in τ ($P < 0.05$) were also found among species from different habitats (Table 10.4). For instance, τ averaged 98.7 for those species inhabiting the driest environments (group 1), 96.4 for those species from intermediate environments (group 2) and 92.6 for those species that do not suffer hot and dry summer seasons (group 3).

Finally, it has been impossible to elucidate any association of τ with phylogeny. From the analysis of variance made within each phylogenetic category (from genera to

classes) no clear pattern could be detected between different phylogenetic branches, in respect to τ (Fig. 10.6).

Figure 10.6

Phylogenetical relationship and τ of species studied. Different letters denote statistical differences at $P < 0.05$ within each criterion by Duncan Analysis.

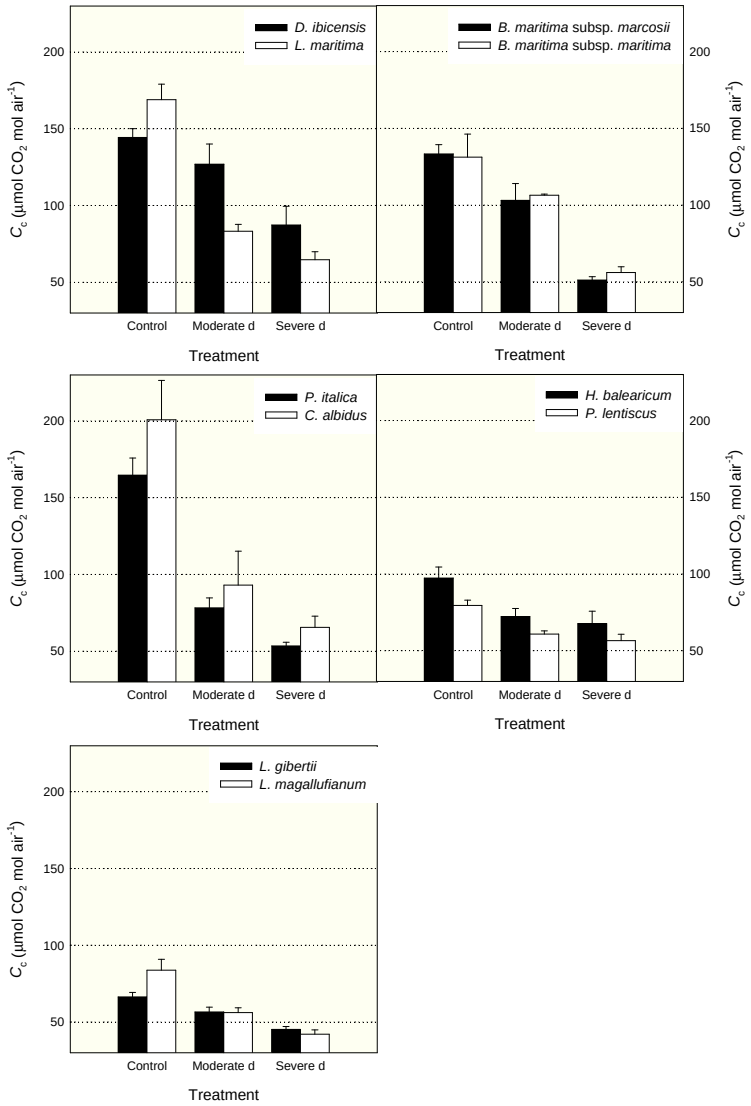


10.4.1.4. CO_2 concentration at the site of Rubisco and carboxylation efficiency

From data of Chapter 7, C_c was estimated in ten Mediterranean species under different degrees of water stress. The drought stress resulted in severe depressions of C_c (Fig. 10.7).

Figure 10.7

CO_2 concentration at the active site of Rubiscos (C_c) from 10 Mediterranean species measured at three different degrees of drought stress: well-watered conditions (control), moderate drought and severe drought. Estimation of C_c was done from according to Epron *et al*. (1995). Values are means ± standard errors (n = 4). These results were obtained from data of Chapter 7.



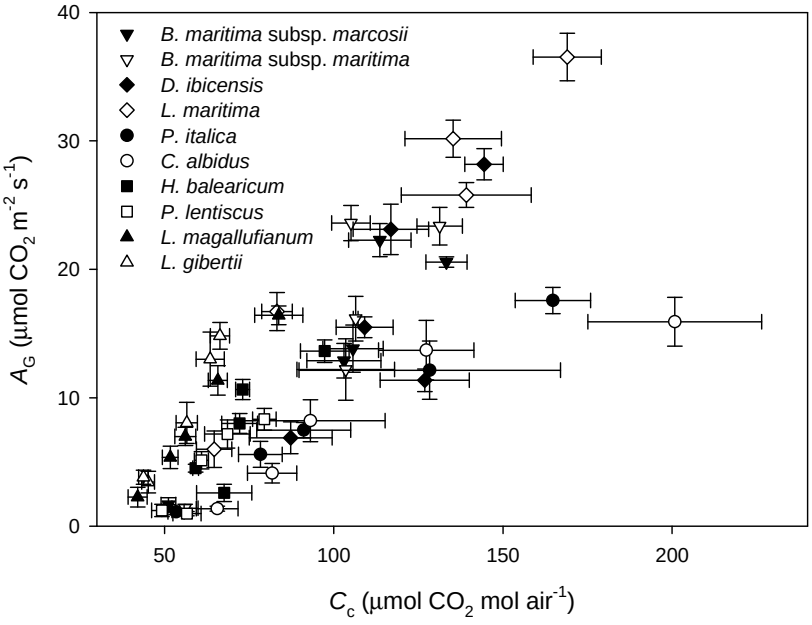
Under well-watered conditions *D. ibicensis*, *L. maritima*, *Beta maritima* subsp. *marcosii*, *Beta maritima* subsp. *maritima*, *P. italica* and *C. albidus* presented C_c values

higher than 130 μmol CO_2 mol air^{-1} (Fig. 10.7). *H. balearicum*, *P. lentiscus* and *L. magallufianum* C_c values ranged between 80 and 100 μmol CO_2 mol air^{-1}. *L. gibertii* showed the lowest value for C_c under optimal conditions, with 66 μmol CO_2 mol air^{-1}. Under severe drought treatment, *D. ibicensis*, *L. maritima*, *C. albidus* and *H. balearicum* showed a C_c higher than 60 μmol CO_2 mol air^{-1}. *Beta maritima* subsp. *marcosii*, *Beta maritima* subsp. *maritima*, *P. italica* and *P. lentiscus* presented intermediate C_c values, between 50 and 60 μmol CO_2 mol air^{-1}. Finally, both *Limonium* species showed the lowest CO_2 concentration levels at the active site of Rubisco, with values even lower than 50 μmol CO_2 mol air^{-1}.

The relationship between gross photosynthesis (A_G) and C_c was plotted for these ten species (Fig. 10.8), considering 5 different degrees of drought: control, mild drought, moderate drought, severe drought and rewatering (24 h after soil water refilling). The slope of this relationship can be considered as the efficiency of carboxylation of the different species, as it reflects the rate of carboxylation per unit CO_2 concentration at the Rubisco active site.

Figure 10.8

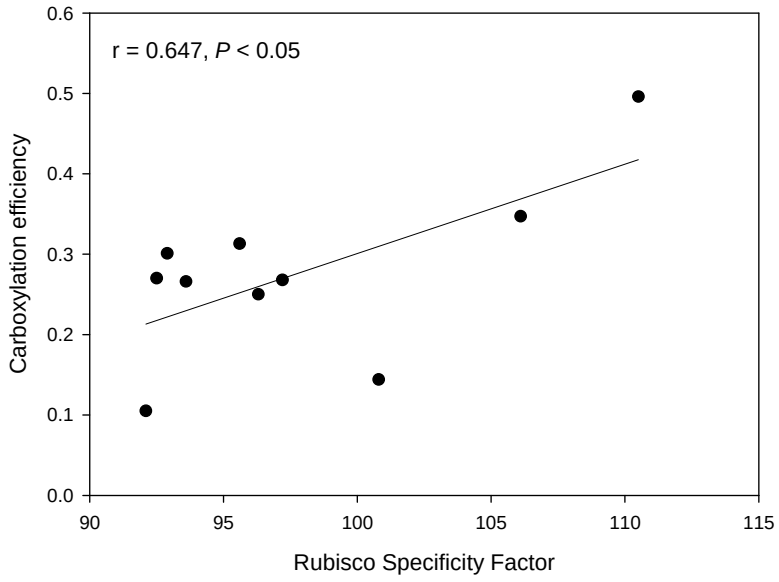
Relationship between gross photosynthetic rates (A_G) and CO_2 concentration at the active site of Rubisco (C_c) from 10 Mediterranean species measured at five different degrees of drought stress: well-watered conditions (control), mild drought, moderate drought, severe drought and re-watering. Estimations of C_c were done according to Epron *et al*. (1995). Values are means ± standard errors (n = 4).



The regression slopes were calculated for each species. The highest carboxylation efficiency was observed for *L. gibertii*, with 0.496, while the lowest for *C. albidus*, with 0.105. Finally, a significant relationship ($P < 0.05$) was found between the carboxylation efficiency and τ (r = 0.647) (Fig. 10.9).

Figure 10.9

Relationship between the carboxylation efficiency and the Rubisco Specificity Factor from 10 Mediterranean species.



10.4.1.5. Temperature dependence of specificity factor among different species

As previously reported by many authors (Jordan & Ogren, 1984; Brooks & Farquhar, 1985; Lorimer *et al.*, 1993; Uemura, 1997), τ decreased with the increase of the temperature of the reaction mixture (T). The relationship between τ and T (in ºK) plotting all the species together resulted in an exponential ($\tau = -231.46 + 4598.01\ e^{-0.0088T}$) or linear regression ($\tau = 975.93 - 2.93\ T$) with high regression coefficients (r^2 = 0.892 and 0.890, respectively). Although no significant differences could be found between these possible patterns of temperature dependence of τ, the relationship seemed to be more fitted by an exponential than by a linear regression, as also found Jordan & Ogren (1984) and Brooks & Farquhar (1985).

Significant differences were found among species in the regression slope, with a range of variation of 24% (data not shown). In this way, species can be classified on the

basis of the temperature dependence of their τ. Thus, Rubiscos from *L. gibertii* and *L. magallufianum* showed the lowest temperature dependence, whereas *L. minoricensis*, *K. sicula* and *U. atrovirens* subsp. *bianorii*, the highest. This species gradation reflected closely the habitats where the species live, those species from cool habitats having the highest regression slope and those from the hottest and driest habitats having the lowest.

When natural logarithms of the τ values were plotted against the reciprocals of the products of the gas constants and the absolute temperature of the reaction mixtures, smaller differences were found among the slopes of the species analysed (16%) (Table 10.6). These slopes represent the difference in the activation energies at the transition states between the oxygenase and the carboxylase reactions for Rubisco ($\Delta G_o^{\ddagger}$-$\Delta G_c^{\ddagger}$). Very close to that reported by Uemura *et al*. (1997) for spinach, the common slope pooling all the species altogether was 5.1 kcal·mol^{-1} with a y-axis intercept of –4.1 and a correlation coefficient of 0.917. Although differences among species were minimum, the highest activation energies found in the *Limonium* species (5.4-5.6 kcal·mol^{-1}) were significantly different ($P < 0.05$) than the smallest ones, found in the *Urtica* species (4.7 kcal·mol^{-1}).

Table 10.6

τ at 15, 25 and 35ºC, and differences in the activation energies of the transition states between the oxygenase and the carboxylase reactions for Rubisco ($\Delta G_o^{\ddagger}$-$\Delta G_c^{\ddagger}$). $\Delta G_o^{\ddagger}$-$\Delta G_c^{\ddagger}$ was calculated from ln τ = $\Delta G_o^{\ddagger}$-$\Delta G_c^{\ddagger}$ /*RT*, where *R* is the gas constant (1.987 cal · mol^{-1} · K^{-1}), and *T* is the absolute temperature of the reaction mixture. Different letters denote statistical differences at $P < 0.05$ within each criterion by Duncan Analysis.

Species	**τ at 15ºC**	**τ at 25ºC**	**τ at 35ºC**	**$\Delta G_o^{\ddagger}$-$\Delta G_c^{\ddagger}$**
Diplotaxis ibicensis	137.2 ^{d}	95.6	73.3 ^{bc}	5.6 ^{d}
Urtica atrovirens subsp. *bianorii*	121.5 ^{ab}	90.2	71.4 ^{b}	4.7 ^{a}
Beta maritima subsp. *marcosii*	130.9 ^{cd}	96.3	73.4 ^{bc}	5.1 ^{abc}
Lysimachia minoricensis	116.8 ^{a}	93.8	65.3 ^{a}	5.2 ^{abc}
Limonium magallufianum	150.7 ^{e}	106.1	81.8 ^{e}	5.4 ^{cd}
Rhamnus ludovici-salvatoris	137.3 ^{d}	94.4	76.1 ^{cd}	5.2 ^{ab}
Urtica membranacea	130.5 ^{cd}	102.4	76.3 ^{cd}	4.8 ^{a}
Kundmannia sicula	126.7 ^{bc}	89.2	71.3 ^{b}	5.1 ^{abc}
Beta maritima subsp. *maritima*	131.1 ^{cd}	92.9	75.6 ^{cd}	4.9 ^{ab}
Mentha aquatica	124.1 ^{bc}	97.2	71.1 ^{b}	4.9 ^{abc}
Limonium gibertii	148.8 ^{e}	110.5	78.8 ^{d}	5.6 ^{d}
Rhamnus alaternus	131.6 ^{cd}	94.7	74.3 ^{bc}	5.0 ^{abc}
Hypericum balearicum	131.6 ^{cd}	93.6	72.1 ^{b}	5.3 ^{bc}
Pistacia lentiscus	129.5 ^{c}	97.2	72.9 ^{bc}	5.1 ^{abc}

10.4.2. Experiment 2

10.4.2.1. Amino acid sequences of *L. gibertii* Rubisco large subunit

Amino acid sequence alignment of the L subunit from *Spinacea oleracea*, *L. gibertii* and other *Limonium* species is shown in the Fig. 10.10 The following 32 amino acid residues were found to be different in the L subunits between spinach and *L. gibertii* (for amino acid symbols see Table 10.7): A8S, S9F, S42T, N95S, C99A, V142P, V145S, A222T, L225I, I235V, C247S, D249E, M251I, V255A, F256C, A276S, T279S, S281A, L320M, S328A, D340E, T354I, S367D, T371M, L375I, T443E, E447Q, T449A, F469X, P470A, V475L. Eight of these amino acid residues are the same in Rubiscos for *L. gibertii* and *Galdieria partita* (C99A, I235V, D249E, S281A, L320M, S328A, T443E, T449A), a thermophilic red algae with a τ value of 238, the highest among the Rubiscos hitherto reported (Uemura *et al*., 1997).

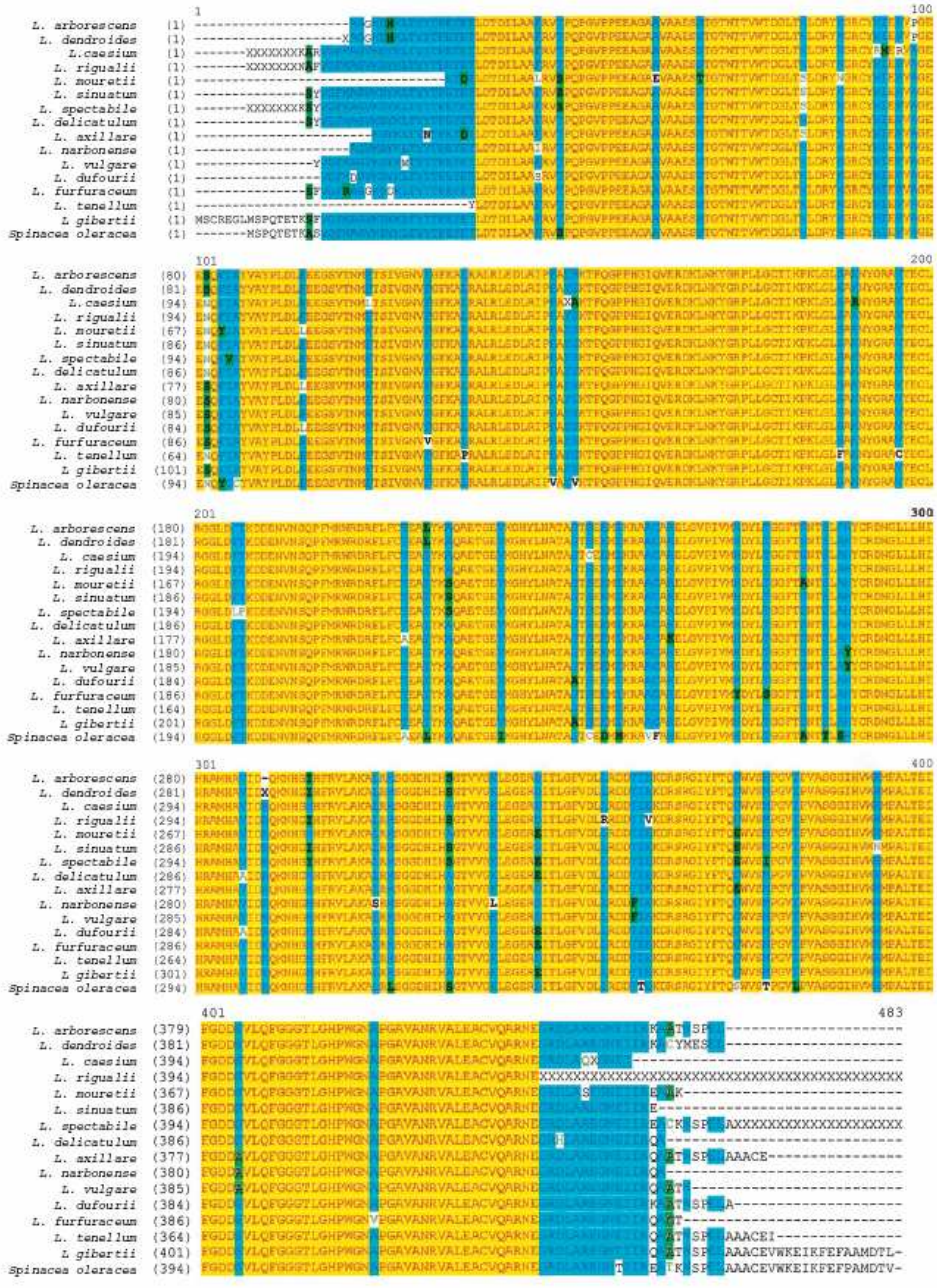
Table 10.7

List of amino acids and their abbreviation and symbol.

Name	Abbreviation	Symbol
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Asparagine + Aspartic acid	Asx	B
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glutamine + Glutamic acid	Glx	Z
Glycine	Gly	G
Hystidine	His	H
Isoleucine	Ilu	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptofan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

Figure 10.10

Alignment (Vector NTI, Invitrogen) of Rubisco large subunit amino acid sequences from *Limonium arborescens* (translation of AF206789), *L. dendroides* (CAB10739), *L. caesium* (CAB10738), *L. rigualii* (CAB10740), *L. mouretii* (CAA76531), *L. sinuatum* (CAA76531), *L. spectabile* (CAB10741), *L. delicatulum* (CAA76533), *L. axillare* (CAB86155), *L. narbonense* (CAB86158), *L. vulgare* (CAA76534), *L. dufourii* (CAB86156), *L. furfuraceum* (CAA76532), *L. tenellum* (CAB86159), *L. gibertii* (translated from AJ786659) and *Spinacia oleracea* (CAB88737) with accession numbers given in parenthesis. Yellow boxes indicate fully conserved residues among species. Blue boxes indicate partially conserved residues, while the exceptions are indicated in green (similar type of amino acids) or white (different type of amino acids). Note that the numeration avobe sequences does not reflect the normalised position number of amino acids in the structure of the protein, which begins with the Met as the first amino acid in the spinach L subunit sequence.

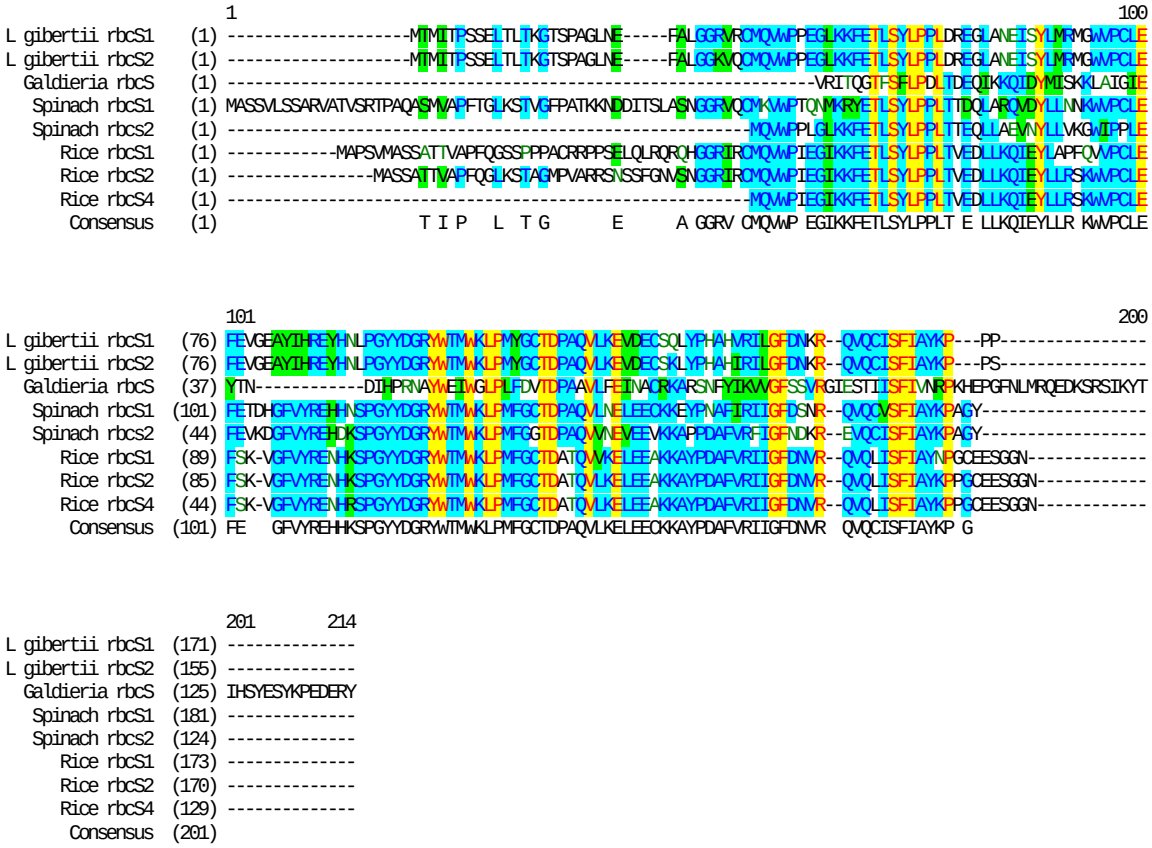


10.4.2.2. Amino acid sequences of *L. gibertii* Rubisco small subunit

The degree of homology between the S subunit of *L. gibertii* and spinach is lower than that of the L subunits, and more than 40 residues were found to be potentially different between both species (Fig. 10.11). From these, up to 27 were different despite the source of information considered for sequences comparision. None of these 27 different residues between *L. gibertii* and spinach S subunits was the same in *Galdieria*.

Figure 10.11

Alignment (Vector NTI, Invitrogen) of Rubisco small subunit amino acid sequences from *Limonium gibertii* (translation of CAH10355 and CAH10356), *Galdieria partita* (BAA75797), *Spinacia oleracea* (AAB81105 and P00870) and *Oryza sativa* (CAA30393, AAA84592 and CAA59218), with accession numbers given in parenthesis. Yellow boxes indicate fully conserved residues among species. Blue boxes indicate partially conserved residues, while the exceptions are indicated in green (similar type of amino acids) or white (different type of amino acids).



10.4.3. Experiment 3

Despite acclimation of the leaves to water deficit, decreased water supply resulted in large decreases in mid morning RWC, from 76.7% in C to 66.9 and 51.7% in MoD and SD treatments, respectively (Table 10.8). As expected, A_N and g_s also decreased with decreasing water availability (Table 10.8). A_N decreased from 23.8 μmol CO_2 m^{-2} s^{-1} in C to 17.4 μmol CO_2 m^{-2} s^{-1} in MoD and 8.9 μmol CO_2 m^{-2} s^{-1} in SD, while the stomatal conductance to CO_2 (g_s) decreased from 209 mmol CO_2 m^{-2} s^{-1} in C to 109 mmol CO_2 m^{-2} s^{-1} in MoD and 31 mmol CO_2 m^{-2} s^{-1} in SD. ETR decreased, from 185 in C to 150 μmol m^{-2} s^{-1} in SD. Acclimation to drought resulted in an increased SLW and a somewhat increased total soluble protein content (Table 10.8). However, the Rubisco specific initial activity and its activation state were similar in all treatments (Table 10.8). R_D remained similar (2.4 ± 0.2 μmol CO_2 m^{-2} s^{-1}) for all treatments.

Table 10.8

Fluorescence, gas exchange and Rubisco assays of tobacco treatments. Relative water content (RWC), specific leaf weight (SLW), light-saturated net photosynthesis (A_N), stomatal conductance (g_s), rate of electronic transport (ETR), Rubisco specific initial activity, total soluble protein and dark respiration (R_D). C: plants maintained at field capacity during all experiment (control). MoD: plants maintained at 40% field capacity (moderate drought treatment). SD: plants maintained at 15% field capacity (severe drought treatment).

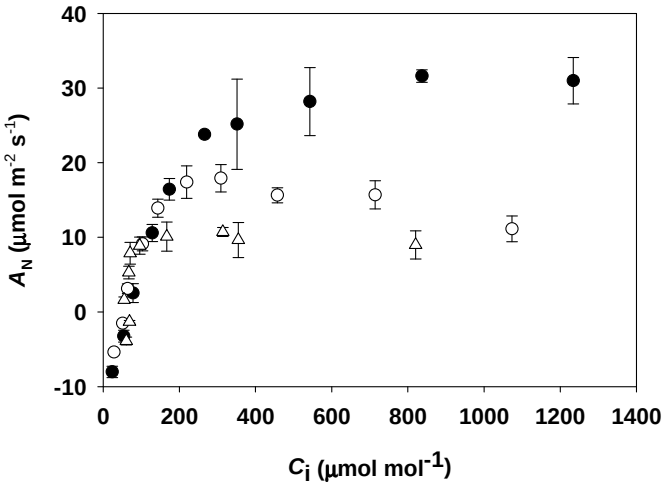
	C	MoD	SD
RWC (%)	76.7 ± 2.5	66.9 ± 0.9	51.7 ± 2.2
SLW (g m^{-2})	30.3 ± 1.4	42.9 ± 0.8	45.9 ± 0.9
A_N (μmol m^{-2} s^{-1})	23.8 ± 0.2	17.4 ± 2.2	8.9 ± 1.1
g_s (mmol CO_2 m^{-2} s^{-1})	209 ± 12	109 ± 20	31 ± 5
ETR (μmol e^- m^{-2} s^{-1})	185 ± 6	175 ± 10	150 ± 11
Rubisco specific initial activity (μmol g^{-1} protein s^{-1})	2.7 ± 0.1	3.2 ± 0.1	3.0 ± 0.1
Total soluble protein (g m^{-2})	7.2 ± 0.6	8.6 ± 0.3	7.8 ± 0.3
R_D (μmol m^{-2} s^{-1})	2.5 ± 0.4	2.1 ± 0.2	2.4 ± 0.2

Water stress also resulted in significantly different A_N-C_i responses (Fig. 10.12). The operational C_i (i.e. the C_i at atmospheric C_a) declined as water stress intensified, from 265 μmol mol^{-1} in C, to 219 μmol mol^{-1} in MoD and 109 μmol mol^{-1} in SD. CO_2 saturated photosynthesis also decreased from 31.6 ± 0.8 in C to 17.9 ± 1.8 and 10.7 ± 0.6 in MoD and SD treatments, respectively. The apparent carboxylation efficiency (i.e.

the initial slope of the A_N-C_i curve) declined slightly but non-significantly with water stress, from 0.18 in C to 0.16 in SD.

Figure 10.12

Relationship between net photosynthesis (A_N) and the internal CO_2 concentration (C_i). Filled circles represent control treatment, empty circles moderate drought and triangles severe drought. Data are means ± standard deviation.



Water stressed plants also presented a lower Γ* (41.4 µmol mol^{-1} in MoD and 21.3 µmol mol^{-1} in SD) than C (55.7 µmol mol^{-1}). Apparent τ (τ∗) was estimated from Γ* according to Brooks & Farquhar (1985), resulting in the following values: 70.1 in C, 94.2 in MoD and 182.2 in SD (Table 10.9). The *in vitro* τ averaged 99.3, which did not differ significantly from that of wheat and was significantly higher than *in vivo* estimations. Therefore, τ∗ underestimated τ in C and MoD and largely overestimated it in SD.

Table 10.9

Apparent Rubisco specificity factor (τ∗) assuming that light respiration (R_L) equals dark respiration (R_D), R_L = 0.5 R_D and R_L = 0.2 R_D, and the *in vitro* Rubisco specificity factor τ (at 25ºC) for the control (C), moderate drought (MoD) and severe drought (SD) treatments.

Treatment	τ* (R_L = R_D)	τ* (R_L = 0.5 R_D)	τ* (R_L = 0.2 R_D)	τ
C	70.2	62.9	59.2	98.1 ± 2.6
MoD	94.2	80.7	74.4	100.9 ± 2.1
SD	182.2	136.3	118.4	98.8 ± 1.9

10.5. DISCUSSION

10.5.1. Species variation of Rubisco specificity factor

There are substantial differences in τ for Rubisco purified from species from widely separated phylogenetic groups (Jordan & Ogren, 1981, 1983) but much less variability of Rubisco τ from higher plants (Delgado *et al*., 1995; Kent & Tomany, 1995). However studies have focused on a small number of species of cultivated plants. The variation in τ for Rubisco from species of higher plants from natural flora remains largely unknown. This work increases the range of known Rubisco τ values for C_3 plants and shows that variation in τ can be found, not only among distant phylogenetic groups, but also even between closely related species (Table 10.3). The magnitude of τ from the plants appears to be related to the environments in which the plants grow, and to their growth forms.

10.5.2. Rubisco adaptation: ecological, phylogenetical and evolutionary influences on specificity factor

The Rubisco τ is greater for plants usually inhabiting hot, arid and saline areas of the Balearic Islands with a xericity index of 1 in Table 10.4. Under these conditions both the high temperature and the low internal CO_2 concentrations, enforced by the need to conserve water, appear to have imposed a selection pressure for high Rubisco τ, in agreement with the hypothesis of Delgado *et al*. (1995) and Kent & Tomany (1995). These results also match the hypothesis of Austin (1999) that the beneficial effects of increasing τ are predicted to be greater at higher temperatures. Carbon isotope composition along climatic gradients in Mediterranean plants tends to show that life-averaged internal CO_2 concentrations are indeed lower in species inhabiting drier areas (Valentini *et al*., 1991; Alessio *et al*., 2004). Gas exchange analysis of the species included in the present study demonstrates that *Limonium* species, with the highest τ values, had the lowest chloroplastic CO_2 concentration among the species analysed (Fig. 10.7). The higher slope in the relationship between A_G and C_c for *Limonium* should be, moreover, considered as reflecting a higher carboxylation efficiency (Fig. 10.8). This is in accordance with the significant, positive relationship between the carboxylation efficiency and τ (Fig. 10.9). The positive significance of the relationship between the carboxylation efficiency and τ is mostly due to the *L. gibertii* value. This

fact adds even more interest to the study of the Rubisco kinetic properties of this species in particular.

Similarly, there is also a trend for an increase in τ from annual herbs, through evergreen herbs to semi-deciduous and to evergreen shrubs (Table 10.4). This trend can be also related to an increased thickness of scherophyll leaves of evergreen shrubs and, as a consequence, to a decreased conductance for CO_2 to the chloroplast stroma. Effectively, low C_c (Fig. 10.7) and relatively high carboxylation efficiency values (Fig. 10.8) have been estimated for evergreen shrubs if compared to the other growth forms. Again, a Rubisco with a higher τ could be regarded as conferring an advantage in supporting faster assimilation relative to photorespiration under such conditions surrounding Rubisco. Moreover, leaves of evergreen shrubs are likely to persist for a longer period of time than those of annual herbs and semi-deciduous shrubs. Therefore, a positive relationship might be also found between the leaf life span and τ, suggesting an improved investment of resources for a better enzyme.

Although Gulías *et al*. (2003) suggested differences in τ between endemic and non-endemic species of the Balearic Archipelago to explain their lower photosynthetic capacities, non-significant differences were observed with the species analysed in the present study. Thus, the evolution in a low competitive environment related to island ecosystems did not result in lower τ values, and therefore, this physiological trait must be discarded as a possible general cause for the decline in the distribution of many Balearic endemic species.

In conclusion, the existence of significant correlation factors between τ and ecological variables and the otherwise non-existence of any correlation with phylogenetic or evolutionary analysis, suggests that, at least for the Mediterranean species studied here, τ is more correlated with the ecologic characteristics of the species than to the phylogenetic ones. It would be interesting to test this hypothesis on a global scale, with a higher number of species, corresponding to a several divergent branches of evolution of higher plants but with convergent ecological traits, and *vice versa*.

10.5.3. Temperature dependence of specificity factor among different species

The coincident different temperature response showed by the Rubiscos of species from different habitats also claim to the existence of some kind of selection pressure on enzyme kinetics, making Rubiscos from driest, hottest areas relatively more independent of variations in temperature, as compared to Rubiscos of species inhabiting wettest, coolest habitats. This differential temperature dependence is reflected by the different relative values of the activation energies to the transition states between the oxygenase and carboxylase reactions for Rubiscos from species inhabiting different environments (Table 10.6). As shown by Uemura *et al*. (1997) for *Galdieria*, *Limonium* Rubisco is likely to adopt the protein structure relatively more unfavourable for the transition state of the RuBP-oxygenation reaction. Since beneficial effects of increasing Rubisco τ are expected to be greater at higher temperatures (Austin, 1999) and τ declines with increasing temperature, these local environmental effects must be considered when planning transformation of crop Rubiscos with Rubiscos from wild species.

10.5.4. Amino acid sequence homologies of *Limonium gibertii* Rubisco

Since the catalytic properties of Rubisco are mainly determined by the structure of the large subunit polypeptide of the enzyme, and this is coded for in the many copies of the chloroplast genome, the selection of particular variants of the gene seem likely to be slow. Nevertheless, the Rubisco large subunit sequences of different *Limonium* species are not identical (Fig. 10.10). For instance, up to 32 amino acid residues of the large subunit were found to be different between spinach and *L. gibertii* Rubiscos. Most importantly, *L. gibertii* large subunit presented eight of these amino acid residues identical to those of *Galdieria partita*. Residue 247 in spinach Rubisco is cysteine and makes disulfide bond between the large subunits composed of a L_2 dimer in higher plants (Newman & Gutteridge, 1993; Schreuder *et al*., 1993; Andersson, 1996; Shibata *et al*., 1996). However, the corresponding residue is serine in *L. gibertii*, as in all the other *Limonium* species, except *L. caesium*. Indeed, *Galdieria* Rubisco also lacks this cysteine, and has a methionine in this position (Sugawara *et al*., 1999). Moreover, while tobacco Rubisco possibly forms disulfide bonds from 449-459 pairs (Curmi *et al*., 1992; Schreuder *et al*., 1993), residue 449 is not cysteine, but alanine, in the *L. gibertii*

enzyme, as in *Galdieria* (Sugawara *et al*., 1999). Amino acid sequences directly interacting with the substrate are completely conserved in *Limonium* species. The flexible loop (loop-6) of the L subunits consists of residues 328-339 and its mobility is necessary to bind the substrate RuBP and stabilize the reaction intermediate. Residues 329-337 are completely conserved except in Rubiscos lacking small sununits (Sugawara *et al*., 1999). As occurs with *Galdieria* Rubisco, *L. gibertii* L subunits have an alanine in position 328, instead of the serine found in spinach and tobacco.

Much less information can be taken from the comparison between S subunit sequences. The lower degree of homology between S subunits is in accordance to the abundance of not estrictly conserved regions, consequence of the multiple different copies of the genes encoding for the S subunit. Moreover, this matches to the still unclear roles of the S subunit in the enzymatic properties of Rubisco (Sugawara *et al*., 1999).

It is very difficult to make a good story from sequences alone because in three-dimensional structure of Rubisco there are interactions between amino acid residues that are very remote from the catalytic site and yet affect catalysis (Andersson, 1996). The conclusion must be that variations in the genes encoding *rbc*L Rubisco gene arise or exist and that the normal process of maternal inheritance (Birky, 2001) allows selection of superior versions over a relatively short timescale. Differences in τ among higher plants are small and the comparison of Rubisco amino acid sequences demonstrate that changes in structure causing them are likely to be minor differences in conformation of the catalytic site due to residue differences distant from that site. Alternatively the differences observed in τ maybe entirely the result of differences in sequence of the Rubisco small subunit polypeptide (Wang *et al*., 2001).

10.5.5. Using *Limonium gibertii* Rubisco to improve crop Rubiscos

The range of variation in the magnitude of τ is sufficiently small (ca. 20%) that there is a need to consider whether the advantage in terms of increased carbon assimilation provides significant selection pressure. Current models of leaf photosynthesis (Farquhar *et al*., 1980) allow for estimations of the effects of τ variation on photosynthesis rates. As an example, the calculations in Table 10.10 show the increases in carbon assimilation that would accrue if the Rubisco from *L. gibertii* replaced the native Rubisco of wheat and tobacco plants. The amounts of any increase

depend on which of the four constants that make up τ is responsible for the difference between Rubisco from the recipient species and Rubisco from *L. gibertii*. The model predicts increases in tobacco net photosynthesis of 26, 30, 16 and 5%, depending whether the difference is due to differences in K_c, V_c, K_o, and V_o, respectively. For wheat, photosynthesis may be increased by 11, 12, 6 and 2%, respectively, for changes due to the same parameters. Table 10.3 shows that there are also significant differences in τ between closely related species e.g. *L. gibertii* and *L. magallufianum* or *U. membranacea* and *U. atrovirens* subsp. *bianorii*, which are less than the difference in τ between the species considered in Table 10.10, but still we can conclude that selection pressures based on Rubisco τ are significant. Table 10.10 illustrates the importance of knowledge of the kinetic constants that make up τ, especially V_c, in predicting the effect of the properties of Rubisco on photosynthetic capacity. Determination of a true V_c (k_c^{cat}) for Rubiscos proved difficult for leaves of the species studied so we cannot comment on the possibility of a negative correlation (see Zhu *et al*., 2004) that may exist over the restricted range of specificity factors observed.

Table 10.10

Predicted increases in light saturated photosynthesis (%) for tobacco and wheat if their native Rubisco could be replaced with Rubisco from *Limonium gibertii*. The concentrations of CO_2 and O_2 at the chloroplast level were considered to be 7 μM and 265 μM, respectively. The calculation used equations 2.17 and 2.23 (von Caemmerer, 2000).

	τ different from wheat because of change in:			
	K_c	V_c	K_o	V_o
	Increase in net photosynthesis (%)			
Tobacco	26	30	16	5
Wheat	11	12	6	2

Even very modest changes may be extremely important, since the difference in the critical steps in the catalytic mechanism, the activation and stabilization of the transition state intermediates of the carboxylation and oxygenation reactions that determine the rate of oxygenation compared to carboxylation, have a free energy difference of less than the energy of one hydrogen bond (Spreitzer, 1993; Spreitzer & Salvucci, 2002). It may be assumed, therefore, that differences of even one amino acid residue could account for observed differences in τ. The identification of sequence

differences in the genes for Rubisco from closely related species (Figs. 10.10 and 10.11), together with differences in the kinetic properties, including τ, needs to be extended so that a cause-effect relationship could be established. Knowledge of gene sequences encoding Rubisco and Rubisco τ for C_3 plants may permit identifying genetic modifications that will have great agronomic importance. This is important because introducing into higher terrestrial plants genes for Rubisco from distantly related cyanobacterial and algal species have thus far failed to yield a fully functional enzyme, whereas the introduction of smaller changes have resulted in catalytically competent enzyme (Spreitzer & Salvucci, 2002; Parry *et al*., 2003; Zhu *et al*., 2004).

10.5.6. Rubisco acclimation to drought

Obviously, adaptation of τ to arid environments would be of lesser importance if plants had also the capacity of acclimate τ, i.e. of adjusting τ values depending on the actual environmental conditions and in the short term. However, no study up to now has addressed this possibility. We analysed this by growing tobacco plants under different conditions of water supply, and allowing new leaves to unfold under these conditions, these leaves may be considered acclimated to drought.

As expected, decreases in mid morning leaf RWC due to water stress were associated to decreases in A_N and g_s (Table 10.8). However, ETR decreased to a much lesser extent than A_N, possibly indicating an increased electron partitioning towards sinks other than photosynthesis, mainly photorespiration (Cornic & Massacci, 1996; Flexas & Medrano, 2002c). Although total soluble protein content increased due to acclimation to drought, the Rubisco specific initial activity and its activation state did not differ significantly between treatments (Table 10.8), as usually observed in water stress experiments, except when stress is very severe (Flexas *et al*., 2004a).

The declining operational C_i as water stress intensified points to an increased stomatal limitation to photosynthesis (Fig. 10.13). Nevertheless, decreases in A_{SAT} suggest an increased non-stomatal limitation to photosynthesis in the stressed leaves (Lawlor, 2002). In addition, both in MoD and SD leaves the maximum A_N value was attained at an intermediate C_i (Fig. 10.12), suggesting that photosynthesis at high C_i was limited by triose phosphate use (Long & Bernacchi, 2003), a situation often observed under water stress (Flexas *et al*., 2004a). All the plants maintained their operational C_i at the breakpoint between CO_2-limited and RuBP- and/or TPU-limited photosynthesis,

which has been interpreted as an acclimation to optimise both the photochemical and biochemical photosynthetic reactions (von Caemmerer & Farquhar, 1984).

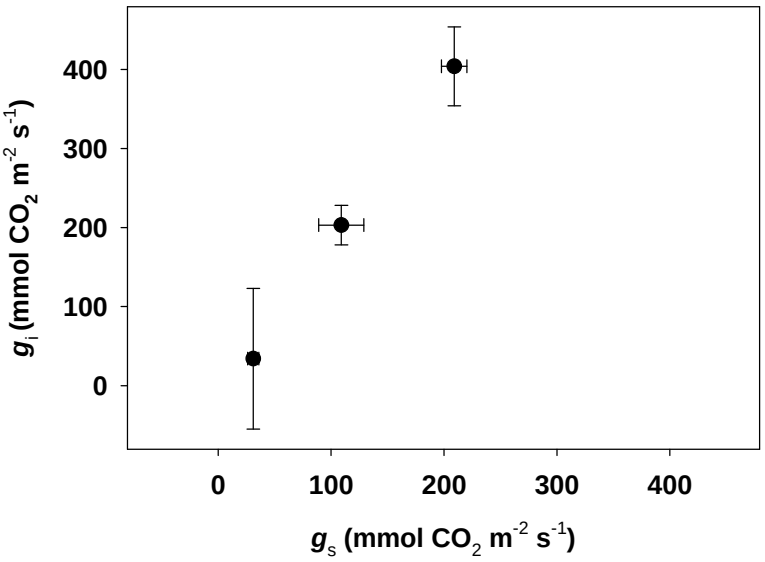
Although Bota *et al*. (2002) and Warren *et al*. (2004) showed an increased Γ* in grapevines and Douglas-fir, respectively, due to water deficit, in the present study water stressed plants presented a lower Γ*. However, in the two previous studies, water stress was applied rapidly and its effects analysed on mature leaves developed during the pre-stress period, while in the present the studied leaves were developed during the stress period, i.e. acclimated. In principle, these results seem to support the hypothesis by Delgado *et al*. (1995) and Kent & Tomany (1995) that under water stress, stomatal closure and low CO_2 concentrations at the site of Rubisco may impose increased selection pressure on Rubisco for improved specificity. However, when τ was measured *in vitro* there were no significant differences between treatments (Table 10.9).

Although our estimates of Γ* (except that in SD) resemble those typically found in other studies (Brooks & Farquhar, 1985; von Caemmerer, 2000; Bernacchi *et al*., 2001), it may be argued that these estimations are strongly dependent on the assumed value for R_L, and that the R_L as measured in the present study may be unrealistic, since mitochondrial respiration in the light is usually lower than in the dark (Brooks & Farquhar, 1985; Villar *et al*., 1994, 1995; Pinelli & Loreto, 2003). However, mitochondrial respiration as measured in the dark did not differ between treatments (Table 10.8). Even assuming that mitochondrial respiration in the light was only 50% of that in darkness (Villar *et al*., 1994, 1995; Pinelli & Loreto, 2003), the estimated τ* values resulted 62.9, 80.7 and 136.2 in C, MoD and SD, respectively. Still, if R_L in the light were as low as 20% of that in darkness (Brooks & Farquhar, 1985; Pinelli & Loreto, 2003), the estimated τ* values resulted 59.2, 74.4 and 118.4 in C, MoD and SD, respectively. Therefore, possible errors in the estimation of R_L do not account for (i) the large difference observed between the *in vitro* and the *in vivo* τ and (ii) the fact that *in vivo* estimations suggest a water-stress effect of Rubisco specificity factor, while *in vitro* estimations show a constant value.

Most likely, *in vivo* estimated τ may be biased due to the misleading assumption that C_i equals the CO_2 concentration at the site of carboxylation. Assuming the validity of the *in vitro* τ estimation for the behaviour of Rubisco *in vivo*, C_c and g_i can be calculated (Fig. 10.13). g_i decreased in parallel with g_s, as already observed in water- and salt-stress experiments (Flexas *et al*., 2002, 2004a; Centritto *et al*., 2003).

Figure 10.13

Relationship between mesophyll conductance (g_i) and stomatal conductance (g_s). Measurements were made at light saturation, 400 ppm CO_2 and 25ºC in plants under control, moderate drought and severe drought treatments. Data are means ± standard deviation.

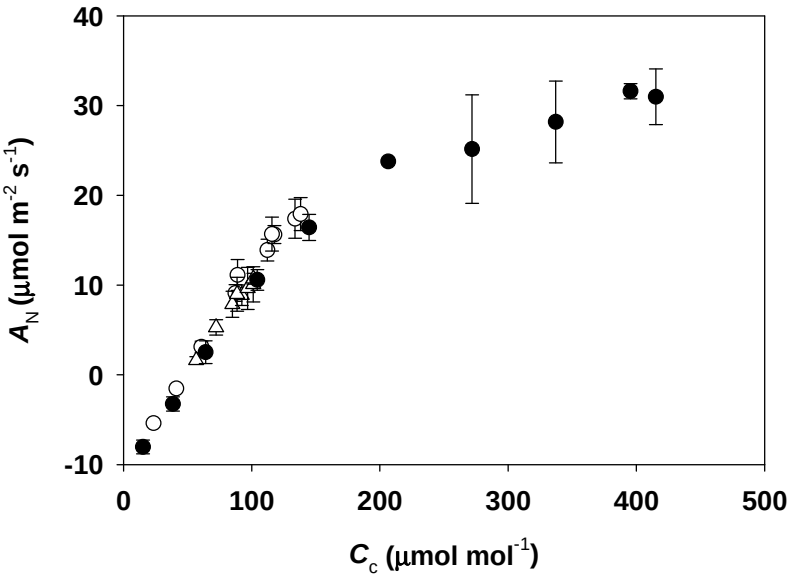


These results are very similar to those often encountered using other methods for estimation of g_i that do not rely on previous knowledge of the *in vitro* τ, which supports the validity of our estimations (Loreto *et al*., 1992; Evans & Loreto, 2000; Flexas *et al*., 2004a). It may be argued, however, that g_i estimations (and A_N-C_i curves) must not be reliable under drought due to incorrect estimations of C_i associated to heterogeneous stomatal closure and/or the influence of cuticular conductance to water and CO_2 (Terashima, 1992; Boyer *et al*., 1997; Buckley *et al*., 1997; Mott & Buckley, 1998). However, from combined gas-exchange and chlorophyll fluorescence measurements, A_N-C_c curves can be obtained that are totally independent of C_i estimations (Sánchez-Rodríguez *et al*., 1999). Clearly, all the differences observed between treatments in the A_N-C_i curves disappeared in the A_N-C_c curves (Fig. 10.14), as already shown by Sánchez-Rodríguez *et al*. (1999) in water stressed *Casuarina equisetifolia*. This is consistent with the constancy of Rubisco specificity factor, initial specific activity and activation state, determined *in vitro* (Tables 10.8 and 10.9). The only difference between treatments is the fact that MoD and SD leaves attain, for the same range of C_a, maximum C_c values much lower than C leaves, due to the superimposed decreasing stomatal and mesophyll conductances to CO_2. It is interesting to note that, in A_N-C_c

curves, CO_2-limited and RuBP-limited regions can be differentiated only in well watered plants, which maintain their operational C_c at the breakpoint between these two limitations. By contrast, MoD and SD operate at the CO_2-limited region, which questions the validity of A_N-C_i curve analysis to separate photosynthetic limitations under water stress. Calculating Γ* from A_N-C_c curves (Γ^*_{Cc}) resulted in a single value for all treatments (39 μmol mol^{-1}) which, obviously, reflected a τ value (96) very close to the that determined *in vitro*.

Figure 10.14

Relationship between net photosynthesis (A_N) and the chloroplastic CO_2 concentration (C_c). Filled circles represent control treatment, empty circles moderate drought and triangles severe drought. Data are means ± standard deviation.



In summary, Rubisco specificity factor does not acclimate to water stress in the short time (weeks-months) in tobacco. Any demonstration of change in the dominant form of functional Rubisco expressed would have been surprising, particularly since it has been demonstrated that there is an adaptation mechanism operating over a much longer time-scale (i.e. generations) (Galmés *et al*., 2005). Acclimation, if it existed, would remove the selective pressure that would lead to adaptation.

The comparison of *in vitro* and *in vivo* estimated τ values clearly support the conclusion by Warren *et al*. (2004) that it would be preferable to determine τ independently of gas-exchange measurements, using an alternative method. *In vitro*

determinations proved to be a suitable means. An alternative method could be determining Γ^*_{Cc} from g_i estimates using a method not relying on previous knowledge of τ, such as the isotope discrimination or the constant fluorescence methods (Harley *et al*., 1992; Loreto *et al*., 1992).

10.5.7. Concluding remarks

1. There is a higher variability in Rubisco τ among higher plants than was at first supposed, with significant differences even between closely related species.
2. This variability is related to environmental pressure factors. Rubiscos from species inhabiting drought prone areas seem to have a catalytic efficiency more suited to the low CO_2 concentrations at the active site imposed by the water limitation. These results suggest that if a larger τ is to be found among C_3 terrestrial species, it probably should be found among evergreen woody species from arid environments.
3. The Rubisco τ of *Limonium gibertii* mean the highest value hitherto reported among higher plant Rubiscos and must be considered as a potential target for genetic engineering of the efficiency of the enzyme, and therefore, for crop productivity improvement.
4. Despite the Rubiscos adaptation to drought stressed conditions, Rubisco τ cannot acclimate to such stress, and therefore the validity of τ estimations by gas-exchange analysis under water stress may be questioned.

Chapter 11

ENDEMICITY CASE 1: *LYSIMACHIA MINORICENSIS*

PHOTOSYNTHESIS AND PHOTOPROTECTION RESPONSES TO WATER STRESS IN THE WILD-EXTINCT PLANT *LYSIMACHIA MINORICENSIS*

11.1. SUMMARY

Lysimachia minoricensis is an endemic species of the Balearic Islands that has become extinct in the wild, but persists in botanical gardens. Attempts of re-introducing the species into its natural habitat, which consisted in temporary dry streams, have failed. Low genetic variability has been reported for the garden individuals, suggesting that a reduced potential to adapt to environmental condition changes could be among the reasons for its extinction. In the present study, we particularly test whether photosynthesis and photoprotection responses of this species to water stress could help explaining the lack of success of this species in its natural habitat.

Plants of *L. minoricensis* were grown in pots in a growth chamber. Soil water depletion was imposed over 20 days by stopping irrigation. Stomata closed early in response to soil water depletion and leaves desiccated progressively. Although net photosynthesis was low in irrigated plants, due to a remarkably low mesophyll conductance to CO_2 (g_i), and decreased in parallel with stomatal closure during drought, substantial photosynthetic activity was kept at severe drought, where leaf relative water content was as low as 50%, suggesting that *L. minoricensis* was very drought-tolerant. In parallel with decreased photosynthesis, thermal dissipation of the excess light and photorespiration progressively increased. The former was linearly related to increased de-epoxidation of the xanthophylls cycle. Photoprotection was effective, as pre-dawn maximum photochemical efficiency (F_v/F_m) was maintained higher than 0.75 through the entire experiment. Moreover, photosynthetic capacity was largely (80%) recovered only 24h after re-watering. These results show that stomatal regulation, photosynthetic metabolism and photoprotection in *L. minoricensis* are well adapted to water stress, suggesting that additional factors may be responsible for its status as a wild-extinct plant.

11.2. INTRODUCTION

The Balearic Islands, located within the Mediterranean basin, are characterised by the richness of its endemic flora (Cardona & Contandriopoulos, 1979), which contributes to the maintenance of a high biodiversity in this area. However, a high proportion of the endemic species is confined to a few geographically restricted populations, what is partially responsible for the high fragility of island ecosystems (Carlquist, 1974). This involves a higher risk of extinction for island species as

compared to those growing in continental areas (Alcover *et al*., 1999; Hylton-Taylor, 2000). *Lysimachia minoricensis* J.J. Rodr. (*Primulaceae*) is, along with *Diplotaxis siettiana* Maire (Alboran Islet) and *Dianthus multinervis* Vis. (Jakuba islet), one of the three Mediterranean island endemic species that has gone extinct in the wild but that is still preserved in botanic gardens. *L. minoricensis* was described from a single population located at the south of the island of Minorca (Rodríguez, 1868), and probably disappeared in the field in the first half of the last century (Ibáñez *et al*., 1999). Fortunately, few individuals were kept in the Botanical Garden of Barcelona and then seeds distributed to several European botanical gardens (Bolós, 1962). Therefore, according to conservation categories developed by the IUCN (1994) this species has been classified as extinct in the wild.

Attempts to re-introduce *L. minoricensis* at its original habitat, which consisted in the vicinity of temporary drying water streams, have failed (Ibáñez *et al*., 1999). The shortage of sample propagules originally recovered in the field before its extinction is probably the cause of the extremely low genetic variability of the individuals nowadays kept in botanical gardens (Ibánez *et al*., 1999; Calero *et al*., 1999). A lack of genetic variability to counteract unfavourable environmental changes has been considered the most decisive factor for the survival of plant species restricted to small areas with low population size (Ellstrand & Elam, 1993; O'Brien, 1994). Therefore, this reason may be argued to explain the unsuccessful re-introduction attempts.

Habitat disturbance, fluctuating environments, niche competition, pests, predation, introgression and hybridization with relatives, and extensive recollection for economic or museistic purposes have been proposed as the main external factors responsible for plant extinction. However, few biological features of this biennial species are known, and the reasons for the extinction of *L. minoricensis* are still not clear. According to Lambers *et al*. (1998), a given species would inhabit a given site if it successfully passes three filters: a historical filter ('does it arrive?'), a physiological filter ('can it germinate, grow, survive and reproduce?') and a biotic filter ('does it successfully compete and defend itself?'). The attempts of re-introduction of *L. minoricensis* ensure passing the historical filter. Then the next cause for its lack of successfulness of this species in the field should be searched among physiological characters of the species that possibly limit overpassing of the physiological filter.

In this sense, a previous study by Rosselló & Mayol (2002) clearly established that fertility and seed viability were not the major causes of extinction and lack of

viability after re-introduction. Nevertheless, there could be many other negative traits underlying the ecophysiological performance that could limit grow and survival of plants in their native habitat. The habitat of *L. minoricensis* has a Mediterranean-type climate, characterized by hot, dry summers alternating with cool, wet winters (Nahal, 1981). From an ecophysiological point of view, the variability and unpredictability of precipitation in such environment imposes strong constraints on plants that could be extremely important for the survival of individuals (Joffre *et al*., 1999). The seasonal fluctuations in soil moisture, particularly summer drought, are considered a limiting factor for growth and productivity of Mediterranean perennial species (Mitrakos, 1980). Indeed, the severity of this stress in Mediterranean areas has increased over the last Century, providing more frequent and longer drought periods (Osborne *et al*., 2000). These changing climatic and environmental conditions may add even more dramatism to the unsuccessfulness of the low genetically diverse populations of *L. minoricensis*.

The aim of the present study was to analyse the photosynthetic responses of *L. minoricensis* to short term drought, as well as its capacity for recovery, to determine whether increased drought incidence in its natural habitat could be the limiting factor for its successfulness. In addition, since water stress in the Mediterranean is often accompanied by excess light which can lead to photoinhibition (Chaves *et al*., 2002), the capacity for thermal dissipation and pigment composition under drought were also examined.

11.3. MATERIALS AND METHODS

11.3.1. Plant material and treatments

Seeds of *Lysimachia minoricensis* J.J. Rodr. (Primulaceae) were obtained from a botanical garden (Jardí Botànic de Sóller, Spain) and germinated on filter paper moistened with deionized water in a controlled environment (germination chamber, at 18ºC in darkness). After germination and emergence of one true leaf, seedlings were transplanted to in large pots (25 L volume, 40 cm height) containing a 40:40:20 mixture of clay-calcareous soil, horticultural substrate and perlite. Plants were grown under natural conditions at the University of the Balearic Islands (Mallorca, Spain). Four weeks before starting the experiment, one year old plants were placed in a controlled growth chamber with a 12h photoperiod (26ºC day/20ºC night) and a photon flux density at the top of the leaves of about 600 μmol m^{-2} s^{-1} (halogen lamps).

Plants were once abundantly supplied with 100% Hoagland's solution, and then irrigated daily prior to the onset of the experiment. Measurements corresponding to control treatments were made during the first day of the experiment, when all the plants were well watered. Thereafter, irrigation was stopped in five plants and measurements made when soil water content reached 65 (mild drought treatment), 58 (moderate drought treatment) and 50% (severe drought treatment) in respect to that of control. Finally, another set of measurements was made one day after re-filling pots (re-watering treatment). Pots were weighted every day to determine the soil water content. Five control plants were watered daily during all the experiment and eventually measured to ensure that they maintained constant values of each parameter during the experiment. The experiment lasted 21 days.

11.3.2. Plant water status

Pre-dawn (Ψ_{MD}) and midday leaf water potential (Ψ_{MD}) was determined with a Scholander chamber (Soil Moisture Equipment Corp., USA) in four replicates per treatment.

The relative leaf water content at pre-dawn (RWC_{PD}) and mid morning (RWC_{MD}) was determined as follows: RWC = (Fresh weight − Dry weight) / (Turgid weight – Dry weight) × 100. To determine the turgid weight of the samples, these were kept in distilled water in darkness at 4°C to minimise respiration losses, until they reached a constant weight (full turgor, typically after 12 h). Their dry weight was obtained after 48 h at 70ºC in an oven. Four replicates per treatment were obtained.

11.3.3. Chlorophyll fluorescence measurements

Chlorophyll fluorescence parameters were measured on attached leaves using a portable pulse amplitude modulation fluorometer (PAM-2000, Walz, Effeltrich, Germany). For each sampling time and treatment, four measurements were made on different plants.

A measuring light of about 0.5 μmol photon m^{-2} s^{-1} was set at a frequency of 600 Hz to determine, at pre-dawn, the background fluorescence signal (F_o), the maximum fluorescence (F_m), and the maximum quantum efficiency of PSII ($F_v/F_m = (F_m-F_o)/ F_m$). At mid-morning the same leaves analysed at pre-dawn were measured with a photon flux density around 1500 μmol m^{-2} s^{-1}, obtained using the halogen lamp of the PAM-

2000, measuring steady state fluorescence signal (F_s). To obtain the steady-state maximum fluorescence yield (F_m'), saturation pulses of about 10000 μmol photon m^{-2} s^{-1} and 0.8 s duration were applied. The PSII photochemical efficiency ($\Delta F/F_m$', Genty *et al*., 1989) was then calculated as:

$$\Delta F/F_m' = (F_m'-F_s)/ F_m'$$

and used for the calculation of the relative linear electron transport rate (ETR) according to Krall & Edwards (1992):

$$ETR = \Delta F/F_m' \cdot PPFD \cdot \alpha \cdot \beta$$

where PPFD is the photosynthetically active photon flux density α is the leaf absorptance and β is a factor that assumes equal distribution of energy between the two photosystems (the actual factor has been described to be between 0.4 and 0.6; Laisk & Loreto, 1996). Leaf absorptances were calculated in ten replicates on leaves of well-irrigated plants with a spectroradiometer coupled to an integration sphere (UniSpec, PP-Systems, USA), and did result in 0.815 ± 0.006. Potential changes in leaf absorptance with drought were not assessed. However, variations are likely too small to induce important bias in the calculations of ETR.

11.3.4. Gas exchange measurements

Light-saturated net CO_2 assimilation rates (A_N) and stomatal conductance (g_s) were measured at mid-morning on one attached and fully developed young leaf of four plants per species and treatment four leaves of different plants per treatment with a gas exchange system (Li-6400, Li-Cor Inc., Nebraska, USA) equipped with a light source (6200-02B LED, Li-Cor). Environmental conditions in the chamber used for leaf measurements were established as: photosynthetic photon fluence rate = 1500 μmol photon m^{-2} s^{-1}; ambient vapour pressure deficit = 1.0-1.5 kPa; leaf temperature = 25ºC; ambient CO_2 concentration (C_a) = 360 μmol mol air^{-1}; and air flux = 300 μmol s^{-1}.

After inducing photosynthesis under the above conditions and once steady-state was reached, photosynthesis response curves to varying sub-stomatal CO_2 concentration (C_i) were performed. First, the C_a was lowered stepwise from 360 to 50 μmol mol^{-1} and then fixed again at 400 μmol mol^{-1} until reaching a steady-state value similar to that obtained at the beginning of the curve. Then, C_a was increased stepwise from 400 to 1500 μmol mol^{-1}. Gas exchange measurements were determined at each step after

maintaining the leaf for at least 5 min at the new C_a. Measurements consisted in 12-13 measurements for each curve.

For dark respiration measurements, four leaf samples per treatment and species were collected during the light period and stored 20 min in the dark in 0.2 mM $CaCl_2$ for membrane stabilization. O_2 uptake rates were measured in the dark, using a liquid-phase oxygen electrode (Hansatech Instruments Ltd., England) in ambient air-equilibrated 10 mM Mes buffer (pH 5.7), as previously described (Delieu & Walker, 1981; Azcón-Bieto *et al*., 1994). Leaf 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, except at low O_2 concentrations. To avoid oxygen-limiting conditions inside the cuvette, all measurements were determined with O_2 concentration above 60% of saturation. Respiration measurements were performed with the oxygen electrode technique to avoid the gasket-related leak with the CO_2 gas exchange measurements (Long & Bernacchi, 2003; Hurry *et al*., 2005). It is well known that the precision of the oxygen electrode techniques for respiration measurements are much higher than techniques based on CO_2 gas-exchange measurements (Hurry *et al*., 2005).

11.3.5. CO_2 concentration at the site of carboxylation and mesophyll conductance estimations

From combined gas-exchange and chlorophyll fluorescence measurements, the chloroplast CO_2 concentration (C_c), according to a model previously described by Epron *et al*. (1995). This model assumes that all the reducing power generated by the electron transport chain is used for photosynthesis and photorespiration, and that chlorophyll fluorescence gives a reliable estimate of the quantum yield of electron transport. Thus, the electron transport rate (ETR) measured by chlorophyll fluorescence can be divided into two components: $ETR = ETR_A + ETR_P$, where ETR_A is the fraction of ETR used for CO_2 assimilation, and ETR_P is the fraction of ETR used for photorespiration. ETR_A and ETR_P can be solved from data of A_N, R_L and ETR, and from the known stochiometries of electron use in photosynthesis and photorespiration, as follows (Epron *et al*., 1995; Valentini *et al*., 1995): $ETR_A = 1/3\ [ETR + 8\ (A_N + R_L)]$; $ETR_P = 2/3\ [ETR - 4\ (A_N + R_L)]$. From ETR_A and ETR_P, τ^* can be calculated according to Laing *et al*. (1974), as follows: $\tau^* = (ETR_A / ETR_P) / (C_c/O)$. The CO_2 concentration at the carboxylation site (C_c) is approximated by C_i, and the oxygen molar fraction at the

oxygenation site (*O*), is assumed to be equal to the molar fraction in the air. Alternatively, if τ as determined *in vitro* is assumed to be valid for the Rubisco *in vivo*, then C_c and g_i can be calculated. In both cases, the corresponding concentrations in the liquid phase were calculated from the published solubility of the gases in water (Harned & Davis, 1943; Harned & Bonner, 1945) at the measured temperature and partial pressure. The mesophyll conductance to CO_2 was then calculated as: $g_i = A_N / (C_i - C_c)$. For such calculations, the Rubisco *in vitro* specificity factor for *L. minoricensis* was considered to be 93.9 (Galmés *et al*., 2005a).

11.3.6. Quantitative limitation analysis

At ambient CO_2 concentration, light-saturated photosynthesis is generally limited by substrate availability, which was verified by A_N–C_i curves in the present data for each species and treatment (not shown). Under CO_2-limited conditions, photosynthesis can be expressed as (Farquhar *et al*., 1980):

$$A_N = \frac{V_{c,\max} \cdot C_c}{C_c + K_c \cdot (1 + O/K_o)} \cdot \left(1 - \frac{\Gamma^*}{C_c}\right) - R_L$$

where $V_{c,max}$ is the maximum rate of carboxylation of Rubisco, K_c and K_o are the Michaelis–Menten constants for CO_2 and O_2, respectively (from Bernachi *et al*., 2001), Γ* is the CO_2 compensation point in the absence of mitochondrial respiration, and R_L is the rate of non-photorespiratory CO_2 evolution in the light. The treatment average of Γ* for the species was obtained, according to Brooks & Fraquhar (1985), as:

$$\Gamma^* = \frac{0.5O}{\tau}$$

from the specific *in vitro* specificity factor measured by Galmés *et al*. (2005) for *L. minoricensis*. Finally, R_L was calculated for the A_N-C_i curve on the same treatment, as in Grassi & Magnani (2005).

Estimations of $V_{c,max}$ were derived from fitting A_N-C_c curves. The A_N-C_c curves were obtained from A_N-C_i curves using the values of g_i, following the method described by Manter & Kerrigan (2004).

To compare relative limitations to assimilation due to drought, photosynthetic limitations were partitioned into their functional components following the approach proposed by Grassi & Magnani (2005). This approach, which requires the measurement of A_N, g_s, g_i and $V_{c,max}$, makes it possible to partition photosynthesis limitations into components related to stomatal conductance (S_L), mesophyll conductance (MC_L) and leaf biochemical characteristics (B_L), assuming that a reference maximum assimilation rate can be defined as a standard. The maximum assimilation rate, concomitantly with g_s and V_{cmax}, was reached under well-watered conditions; therefore the control treatment was used as a reference.

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

11.3.7. Pigment analyses

Immediately after chlorophyll fluorescence measurements (at predawn and midday), discs were punched from leaves of the same plants showing the same orientation as those used for fluorescence measurements and submersed into liquid nitrogen. Four samples per treatment were taken from different plants (four leaves per sample). Pigments were extracted by grinding leaf tissue in a mortar with acetone in the presence of sodium ascorbate. Pigments were identified and quantified by high performance liquid chromatography according to Abadía & Abadía (1993), with modifications as described in Larbi *et al*. (2004).

11.3.8. Statistical analysis

Statistical analyses of the data were performed with the SPSS 12.0 software package (SPSS, Chicago, USA). One-way ANOVAs, with the treatment as factors, were performed for the studied parameters. Differences between means were revealed by Duncan analyses ($P < 0.05$).

11.4. RESULTS

Soil water depletion from 100% to 50% SWC resulted in large decreases in leaf RWC and Ψ (Table 11.1). RWC_{PD} decreased from 91.4% in well-watered plants to approximately 50% in the severe drought treatment. Similar decreases in RWC were recorded in leaves sampled at midday. Ψ_{PD} and Ψ_{MD} also decreased as water stress increased, ranging from -0.23 to -2.10 MPa at predawn and from -0.57 to -3.23 MPa at midday, for well-watered and severe drought conditions, respectively. Despite the very low RWC achieved, plants largely recovered their water status 24h after re-filling water in pots, reaching RWC values of 71.8 and 84.4% at predawn and midday, respectively. Consequently, Ψ_{PD} and Ψ_{MD} also recovered to lower values of -0.64 and -0.70 MPa, respectively.

Table 11.1

Soil water content (SWC), predawn leaf relative water content (RWC_{PD}), midday leaf RWC (RWC_{MD}), predawn leaf water potential (Ψ_{PD}) and midday leaf water potential (Ψ_{MD}) measured under different irrigation treatments: control (C), mild drought (MiD), moderate drought (MoD), severe drought (SD) and re-watering (R). Values are given in means ± standard deviations. Different letters denote statistical differences by Duncan test ($P < 0.05$) among treatments.

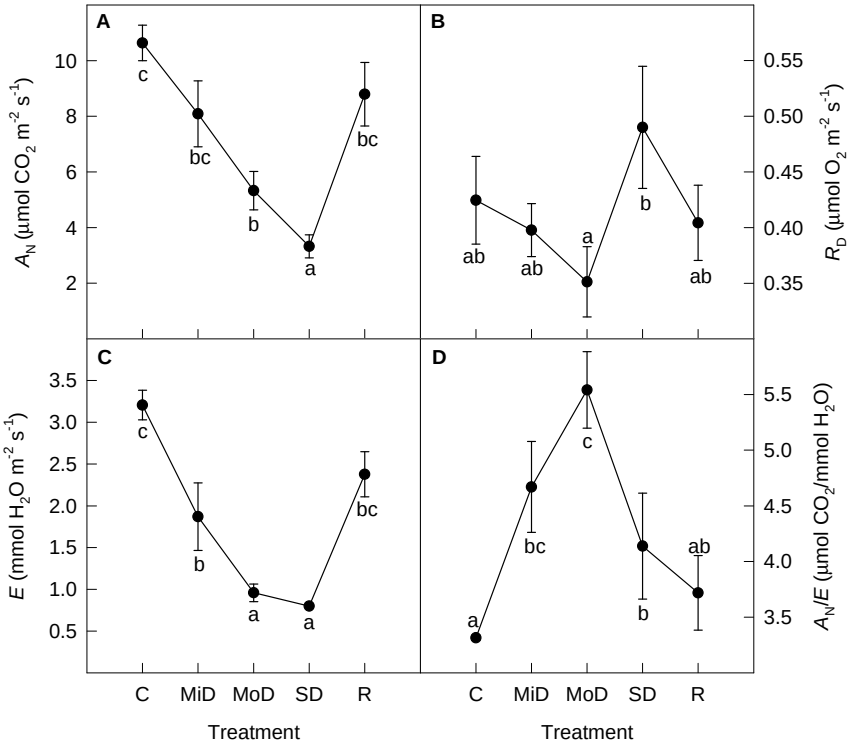
	SWC (% control)		RWC_{PD} (%)		RWC_{MD} (%)		Ψ_{PD} (MPa)		Ψ_{MD} (MPa)	
C	100.0 ± 0.0	c	91.4 ± 0.4	c	76.1 ± 0.9	b	-0.23 ± 0.01	c	-0.57 ± 0.08	c
MiD	65.6 ± 2.6	b	80.3 ± 3.6	bc	74.5 ± 3.8	b	-0.78 ± 0.33	bc	-1.05 ± 0.28	c
MoD	58.4 ± 3.6	ab	73.2 ± 4.1	b	61.9 ± 4.0	a	-1.33 ± 0.17	b	-2.00 ± 0.09	b
SD	49.5 ± 3.6	a	49.8 ± 5.2	a	51.2 ± 5.5	a	-2.10 ± 0.27	a	-3.23 ± 0.23	a
R	90.6 ± 5.7	c	71.8 ± 3.6	b	84.4 ± 2.0	b	-0.64 ± 0.08	c	-0.70 ± 0.06	c

Net CO_2 assimilation rates were decreased by water stress (Fig. 11.1A). Plants responded to a 50% reduction in water availability by gradually decreasing their A_N up to approximately a 30% of that observed under control treatment. Re-watered plants recovered their photosynthetic capacity by 8 %. Dark respiration (R_D) was less affected by water stress, ranging from 0.35 to 0.50 μmol O_2 m^{-2} s^{-1} during the entire experiment (Fig. 11.1B). Only under severe water stress R_D was significantly increased, but 24h after re-watering plants recover the initial rates.

Transpiration rates (*E*) were reduced by water stress from 3.2 to 0.8 mmol H_2O m^{-2} s^{-1} (Fig. 11.1C). Re-watered plants recovered only partially *E* (2.4 mmol H_2O m^{-2} s^{-1}). Instantaneous water-use efficiency (A_N/E) in well-watered plants was found to be 3.3 mmol CO_2 mol^{-1} H_2O, and increased to 5.5 mmol CO_2 mol^{-1} H_2O when water availability decreased to moderate levels (Fig. 11.1D). When water stress became more severe, A_N/E was decreased again to 4.1 mmol CO_2 mol^{-1} H_2O. After re-watering, A_N/E recovered control values.

Figure 11.1

(A) Net CO_2 assimilation (A_N), (B) and dark respiration rates (R_D), (C) transpiration rates (*E*), and (D) instantaneous water use-efficiency (A_N/E) under different irrigation treatments: control (C), mild drought (MiD), moderate drought (MoD), severe drought (SD) and re-watering (R). Values represent means ± standard deviations. Different letters denote statistical differences by Duncan test ($P < 0.05$) among treatments.

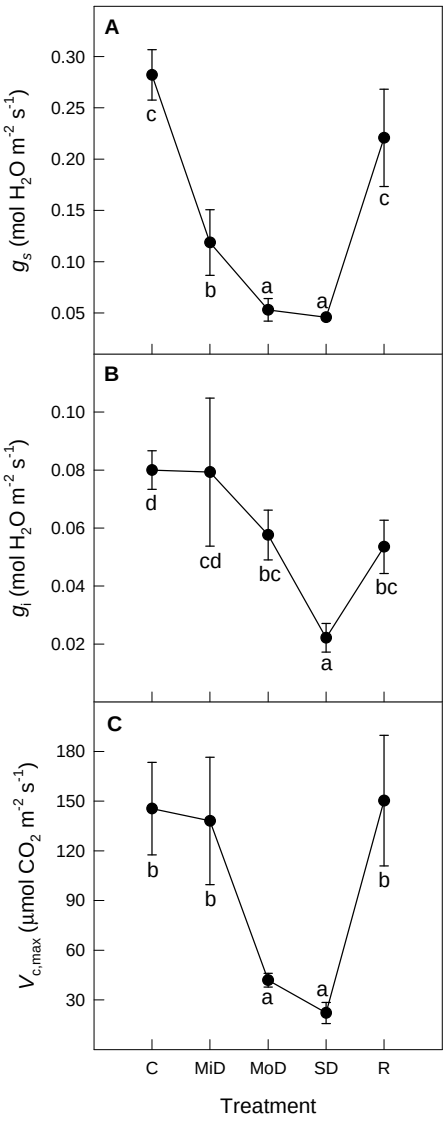


Under ambient CO_2 concentrations, three factors can be limiting photosynthesis during drought stress development: stomatal and mesophyll conductances to CO_2 and carboxylation capacity (Flexas *et al*., 2004a; Grassi & Magnani, 2005). The first two are considered diffusional limitations, while a decreased carboxylation capacity is usually related to a decrease in Rubisco activity and, therefore, is considered a biochemical

limitation. Stomatal conductance (g_s) was largely decreased even at mild stress, dropping from ca. 0.30 to 0.12 mol H_2O m^{-2} s^{-1} (Fig. 11.2A). Moderate and severe water stress resulted in a further decrease of g_s to values close to 0.05 mol H_2O m^{-2} s^{-1}. By contrast, mesophyll conductace (g_i) and the carboxylation capacity ($V_{c,max}$) were not affected by mild water stress (Fig. 11.2B, C), but they were at moderate and severe water stress. Re-watering resulted in a total recovery of $V_{c,max}$, but g_s and g_i were recovered only by 85% and 75%, respectively.

Figure 11.2

(A) Stomatal conductance (g_s), (B) mesophyll conductance (g_i), and (C) maximum rate of carboxylation ($V_{c,max}$) under different irrigation treatments: control (C), mild drought (MiD), moderate drought (MoD), severe drought (SD) and re-watering (R). Values represent means ± standard deviations. Different letters denote statistical differences by Duncan test ($P < 0.05$) among treatments.



When using these data for a limitation analysis (Table 11.2), it resulted that a 21% of total photosynthetic limitation under mild water stress was fully due to stomatal limitation (S_L). Under moderate and severe water stress, mesophyll conductance (MC_L) and biochemical (B_L) limitations also contributed significantly to the larger total limitations (50% and 69% under moderate and severe stress, respectively). At moderate stress, non-stomatal limitations (i.e. MC_L plus B_L) were of similar magnitude as stomatal limitations, but under severe stress non-stomatal limitations were much larger than stomatal limitations, accounting for up to 2/3 of the total limitations. However, the total diffusional limitations (i.e. S_L plus MC_L) were larger than biochemical limitations, accounting from 60% to 100% of the total limitations during the entire experiment. 24h after re-watering, a total photosynthesis limitation of 18% persisted. This was largely accounted for MC_L (13%), and much less for S_L (5%), with no contribution of B_L remaining (Table 11.2).

Table 11.2

Limitations of A_N, expressed as a percentage as compared to the control maximum values, under the different irrigation treatments: mild drought (MiD), moderate drought (MoD), severe drought (SD) and re-watering (R).

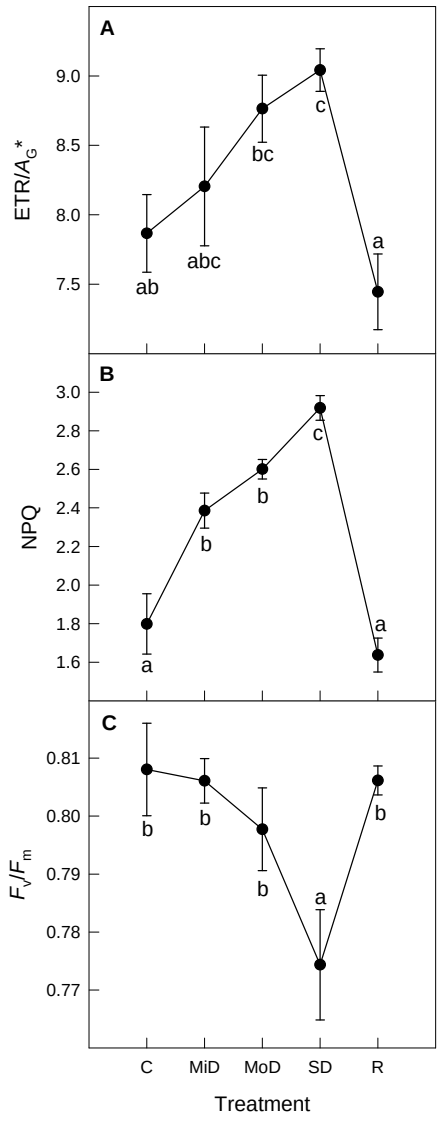
Treatment	Total limitations	Stomatal limitation (S_L)	Mesophyll conductance limitation (MC_L)	Biochemical limitation (B_L)	Non-stomatal limitations (NS_L)	Diffusional limitations (D_L)
MiD	24	21	1	2	3	22
MoD	50	25	6	19	25	31
SD	69	21	25	23	48	46
R	17	5	13	-1	13	18

Under water stress conditions, the light which is in excess of what can be used in photosynthesis increases, which can result in photoprotection and/or photoinhibition. Regarding photoprotection, ETR/A_G* (an indicator of photorespiration, see Flexas & Medrano, 2002c) and NPQ (an indicator of thermal dissipation in PSII antennae, see Björkman & Demmig-Adams, 1994) both increased progressively during water stress development (Fig. 11.3A, B). In respect to photoinhibition, while A_N was progressively reduced by ca. 70% during water stress, the maximum photochemical efficiency of PSII

(F_v/F_m) was only depressed by 5% under the most severe water stress situation, which fully recovered after re-watering (Fig. 11.3C).

Figure 11.3

(A) Ratio of electron transport rate to gross CO_2 assimilation accounting for dark and photorespiration (ETR/A_G*), (B) non-photochemical quenching (NPQ), (C) maximum quantum yield of PSII measured at predawn (F_v/F_m) under different irrigation treatments: control (C), mild drought (MiD), moderate drought (MoD), severe drought (SD) and re-watering (R). Values represent means ± standard deviations. Different letters denote statistical differences by Duncan test ($P < 0.05$) among treatments.



Area-based total chlorophyll concentration differed among treatments (Table 11.3), being maximal under non-stressed conditions (521 μmol m^{-2}) and minimal under

severe drought treatment (395 μmol m^{-2}). In contrast, β-carotene was unaffected by drought stress. The total pool of xanthophylls cycle pigments (VAZ, sum of violaxanthin, antheraxanthin and zeaxanthin) expressed on a chlorophyll basis was only marginally increased under drought, and this was largely a consequence of decreased chlorophyll and not increased VAZ on an area basis. However, the sum of A+Z progressively increased as soil water depletion increased, due to the conversion of violaxanthin to zeaxanthin. The other xanthophylls, taraxanthin and lutein, they did not differ significantly ($P > 0.05$) among treatments.

Table 11.3

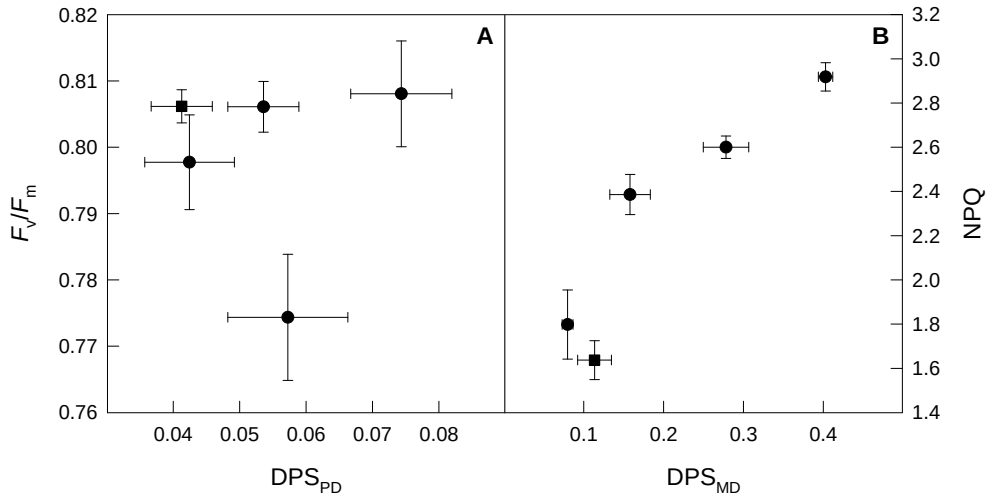
Chlorophyll and xanthophylls concentrations at mid-morning and de-epoxidation state at predawn (DPS_{PD}) and mid-morning (DPS_{MD}), under different irrigation treatments: control (C), mild drought (MiD), moderate drought (MoD), severe drought (SD) and re-watering (R). Units for chlorophyll are μmol m^{-2} and for xanthophylls, mmol mol^{-1} Chl. Values are given in means ± standard deviations. Different letters denote statistical differences by Duncan test ($P < 0.05$) among treatments. Abbreviations: V = violaxanthin; A = anteraxanthin; and Z = zeaxanthin.

	C		MiD		MoD		SD		R	
Chl a + b	521 ± 46	b	407 ± 40	ab	442 ± 38	ab	395 ± 29	a	436 ± 31	ab
β-carotene	85.3 ± 1.8	ab	88.3 ± 2.2	b	81.1 ± 2.5	a	86.4 ± 1.0	ab	87.2 ± 2.0	ab
Neoxanthin	30.0 ± 0.5	ab	31.2 ± 1.0	abc	34.2 ± 1.5	c	32.3 ± 0.6	bc	28.8 ± 1.1	a
Violaxanthin	45.7 ± 3.7	bc	48.2 ± 4.7	bc	40.2 ± 3.5	ab	33.2 ± 1.1	a	52.9 ± 4.1	c
Taraxanthin	2.3 ± 0.5	a	2.4 ± 0.5	a	2.0 ± 0.3	a	1.3 ± 0.3	a	2.3 ± 0.1	a
Antheraxanthin	6.6 ± 1.3	a	9.5 ± 0.3	ab	14.6 ± 1.9	c	13.4 ± 1.9	bc	7.7 ± 0.6	a
Lutein	113.2 ± 4.4	a	114.8 ± 3.6	a	117.5 ± 4.8	a	121.9 ± 2.9	a	111.8 ± 2.6	a
Zeaxanthin	0.9 ± 0.6	a	5.2 ± 1.6	b	11.0 ± 1.8	c	20.2 ± 1.0	d	3.2 ± 0.9	ab
Σ xanthophylls	198.6 ± 7.2	a	211.4 ± 7.6	ab	219.5 ± 10.2	ab	222.4 ± 4.8	b	206.8 ± 4.4	ab
VAZ	53.2 ± 3.4	a	62.9 ± 5.1	ab	65.8 ± 4.7	ab	66.8 ± 1.9	b	63.8 ± 3.6	ab
Z+A	7.5 ± 0.7	a	14.7 ± 1.8	b	25.6 ± 3.2	c	33.6 ± 1.4	d	10.9 ± 1.2	ab
DPS_{PD}	0.07 ± 0.01	b	0.05 ± 0.01	ab	0.04 ± 0.01	a	0.06 ± 0.01	ab	0.04 ± 0.00	a
DPS_{MD}	0.08 ± 0.01	a	0.16 ± 0.03	b	0.28 ± 0.03	c	0.40 ± 0.01	d	0.11 ± 0.02	ab

De-epoxidation state at midday (DPS_{MD}) increased gradually with increasing water stress, with a maximum value of 0.4 found in severely stressed plants. However, this was not correlated with a drought-induced sustained DPS_{PD}. A lack of correlation was found between DPS_{PD} and F_v/F_m (Fig. 11.4A). In contrast, DPS_{MD} was highly correlated with NPQ ($r^2 = 0.845$, $P < 0.01$) (Fig. 11.4B).

Figure 11.4

(A) Relationship between the maximum quantum yield of PSII measured at predawn (F_v/F_m) and predawn de-epoxidation state of the xanthophylls cycle (DPS_{PD}), and (B) relationship between the mid-morning non-photochemical quenching (NPQ) and the mid-morning DPS (DPS_{MD}) for the five treatments studied. Measurements corresponding to re-watering treatment are indicated by square symbols.



11.5. DISCUSSION

11.5.1. Photosynthetic capacity and water stress-induced down-regulation

According to Gulías *et al*. (2003), Balearic endemic species presented a low photosynthetic capacity per leaf mass as compared to widespread Mediterranean species of similar specific leaf area (SLA), and this was hypothesised to contribute to the declining distribution of such species. *L. minoricensis* was not included in that study, but here we show that its photosynthetic capacity was as low as 10.6 μmol CO_2 m^{-2} s^{-1}, with a SLA of 222 cm^2 g^{-1}, which results in a photosynthetic capacity per leaf mass of 235 nmol g^{-1} s^{-1}. According to the regression line found by Gulías *et al*. (2003) for

widespread Mediterranean species, a plant with a SLA of 222 cm^2 g^{-1} should have a photosynthetic capacity of 475 nmol g^{-1} s^{-1}. Therefore, the photosynthetic capacity of *L. minoricensis* is considerably low, similar to that typically found in Mediterranean schlerophyll evergreens, and it falls even below the average relationship found by Gulías *et al*. (2003) for Balearic endemics. The low photosynthetic capacity exhibited by *L. minoricensis* is not due to stomatal conductance, which is quite high (up to 0.3 mol H_2O m^{-2} s^{-1}) nor to its biochemical capacity to fix CO_2 (a $V_{c,max}$ of ca. 150 μmol m^{-2} s^{-1} is a very large value for a C_3 species, see Wullschleger, 1993; Manter & Kerrigan, 2004). Rather, the low photosynthetic capacity seems related to the low mesophyll conductance to CO_2 exhibited by this species, which is remarkably less than 1/3 of g_s (Fig. 11.2). As a consequence, the estimated CO_2 concentration at the Rubisco locus was as low as 91.8 μmol CO_2 mol^{-1}. A g_s/g_i ratio below 1 is sometimes found in sclerophyllous species (Niinemets *et al*., 2005; Peña-Rojas *et al*., 2005; Galmés, unpublished) and conifers (De Lucia *et al*., 2003; Warren *et al*., 2004), i.e. in species with low SLA. This low g_s/g_i ratio in a species with a high SLA adds another milestone to the increasing evidence that the importance of g_i in setting the photosynthetic capacity cannot be neglected (Ethier & Livingston, 2004; Flexas *et al*., 2004a; Manter & Kerrigan, 2004; Warren & Adams, 2006).

In principle, a low photosynthetic capacity for a biennial species like *L. minoricensis* may severely compromise a positive carbon balance throughout the year, because short-lived species are obviously unable to develop a root system enough large to explore deeply water soil resources, and therefore their carbon gain rely mostly on the photosynthetic activity when water is available. However, this statement may vary if the plant is capable of maintaining a substantial photosynthetic activity during temporary dry periods. We therefore aimed to analyse the response of photosynthesis during drought.

In response to water stress, and despite the fact that stomatal conductance (g_s) and transpiration were rapidly adjusted in response to mild water stress, *L. minoricensis* responded as an anisohydric species, gradually decreasing its water status (both RWC and Ψ) as the severity of water stress increased (Table 11.1). At severe stress, a very low leaf RWC (50%) was achieved, as compared with values found in other Mediterranean species (Lo Gullo & Salleo, 1988; Gulías *et al*., 2002). Despite this very low RWC, *L. minoricensis* maintained a substantial photosynthetic activity (ca. 30% of

control values) at severe stress. This fact, together with an almost 80% of photosynthetic recovery only 24h after re-watering, suggests that *L. minoricensis* is a drought tolerant species. This drought tolerant strategy may be a clearly advantageous feature for a plant like *L. minoricensis*, whose natural habitat consisted in the vicinity of temporary dry water streams.

Down-regulation of photosynthesis and its components during progressive drought was very similar to that typically observed in C_3 plants (Flexas & Medrano, 2002a; Flexas *et al*., 2004a; Grassi & Magnani, 2005). In response to a mild water stress, only g_s declined while g_i and $V_{c,max}$ were kept at control values. At moderate and severe drought stress, g_i and $V_{c,max}$ also declined (Fig. 11.2). A limitation analysis showed that stomatal closure accounted for the entire reduction of photosynthesis at mild drought. At moderate drought the non-stomatal limitations (i.e. the sum of contributions by mesophyll conductance and biochemical capacity) were of the same magnitude as stomatal limitations, and at severe drought 2/3 of the total photosynthetic limitation was due to non-stomatal factors (Table 11.2). The similarity of this pattern to that found in many C_3 plants suggests that *L. minoricensis* presents no particular disadvantage in its photosynthetic response to water stress. It is remarkable that incomplete recovery of photosynthesis after re-watering is largely due to a low recovery of mesophyll conductance to CO_2. This fact points out again for the importance of g_i in controlling the photosynthetic capacity of plants. Understanding the molecular and physiological mechanisms underlying the observed g_i responses may be a research priority for the near future.

Regarding respiration, the other component of plant carbon balance, the leaf respiration rates measured in the dark (R_D) were quite low in this species (Fig. 11.1). Moreover, R_D in *L. minoricensis* is maintained within a narrow range (0.35 to 0.50 μmol m^{-2} s^{-1}) during drought, although it certainly follows the typical pattern described by Flexas *et al*. (2005), consisting in decreasing during the initial stages of drought (RWC > 60%) and raising as RWC decreases below 50%. The initial decrease in R_D would be related to the immediate stoppage of leaf growth and, consequently, the reduction of the growth respiration component. The latter increase may be related to increased metabolism as the plant induces acclimation mechanisms to resist drought (Flexas *et al*., 2005). As a result of these R_D responses, even under severe drought the rates of R_D were only 15% of photosynthetic rates. Respiration in the light (not measured) is expected to be even lower (Villar *et al*., 1994, 1995). While leaf R_D may not be representative of the

total plant respiration, the low values measured during the entire experiment suggest that reduced carbon loss in respiration may partly compensate for the low photosynthetic capacity of *L. minoricensis*.

11.5.2. Photoprotection responses to water stress

Under water stress conditions, the light which is in excess of what can be used in photosynthesis increases, which can result in photoinhibition (Osmond *et al*., 1999). Plants can avoid photoinhibition either decreasing the absorption of light or increasing the dissipation of excess absorbed light through photochemical and non-photochemical mechanisms (Björkman & Demmig-Adams, 1994). Photoprotection mechanisms have been recognised as an adaptive trait to the Mediterranean environment (Chaves *et al*., 2002). *L. minoricensis* presented steep leaf angles and severely rolled its leaves in response to drought, which may have resulted in a larger decrease in light absorption by the leaves (Valladares & Pugnaire, 1999). On the other hand, leaves of *L. minoricensis* showed an early decrease in chlorophyll content in response to mild drought (Table 11.3), far before any change in the maximum photochemical capacity was observed (Fig. 11.3). In the Mediterranean semi-deciduous shrub *Phlomis fruticosa*, a chlorophyll loss during summer which is not accompanied by decreased photochemical capacity has been suggested as a means of reducing leaf absorptance, therefore contributing to photoprotection (Kyparissis *et al*., 1995).

In addition to possible reductions in leaf absorptance, increased photorespiration and thermal dissipation are known to be the major photoprotective responses to avoid photoinhibition under water stress (Flexas & Medrano, 2002c). ETR/A_G* (an indicator of photorespiration, see Flexas *et al*., 2002c) and NPQ (an indicator of thermal dissipation in PSII antennae, see Björkman & Demmig-Adams, 1994) both increased progressively during water stress development (Fig. 11.3A, B). As previously described for other Mediterranean species (García-Plazaola *et al*., 1997; Faria *et al*., 1998; Martínez-Ferri *et al*., 2000; Gulías *et al*., 2002), there was a high correlation between DPS_{MD} and NPQ (Fig. 11.4), suggesting a tight regulation of the xanthophylls cycle in response to drought-induced excess light, resulting in safe energy dissipation (Demmig-Adams & Adams, 1996; Demmig-Adams, 1998).

As a consequence of the activation of these photoprotective mechanisms, while A_N was progressively reduced by ca. 70% during water stress, the maximum

photochemical efficiency of PSII (F_v/F_m) was only depressed by 5% under the most severe water stress situation, which fully recovered after re-watering (Fig. 11.3C). The fact that this decrease in F_v/F_m did not correlate with DPS during the night (Fig. 11.6A) supports the idea that it reflected in a small induction of photodamage to PSII when water becomes severely scarce (Osmond *et al*., 1999).

The combined operation of photoprotective mechanisms in *L. minoricensis* during progressive drought allow reducing photodamage to a minimum restricted to severe drought, and therefore help preserving the integrity of the photosynthetic apparatus. This may be important for the rapid photosynthetic recovery observed after re-watering.

11.5.3. Concluding remarks

In summary, the present ecophysiological analysis of *L. minoricensis* shows that, although this species presents a very low photosynthetic capacity – due to an intrinsically low mesophyll conductance to CO_2 – as compared to possible competitors with similar SLA, it also presents relatively low and invariable respiration rates, which may partly compensate the photosynthetic limitation for carbon balance. On the other hand, *L. minoricensis* shows a drought-tolerant behavior, related to very efficient photoprotection mechanisms, which results in the maintenance of substantial photosynthesis even under severe drought, and in a rapid recovery upon re-watering. All together, these ecophysiological features seem quite adaptive to the natural environment of the species, which consisted in temporary drying water streams.

Chapter 12

ENDEMICITY CASE 2: *DIGITALIS MINOR*

PHOTOSYNTHESIS AND PHOTOINHIBITION IN RESPONSE TO DROUGHT IN A PUBESCENT (VAR. *MINOR*) AND A GLABROUS (VAR. *PALAUI*) VARIETY OF *DIGITALIS MINOR*

12.1. SUMMARY

Ecophysiological differences on morphological and photosynthetic traits were studied in two varieties of a Mediterranean endemic species, *Digitalis minor*. These varieties are distinguished according to their differences in leaf pubescence: var. *minor* is pubescent while var. *palaui* is glabrous. Water status, specific leaf weight, gas-exchange and chlorophyll fluorescence measurements were performed under two different water availability treatments: field capacity and 25% field capacity. The presence of leaf trichomes in var. *minor* is shown as an efficient mechanism in preserving photochemistry apparatus, when compared to the glabrous var. *palaui*. However, such differential pubescence did not imply a lower leaf water loss, probably because of divergent leaf structure, as suggested by a higher specific leaf weight in var. *palaui*. Other intraspecific differences are found both in the photosynthetic behaviour and in leaf morphological traits between both varieties, especially higher photosynthetic and mesophyll conductance capacities for var. *minor* in respect to var. *palaui*. These results are discussed in terms of selection pressures and ecological plasticity to link with actual and future scenarios derived from the climatic change.

12.2. INTRODUCTION

The Mediterranean flora presents a high level of endemic species richness, which contributes to the maintenance of a high biodiversity in such ecosystems (Cardona & Contandriopoulos, 1979). *Digitalis minor* L. (*Scrophulariaceae*) is an endemic long-lived herbaceous species of the Balearic archipelago (Contandriopoulos & Cardona, 1984). *Digitalis minor* shows a high degree of morphological polymorphism, but only two infraspecific taxa are currently recognized according to its differences in pubescence (Hinz, 1987): *D. minor* var. *minor* (pubescent) and *D. minor* var. *palaui* (glabrous).

The Mediterranean-type climate is characterized by hot, dry summers alternating with cool, wet winters (Nahal, 1981). From an ecophysiological point of view, the variability and unpredictability of precipitation imposes strong constraints on plants that are considered a limiting factor for growth and productivity of Mediterranean perennial species (Mitrakos, 1980). Moreover, the global change effects on the Mediterranean climate likely increase the severity of this stress, providing more frequent and longer drought periods (Osborne *et al.*, 2000). Plants respond to water stress by closing

stomata, which reduces CO_2 availability in the chloroplasts, and thus photosynthesis and photosynthetic capacity are progressively decreased under drought. In consequence, a lower light intensity is required to saturate photosynthesis under drought than under well-watered conditions (Cornic, 1994; Lawlor, 1995). Therefore, at high light intensity, more light is in excess of what can be used for photosynthesis as drought progresses. Thus, the susceptibility of drought plants to photoinhibition may be increased (Osmond, 1994), especially in those environments where plants are exposed to a combination of low water availability and high solar irradiance. In such conditions, as those typically found under Mediterranean summer climate, protection against photoinhibition may confer plants adaptation to the environment (Ripley *et al*., 1999; Morales *et al*., 2002).

Such photoprotective mechanisms involve physiological adjustments in leaf morphology and anatomy, leaf biochemistry and photochemistry (Lovelock & Clough, 1992; Havaux & Niyogi, 1999). Regarding leaf morphology and among other functions (Johnson, 1975; Zvereva *et al*., 1998), non-glandular leaf hairs are known to modify the internal radiation environment of a leaf (Karabourniotis & Bornman, 1999). A hair mat may act as a reflector in the visible and infra-red part of the spectrum, reducing the radiant energy absorbed by the leaf and affecting its energy balance (Ehleringer, 1984). Therefore, leaf pubescence has already been reported to be an adaptation to the Mediterranean environment by reducing transpiration, increasing the probability of water uptake by leaves, maintaining favourable leaf temperature, and protecting against UV-B radiation responsible for photosynthetic inhibition (Savé *et al*., 2000). Furthermore, increased pubescence reduces the heat load, which helps maintaining leaf temperatures within the photosynthetically stable range while also decreasing reliance on transpirational cooling for avoiding high leaf temperatures (Ehleringer & Mooney, 1978). This developmental response has been considered adaptive in water-limited environments because it reduces water loss and permits the maintenance of photosynthetic activity longer into the drought period (Smith & Nobel, 1977; Ehleringer & Mooney, 1978; Ehleringer, 1983). Finally, the density of trichomes has been documented to be involved in defence strategies related to the presence or absence of herbivores (Levin, 1973; Becerra & Ezcurra, 1986).

The variability of leaf absorptance within a species may result from the balance between costs and benefits over regional differences in precipitation and drought. When water availability is greater, or when drought is ameliorated by rainfall, the benefit of leaf hairs may be set by costs and constraints associated with their production

(Ehleringer & Mooney, 1978). Leaf hairs can have high one-time construction costs, and they also continuously reduce photosynthesis by reflecting photosynthetically active radiation (Sandquist & Ehleringer, 2003).

The aim of this work was to compare the two varieties of the Balearic endemic *D. minor*, to check if the presence of a high density of trichomes in *D. minor* var. *minor* provides a successful photoprotective mechanism when compared to the glabrous *D. minor* var. *palaui*. We also studied if such morphological differences between both varieties were accompanied by divergences developed at the photosynthetic level. According to the major influencing stress on plant development and survival under the Mediterranean conditions, such potential ecophysiological differences were discussed on the basis of their adaptation to water deficit conditions.

12.3. MATERIALS AND METHODS

12.3.1. Plant material

Plant material was obtained from seeds collected from natural populations of both varieties of *Digitalis minor* in Mallorca: *D. minor* var. *minor* from Torrent de Mortitx (N 39º52' E 02º54') and *D. minor* var. *palaui* from Son Fortuny (N 39º38' E 02º27') populations.

Seeds were germinated on filter paper moistened with deionized water in a controlled environment (germination chamber, at 18ºC in darkness). On the day following radicle emergence, seedlings were planted individually in pots (20 cm height, 4.1 L volume) containing a 40:40:20 mixture of clay-calcareous soil, horticultural substrate and perlite. A total of ten plants per variety of about six months old and with similar size (total leaf area of about 0.8 m^2) were selected to perform the study in June 2003, outdoors at the University of the Balearic Islands (Mallorca, Spain).

The environmental conditions during the experiment were characteristic of the typical late spring-summer Mediterranean climate, with high temperatures and low relative humidity which generate considerable water losses by evapotranspiration. The maximum average temperature was 25.3ºC, with a total monthly irradiance of about 740 MJ m^{-2}.

The transplanted seedlings were equally well-watered during the first 90 days prior to exposure to water stress. During this period, plants were fertilised weekly with Hoagland's solution at 50%. After that, seedlings were randomly assigned to two

irrigation treatments: a) plants maintained at field capacity (control), and b) plants maintained at soil water deficit (25% of field capacity). Desired moisture levels were attained by allowing the soil to dry until close to the selected moisture level (5 days), as determined gravimetrically on each pot.

12.3.2. Plant and soil water status

The predawn relative water content (RWC) was determined on leaves as follows: RWC = (Fresh weight - Dry weight) / (Turgid weight – Dry weight) × 100. To determine the turgid weight of the samples, these were kept in distilled water in darkness at 4°C to minimise respiration losses, until they reached a constant weight (full turgor, typically after 12 h). Their dry weight was obtained after 48 h at 70ºC in an oven. Ten replicates per variety and treatment were obtained.

To determine the soil water capacity, soil samples were watered at saturation and, after over-night drainage under high atmospheric humidity, their weight was determined and considered as the pot weight at field capacity. Then, the same sample was desiccated in an oven at 70ºC for at least five days. After reaching constant weight (five days) this value was taken as the pot weight at zero soil water content. Finally, the soil water content capacity was determined as the difference between the soil weight at field capacity and the soil weight at zero water content.

12.3.3. Specific leaf weight

Specific leaf weight (SLW) was calculated, in ten replicates per treatment and variety, as the ratio of dry mass to leaf area. Leaf area was determined in fresh leaves using an AM-100 leaf area meter (ADC, Herts, UK).

12.3.4. Gas exchange and chlorophyll fluorescence measurements

Leaf gas exchange parameters were measured simultaneously with measurements of chlorophyll fluorescence using an open gas exchange system (Li-6400; Li-Cor, Inc., Nebraska, USA) with an integrated fluorescence chamber head (Li-6400-40 leaf chamber fluorometer; Li-Cor, Inc.).

All gas exchange and chlorophyll measurements were made in ten replicates in ten attached fully expanded leaves (i.e., one leaf per plant).

To evaluate the presence of fotoinhibition processes, maximum quantum yield of PSII (F_v/F_m) was measured on leaves adapted at predawn and at mid-morning after 1 and 30 min darkness for both varieties under well-watered and severe drought treatments. Predawn F_v/F_m was considered as an estimation of the permanent photoinhibition (Long *et al*., 1994). 30 min is an adequate time to allow complete re-oxidation of the PSII reaction centres and to ensure that all energy dependent quenching is relaxed in similar plant species (Werner *et al*., 1999) and therefore any decrease in F_v/F_m after 30 min darkness was considered indicative of chronic photoinhibition (Osmond & Grace, 1995). Finally, dynamic photoinhibition processes were detected by measuring F_v/F_m immediatelly (1 min) after darkening the leaves (Osmond & Grace, 1995).

At mid-morning, the actual photochemical efficiency of photosystem II ($\Delta F/F_m'$) was determined according to Genty *et al*. (1989). The electron transport rate (ETR) was then calculated as:

$$ETR = \Delta F/F_m' \cdot PPFD \cdot \alpha \cdot \beta$$

where PPFD is the photosynthetically active photon flux density, α is the leaf absorptance, and β is a factor that assumes equal distribution of energy between the two photosystems (the actual factor has been described to be between 0.4 and 0.6; Laisk & Loreto, 1996). Leaf absorptances were calculated in ten replicates on leaves of well-irrigated plants with a spectroradiometer coupled to an integration sphere (UnisSpec, PP-Systems, USA), and did result in significant differences between both varieties ($P < 0.05$): 0.76 and 0.83 for *D. minor* var. *minor* and *D. minor* var. *palaui*, respectively. Potential changes in leaf absorptance with drought were not assessed. However, since the stress was applied rapidly and changes in leaf coloration were nor apparent, variations are likely too small to induce important bias in the calculations of ETR.

The experimental conditions for gas-exchange mid-morning measurements were set as follows: 25ºC of cuvette temperature, 1,000 μmol photon m^{-2} s^{-1} to ensure light saturation (with 10% blue light), C_a of 400 μmol CO_2 mol air^{-1} and vapour pressure deficit between 0.5 and 2.0 kPa. After inducing photosynthesis under the above conditions and once steady-state was reached, the following parameters were measured: the net CO_2 assimilation rate (A_N), stomatal conductance (g_s), internal CO_2 concentration (C_i) and transpiration rates (E). In the same leaves, dark respiration rate (R_D) was measured at predawn at a CO_2 concentration (C_a) of 400 μmol CO_2 mol air^{-1}.

From combined gas-exchange and chlorophyll fluorescence measurements, the CO_2 concentration at the site of carboxylation (C_c) and the mesophyll conductance (g_i) were estimated in both varieties under the two irrigation treatments as described by Epron *et al*. (1995). For such calculations, the *in vitro* Rubisco specificity factor was considered to be 91.0 and 97.1 for *D. minor* var. *minor* and *D. minor* var. *palaui*, respectively (Galmés *et al*., 2005).

12.3.5. Statistical analysis

Statistical analyses of the data were performed with the SPSS 12.0 software package (SPSS, Chicago, IL). Two-way ANOVAs, with the treatment and varieties as factors, were performed for the studied parameters. Differences between means were revealed by Duncan analyses ($P < 0.05$).

12.4. RESULTS AND DISCUSSION

The most accepted systematic positioning of *D. minor* takes into account the plasticity of the species and considers two infraspecific taxa, varieties *minor* and *palaui*, in virtue of their differences in pubescence (Hinz, 1987). According to this character, in the present survey we compare photosynthetic and photoprotective characters between both varieties to find differential traits underlying the competitiveness and survivorship capacities under the most typical Mediterranean stress. A recent population genetic study has demonstrated that there were no significant differences when the natural populations of *D. minor* were subdivided according to their corresponding infraspecific taxon (Sales *et al*., 2001). This fact adds more interest to the comparison of the photosynthetic and photoprotective behaviour between both varieties.

A summary of the ANOVA on the effects of variety, drought and drought treatment × variety interaction on several water status, gas-exchange and photochemistry measurements is shown in Table 12.1.

Despite of the high genetic homology between both varieties, significant differences ($P < 0.05$) were found in A_N, ETR, P_r, g_i, C_c, SLW and F_v/F_m between varieties, suggesting that differential traits in both photosynthetic and photoprotective mechanisms exist. Moreover, such differences were maintained or even increased if the combined treatment × varieties interaction was considered. On the other hand, neither

variety nor drought treatment × variety had significant effect ($P < 0.05$) on midday RWC, g_s, E, R_D and NPQ.

Table 12.1

Two-way ANOVA of the effects variety and treatment × varieties interaction on relative water content (RWC), stomatal conductance (g_s), transpiration rate (E), net CO_2 assimilation (A_N), electron transport rate (ETR), dark respiration (R_D), photorespiration rate (P_r), mesophyll conductance (g_i), CO_2 concentration at the site of carboxylation (C_c), specific leaf weight (SLW), maximum quantum yield of PSII measured at predawn (predawn F_v/F_m), maximum quantum yield of PSII measured on leaves adapted at darkness for 30 min (30 min F_v/F_m), maximum quantum yield of PSII measured on leaves adapted at darkness for 1 min (1 min F_v/F_m) and non-photochemical quenching (NPQ).

Parameter	Variety *P*-value	Treatment × variety *P*-value
RWC	0.108	0.542
g_s	0.923	0.773
E	0.900	0.688
A_N	0.030	0.028
ETR	0.001	0.003
R_D	0.206	0.763
P_r	0.027	< 0.001
g_i	0.010	0.008
C_c	< 0.001	0.142
SLW	< 0.001	0.215
Predawn F_v/F_m	0.001	0.010
30 min F_v/F_m	0.003	0.080
1 min F_v/F_m	< 0.001	< 0.001
NPQ	0.120	0.416

The similar soil water content (SWC) values resulted in non-significant differences in RWC between varieties under any treatment (Table 12.2). The stomatal conductance (g_s) was found to be similar between varieties under well-watered conditions (257 and 251 mmol H_2O m^{-2} s^{-1} for var. *minor* and var. *palaui*, respectively). The severe drought treatment leaded to an almost completely stomatal closure for both varieties. Although a higher trichome density has been shown to decrease rates of water loss in several species (Ehleringer *et al*., 1976; Ehleringer & Björkman, 1978; Ehleringer & Mooney, 1978; Ehleringer, 1981; Pérez-Estrada *et al*., 2000), this was not the case of *D. minor* var. *minor*, and rates of transpiration (E) were shown to be non-significantly different between varieties (4.25 and 4.15 μmol H_2O m^{-2} s^{-1} for var. *minor*

and var. *palaui*, respectively) (Table 12.2). This fact could be due to the higher specific leaf weight (SLW) of var. *palaui* than var. *minor*, especially under severe drought treatment. According to the relative importance of trichomes weight in determining the total SLW, such differences in SLW might be even higher if we refer exclusively to the 'real' SLW, as the SLW without considering trichomes. A higher SLW is mainly due to a higher leaf mass density (Castro-Díez *et al*., 2000), and probably a higher cuticule thickness in var. *palaui*. Both effects of the higher SLW may compensate in var. *palaui* the effects of trichomes lowering non-stomatal water losses in var. *minor*. Obviously, the equal *E* implied non-significant differences in leaf temperature, which were measured to be close to 26ºC and 32ºC for both varieties under control and severe drought treatments.

Table 12.2

Mean values for the study varieties and treatments of most of the parameters studied. Different letters indicate significantly ($P < 0.05$) different means among treatments and varieties. Abbreviations: SWC (mg H_2O) = soil water content; RWC (%) = relative water content; g_s (mmol H_2O m^{-2} s^{-1}) = stomatal conductance; E (µmol H_2O m^{-2} s^{-1}) = transpiration rate; SLW (g m^{-2}) = specific leaf weight; A_N (µmol CO_2 m^{-2} s^{-1}) = net CO_2 assimilation; A_N/g_s (µmol CO_2 mol^{-1} H_2O) = intrinsic water-use efficiency; A_N/E (µmol CO_2 μmol^{-1} H_2O) = instantaneous water-use efficiency; R_D (µmol CO_2 m^{-2} s^{-1}) = dark respiration; P_r (µmol CO_2 m^{-2} s^{-1}) = photorespiration rate; ETR (µmol e^- m^{-2} s^{-1}) = electron transport rate; g_i (mmol H_2O m^{-2} s^{-1}) = mesophyll conductance; C_c (µmol CO_2 mol^{-1} air) = CO_2 concentration at the site of carboxylation; A_G/C_c = carboxylation efficiency. Treatments: C = control (100% field capacity); and SD = severe drought (25% field capacity). n.d.: not determined due to a high noise/signal ratio.

	D. minor var. *minor*		*D. minor* var. *palaui*	
	C	SD	C	SD
SWC	2.08 ^{b}	0.51 ^{a}	2.07 ^{b}	0.46 ^{a}
RWC	87.3 ^{b}	40.5 ^{a}	85.6 ^{b}	37.2 ^{a}
g_s	257 ^{b}	5 ^{a}	251 ^{b}	2 ^{a}
E	4.25 ^{b}	0.11 ^{a}	4.15 ^{b}	0.04 ^{a}
SLW	114.3 ^{a}	139.6 ^{c}	127.0 ^{a}	159.5 ^{d}
A_N	18.4 ^{c}	-0.1 ^{a}	15.7 ^{b}	-0.5 ^{a}
A_N/g_s	75.0 ^{b}	n.d.	66.6 ^{b}	n.d.
A_N/E	4.45 ^{a}	n.d.	3.89 ^{a}	n.d.
P_r	5.7 ^{b}	5.2 ^{b}	6.4 ^{c}	3.4 ^{a}
R_D	2.1 ^{ab}	2.9 ^{c}	1.7 ^{a}	2.7 ^{bc}
ETR	131.7 ^{a}	50.4 ^{b}	130.1 ^{a}	28.5 ^{c}
g_i	237 ^{b}	1 ^{a}	111 ^{b}	1 ^{a}
C_c	179.7 ^{d}	24.1 ^{b}	134.8 ^{c}	1.1 ^{a}
A_G/C_c	0.117 ^{a}	n.d.	0.129 ^{b}	n.d.

It has been argued that the positive or negative traits derived from leaf trichomes for enhancing productivity and fitness strongly depended on environmental constraints, such as the degree of drought, because of productivity trade-offs associated with pubescence (Sandquist & Ehleringer, 2003). These include the additional costs for construction of the pubescence and lower rates of photosynthesis from leaf hairs reflecting photosynthetically active radiation (PAR) (Ehleringer & Björkman, 1978; Ehleringer & Werk, 1986). Such negative traits associated to leaf trichomes were not found in var. *minor* photosynthetic capacity. In fact, *D. minor* var. *minor* presented significantly ($P < 0.05$) higher A_N (18.4 μmol CO_2 m^{-2} s^{-1}) than var. *palaui* (15.7 μmol CO_2 m^{-2} s^{-1}) under non-drought stressed conditions (Table 12.2). This might have to be linked to a somewhat higher photon quantum yield for var. *minor* compared to var. *palaui*. Moreover, this higher light-use efficiency was followed by a lower photorespiration rates (P_r) for var. *minor* in relation to those found for var. *palaui* (5.7 and 6.4 μmol CO_2 m^{-2} s^{-1}, respectively).

Such differences in A_N were not sufficient to result in significant differences neither in intrinsic water-use efficiency (A_N/g_s) nor in instantaneous water-use efficiency (A_N/E), although var. *minor* showed slightly higher values than var. *paluii* (Table 12.2).

Regarding additional costs derived from construction of trichomes, these, if existed, would be related to higher respiration rates. Effectively, dark respiration rates (R_D), although non-significantly ($P > 0.05$), tended to be higher in the pubescent variety (Table 12.2). Both varieties increased R_D in response to the extreme water deficit, may be as a consequence of increased metabolism as the plant induces acclimation mechanisms to resist drought, as hypothesized by Flexas *et al*. (2005).

The electron transport rate was found to be similar in both varieties under well-watered conditions (approximately 130 μmol e^- m^{-2} s^{-1}) (Table 12.2). However, var. *palaui* decreased ETR under drought treatment to a higher extent than var. *minor* (28.5 and 50.4 mol e^- m^{-2} s^{-1}, respectively). Electron transport is determined by the capacity of sinks, A_N, P_r and possibly the Mehler ascorbate peroxidase reaction (Lawlor, 2002). Regarding A_N, under severe drought conditions it resulted negative and non-different between varieties. Nevertheless, P_r was found to be higher in var. *minor* than var. *palaui*, especially when water stress became severe (Table 12.2), possibly explaining the maintenance of relatively higher ETR in this variety. Although the importance of the

Mehler reaction was not estimated, it seems to contribute very little to energy dissipation, and mostly experiments show that even under drought the relative contribution of Mehler reaction total energy dissipation is just up to 10 % (Flexas & Medrano, 2002b; Lawlor & Cornic, 2002)

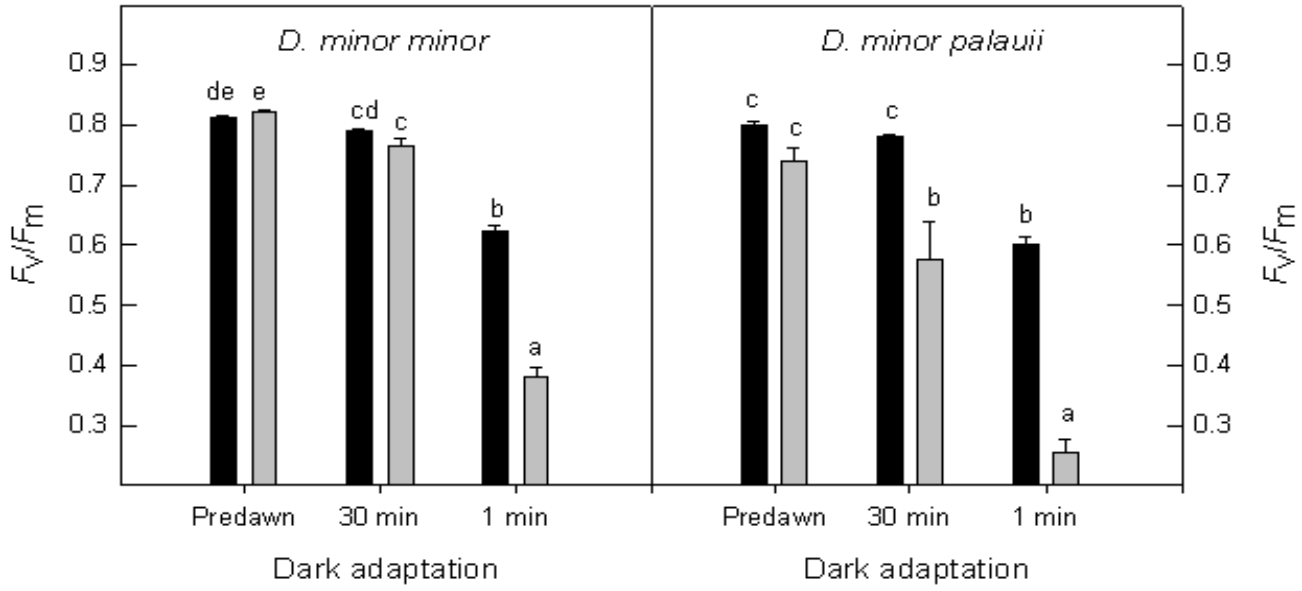
One of the most obvious differences between *D. minor* var. *minor* and *D. minor* var. *palaui* was two-fold higher mesophyll conductance (g_i) for var. *minor* if compared to var. *palaui* under control treatment (Table 12.2). As mentioned above, var. *palaui* presented a somewhat higher SLW, which could partly explain the observed differences in g_i. The internal gas conductance of leaves has been shown to be inversely proportional to SLW (Syversten *et al*., 1995). Obviously, such differences in g_i determined differences in the concentration of CO_2 in the chloroplast (C_c). In well-watered plants var. *minor* showed a C_c of 179.7 μmol CO_2 mol^{-1} air, while C_c was only 134.8 μmol CO_2 mol^{-1} air in var. *palaui* (Table 12.2). Since differences in C_c were higher than those found in A_N, the carboxylation efficiency (A_G/C_c) resulted to be significantly higher in var. *palaui* (0.129) than in var. *minor* (0.117), which was probably due to the higher *in vitro* Rubisco specificity factor (τ) measured for this variety when compared to var. *minor* (Galmés et al., 2005).

Any stress that reduces the rate of photosynthetic electron transport and the light-saturated rate of photosynthesis, either directly or mediated by stomatal closure, enhances the amount of excess energy (Björkman, 1989). Exposure to excessive energy, arising either from high irradiance on leaf or as a consequence of drought stress, is potentially harmful and can induce photoinhibition of PSII reaction centres and enhanced production of reactive oxygen species. However, it is now widely accepted that plants are protected by several mechanisms capable of preventing potential damage. In this way, the highly reflective pubescence trait is related to be an important adaptation to arid and semi-arid conditions because decreases absorption of solar radiation (Ehleringer & Clark, 1988), which under the hot and dry Mediterranean summer is in excess of that which can be used for photosynthetic assimilation (Flexas & Medrano, 2002c). In the present survey, leaf absorption was found to be significantly ($P < 0.05$) lower in the pubescent *minor* variety (0.76) than in the glabrous *palaui* variety (0.83). Under well-watered conditions, the decline in the intrinsic efficiency of PSII photochemistry, monitored as F_v/F_m, as decreased the dark-exposure time from predawn conditions up to 1 min exposition to darkness, was identical for both varieties (Fig.

12.1). Under such control conditions, only dynamic photoinhibition was recorded. However, similarities between varieties disappeared when the comparison was made under severe drought conditions. Effectively, chronic photoinhibition was found in severely stressed plants, in relation to control plants, for var. *palaui*, but not for var. *minor* (Fig. 12.1). Finally, regarding dynamic photoinhibition, var. *palaui* also presented a lower F_v/F_m than var. *minor* after exposure to 1 min darkness. Therefore, the presence of trichomes in var. *palaui* is demonstrated to be an important mechanism to decrease susceptibility to photodamage. Furthermore, it has to be noted the strong resistance underlying the photochemistry behaviour of this species, preserving a good PSII efficiency, especially in the case of var. *minor*, in which the presence of permanent photoinhibition events can be discarded even under the extreme water-stressing conditions to which plants were subjected in the present experiment. This fact adds even more arguments to define pubescence as an efficient way to enhance survival probabilities in extremely low water-availability environments.

Figure 12.1

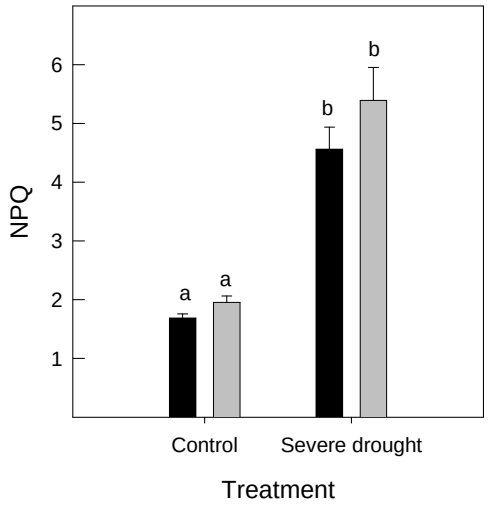
Maximum quantum yield of PSII (F_v/F_m) measured on leaves adapted at predawn, 30 minutes and 1 min darkness for both varieties under well-watered (black bars) and severe drought (grey bars) treatments. Different letters indicate significantly ($P < 0.05$) different means among treatments and dark adaptation (i.e. within each variety).



Interestingly, despite such differences in maximum quantum yield of PSII, non-significant differences ($P > 0.05$) were found in the non-photochemical quenching (NPQ) between both varieties, although var. *palaui* presented slightly higher values either under control and drought conditions (Fig. 12.2). This fact suggests that for this species, adaptation traits to cope with the excess of radiation have been developed in the direction of avoiding its capture, i.e. preserving its photochemistry apparatus, rather than in the direction of enhancing dissipation mechanisms, i.e. to cope with the absorbed light.

Figure 12.2

Non-photochemical quenching for *D. minor* var. *minor* (black bars) and *D. minor* var. *paluii* (grey bars) under well-watered and severe drought treatments. Different letters indicate significantly ($P < 0.05$) different means among treatments and varieties.



The observed differences due to the presence of leaf trichomes in var. *minor* matches with the geographical distribution of both varieties. Although the microhabitat is similar for the two varieties, var. *minor* presents a higher plasticity when compared to var. *palaui*, and their populations reach semi-arid areas with an annual precipitation of about 300 mm (Mus, 1992). The positive effects of the leaf pubescence on photoprotection may have played an important role determining the higher ecological success and geographical plasticity of var. *minor* in respect to var. *palaui*.

In conclusion, the presence of leaf trichomes in var. *minor* has been shown to be an efficient mechanism in preserving photochemistry apparatus in *D. minor*, when compared to the glabrous var. *palaui*, although such pubescence has not found to be related to a lower leaf water loss. Moreover, other differences in both the photosynthetic characters and leaf morphological traits between the two varieties have been found, especially a higher photosynthetic and mesophyll conductance capacities for var. *minor* in respect to var. *palaui*.

Chapter 13
GENERAL DISCUSSION

The Results of the present thesis have been structured in Chapters that correspond to each of the different traits or processes analysed. The relevance of the findings for the understanding of these processes and their response to water stress is profusely discussed in each of these Chapters. Therefore, the present General Discussion chapter focuses only on aspects including a general view of all the processes together, highlighting the variability among species and the effects of growth forms and evolutionary history. For proper discussion on specific processes, readers are addressed to the specific Discussion in each chapter.

13.1. BIODIVERSITY OF ECOPHYSIOLOGICAL TRAITS AND THEIR RESPONSES TO DROUGHT IN MEDITERRANEAN SPECIES WITH DIFFERENT GROWTH FORMS AND EVOLUTIONARY HISTORY

A high biodiversity may be linked to large inter-specific differences in the ecophysiological responses to environmental conditions (Schulze, 1988; Gaston, 1996). As pointed out in the Introduction, the Mediterranean basin, and very especially some specific areas within it, are characterised by their biological diversity richness (Heywood, 1977). Therefore, the Mediterranean vegetation may be viewed as an excellent material to explore how biological diversity is translated in a diversity of ecophysiological adaptive traits in response to environmental conditions, particularly drought.

The results obtained in the present Thesis largely confirm such wide diversity of ecophysiological traits in Mediterranean plants, from seed germination and establishment to adult plant traits, and at different levels of organisation, from whole plant processes to molecular biology. Table 13.1 summarises the main physiological parameters measured and their dependence on growth forms and endemicity. Clearly, about half of the parameters analysed did not depend on growth form. In other words, Mediterranean plants present a diversity of ecophysiological traits irrespective of their growth form. This is consistent with the idea of a high biodiversity in Mediterranean floras, as well as with the fact that any Mediterranean species – regardless of its phenological cycle or growth form – may have to endure drought periods, due to the highly episodic distribution of precipitation in this climate. By contrast, endemicity as a

factor did not significantly affect any of the analysed traits and responses, therefore showing that the patterns found by Gulías *et al*. (2003) regarding maximum photosynthesis cannot be generalised either to other species or physiological traits. There are some possible explanations for the discrepancy between the present survey and others considering non-endemic and endemic species form islands (Pattison *et al*., 1998; Baruch & Goldstein, 1999; Fogarty & Facelli, 1999; Matsumoto *et al*., 2000; Durand & Goldstein, 2001; Gulías *et al*., 2002; Gulías *et al*., 2003). First, it has to be considered the typology of the island where the study was performed. For instance, the low competitiveness of endemic plants as compared to non-endemics is likely to be more accentuated in those islands with a higher degree of isolation. i.e. oceanic islands, with respect to continental islands. Second, the species included in the analysis may explain the discrepancy of these results with those of Gulías *et al*. (2002, 2003). The fact that the results and conclusions are strongly dependent on the species considered, leads to the conclusion that the source of variability is mostly determined by the species itself. Hence, Lefi *et al*. (2004) comparing *Medicago citrina*, endemic of the Balearic Islands, and *M. arborea*, widespread in the Mediterranean region, showed that *M. citrina* presented a higher water use efficiency than *M. arborea*. Certainly, the analysis needs to be focussed in terms of specific disadvantageous ecophysiological characters of a specific species, especially when considering islands with weak isolation history, rather than looking for general trends characteristic of endemic species. Even so, it is noticeable that some of the *a priori* most suitable species for presenting disadvantageous photosynthetic drought-response traits, such as the wild-extinct *Lysimachia minoricensis*, no particular disadvantage was found when compared to other Mediterranean species with a wide geographical distribution.

Coming back to the high diversity of ecophysiological traits and responses to the environment, all the possible patterns in the seed germination were registered when comparing different species. This occurred either in terms of seed viability, germination capacity, temperature dependence of germination, time response or dormancy period, among each only the first was growth form-dependent. On the other hand, only the optimum temperature for germination, which was found to be 10-20ºC, displayed a general pattern, similar to that reported for many Mediterranean species (Ayerbe & Ceresuela, 1982; Lagarda *et al*., 1983; Baskin & Barskin, 1998; Barragán *et al*., 1999; Thanos, 2000) but not all (Mitrakos, 1981).

Table 13.1

Significant (YES) and non-significant (NO) effects of growth form and endemicity on the main parameters considered in the present thesis.

	Parameter	Growth Form	Endemicity
Seed germination	%G	YES	NO
	%G *vs.* temperature	NO	NO
	T50	NO	NO
	D	NO	NO
	% ES	NO	NO
Seedling growth	RGR optimal conditions	YES	NO
	RGR drought effects	YES	NO
	NAR optimal conditions	YES	NO
	LAR optimal conditions	YES	NO
	LMR optimal conditions	YES	NO
	SLA optimal conditions	YES	NO
	Biomass allocation optimal conditions	YES	NO
	GRCs drought effects	YES	NO
Water relations and stomatal regulation	Minimal Ψ	NO	NO
	Ψ *vs.* SWC	NO	NO
	$\Psi\pi_{100}$	YES	NO
	$\Psi\pi_{0}$	NO	NO
	RWC_0	YES	NO
	ε	YES	NO
	ε *vs.* SLW	NO	NO
	$g_{s\,max}$	YES	NO
	g_s range	YES	NO
	$g_{s\,max}$ *vs.* SLW	NO	NO
	$g_{s\,max}$ *vs.* SD	NO	NO
	SD	NO	NO
	Stomatal length	NO	NO
	Stomatal lenght *vs.* SD	NO	NO
	SAI *vs.* SD	NO	NO
	SR	NO	NO
	SR *vs.* SLW	NO	NO
	SR *vs.* ε	NO	NO
	SR *vs.* SD	NO	NO
	K_L optimal conditions	NO	NO
	K_L drought effects	NO	NO
	g_s recovery	YES	NO
	Ψ recovery	YES	NO
	K_L recovery	YES	NO
Photosynthetic limitations	A_N optimal conditions	YES	NO
	$V_{c,max}$ optimal conditions	YES	NO
	g_i optimal conditions	YES	NO
	ETR optimal conditions	YES	NO
	A_N *vs.* g_s	NO	NO
	ETR *vs.* g_s	NO	NO
	g_i *vs.* g_s	NO	NO
	$V_{c,max}$ *vs.* g_s	NO	NO
	A_N limitation components *vs.* g_s	NO	NO
	A_N recovery	YES	NO

Photoinhibition and photoprotection	Pigment contents optimal conditions	NO	NO
	Pigment contents drought effects	NO	NO
	P_r optimal conditions	NO	NO
	P_r drought effects	NO	NO
	Fv/Fm drought effects	NO	NO
	NPQ drought effects	NO	NO
	F_v/F_m *vs*. DPS_{PD}	NO	NO
	NPQ *vs*. DPS_{MD}	NO	NO
Rubisco	τ	YES	NO
	$\Delta G_o^{\ddagger}$-$\Delta G_c^{\ddagger}$	NO	NO
Leaf respiration and carbon balance	R_{Dm} optimal conditions	YES	NO
	R_{Dm} drought effects	YES	NO
	A_{Nm} optimal conditions	YES	NO
	A_{Nm} drought effects	NO	NO
	A_{Nm}/R_{Dm} optimal conditions	YES	NO
	A_{Nm}/R_{Dm} drought effects	YES	NO
	A_{Nm}/R_{Dm} *vs*. A_{Nm} slope	YES	NO
	R_{Dm} *vs*. g_s	NO	NO
	R_{Dm} *vs*. RWC_{PD}	NO	NO
	R_{Dm} *vs*. A_{Nm}	YES	NO

After seed germination, seedling establishment is a critical developmental stage that may strongly depend on water availability (Moles & Westoby, 2004). This was, along with respiration and carbon balance, the experiment whose parameters presented the highest growth form-dependency. However, even within each growth form, a significant differentiation was found. For instance, although the analysis of the decrease in RGR due to water stress in terms of the relative contribution of its components resulted strongly dependent on the growth form, in the group of semi-deciduous shrubs the decreases in RGR under water stress were found to be the result of decreases in SLA alone or a combined effect of decrease in SLA and NAR, depending on the species. This means that, irrespective of the diversity of drought incidence on the life span for the different growth forms, the capacity to overcome such limitations is feasible by different ways.

Regarding to plant water relations and stomatal regulation, a high variability was found among Mediterranean plants. The most variable parameters included: stomatal density and cell guard length, stomatal conductance, stomatal responsiveness to water stress, leaf water relations (pre-dawn and midday leaf water potential and relative water content), leaf specific hydraulic conductance and the bulk modulus of elasticity. Only few parameters were found to present a general trend, such as the dependency of stomatal responsiveness on stomatal density or the bulk modulus of elasticity. The responses of other parameters, particularly that of leaf water potential to progressive

drought, were not in accordance with the patterns usually described (Duhme & Hinckley, 1992; Correia & Catarino, 1994; Werner *et al*., 1999; Mediavilla & Escudero, 2003). Such discrepancy, while it could be partly attributed to differences between field- and pot-grown plants, largely supports that a high variability is present among Mediterranean plants, reflecting a continuum of leaf water relations and stomatal behaviour in response to water stress which is mostly independent on growth forms or functional groups, similar to what has been shown regarding leaf economics under optimum conditions (Wright *et al*., 2004).

The analysis of the photosynthetic limitations revealed that most of the relationships established among different parameters, although irrespective of the growth form, followed a common trend for all the species. The existence of such general trends (i.e. photosynthetic and electron transport rates, maximum velocity of carboxylation, mesophyll conductance and the extent of the limitation components of photosynthesis under drought against the stomatal conductance) confirms the observations reported in previous works of our research group (Flexas *et al*., 2002; Flexas & Medrano, 2002a; Bota *et al*., 2004; Flexas *et al*., 2004a; Flexas *et al*., 2006) and others (Warren *et al*., 2004; Ennahli & Earl, 2005: Grassi & Magnani, 2005; Correia *et al*., 2006). Still inside this general trend, a few considerations may be made regarding ecophysiology of photosynthesis in Mediterranean plants and its response to drought:

- First, although it was previously considered that a relatively low mesophyll conductance and a low carboxylation capacity were typical of evergreen sclerophylls (Syversten *et al*., 1995; Niinemets *et al*., 2005), the present results clearly show that other, non-sclerophyll Mediterranean plants may also present these traits.
- Second, and despite general response patterns to drought, a 6-fold range was observed regarding the *actual* mesophyll conductance achieved by different Mediterranean species which, as will be discussed, results in a driving force for other physiological adjustments in the photosynthetic machinery, such as the specificity factor of Rubisco.
- Third, the present results demonstrate for the first time that, although different species and growth forms follow general patterns of photosynthetic response to *progressively imposing drought*, there are strong inter-specific differences in the degree of recovery *after drought*, and the latter are actually strongly dependent

on growth forms. The extent of recovery seems mostly dependent on the extent of mesophyll conductance recovery after drought, which may deserve further attention in the near future.

The study of photoinhibition and photoprotection processes under drought, along with the study of water relations and stomatal regulation, revealed the highest variability among species. The parameters that differed strongly among species – but not among growth forms – included: total pigment composition and content, operation of the xanthophylls cycle, photorespiration rates, maximum quantum efficiency of PSII, non-photochemical quenching, and the effects of drought on all these parameters. The diversity in the response of some of these parameters and their inter-relationships, such as the one between non-photochemical quenching and the concentration of zeaxanthin or the de-epoxidation state of the cycle, was to some extent expected attending to the discrepancies found in the results from literature (García-Plazaola *et al*., 1997; Demmig-Adams, 1998; Faria *et al*., 1998; Gulías *et al*., 2002; Munné-Bosch & Alegre, 2000c). Nevertheless, the existence of some general responses with respect to photoinhibition and photoprotection processes in the Mediterranean vegetation cannot be obviated, such as the confirmation of the operation of the xanthophyll cycle in Mediterranean plants regardless of their growth form (Demmig *et al*. 1988; Demmig-Adams *et al*. 1989; García-Plazaola *et al*. 1997; Gulías *et al*. 2002; Munné-Bosch & Alegre 2000a, b, c; Balaguer *et al*. 2002), a high stability of dark-adapted PSII efficiency under mild to moderate drought (Chaves *et al*., 1992; Epron & Dreyer, 1992, 1993; Méthy *et al*., 1996; García-Plazaola *et al*., 1997; Epron, 1997; Faria *et al*., 1998; Werner *et al*., 1999; Martínez-Ferri *et al*., 2000; Flexas *et al*., 2001; Gulías *et al*., 2002b), and again a general pattern when stomatal conductance (g_s) is used as a reference for drought intensity (Flexas & Medrano 2002a; Flexas *et al*. 2004a).

Although differences in the Rubisco capacity to distinguish between CO_2 and O_2, i.e. the Rubisco specificity factor, among distant phylogenetic groups were generally accepted, still nowadays it is well-assumed that differences among C_3 plants are minimal and for instance current models of leaf photosynthesis assume constant values among C_3 plants (Wilson *et al*., 2000; Xu & Baldocchi, 2003; Peña-Rojas *et al*., 2004; Warren *et al*., 2004). A previous survey by Delgado *et al*. (1995) suggested, after analysing few Mediterranean species, a higher variability in the catalytic properties of Rubisco than usually thought. This hypothesis has been largely confirmed by the results achieved in the present thesis, moreover suggesting that the dry and hot Mediterranean

summer conditions have imposed a selection pressure for an improved specificity of the enzyme. Whether such variability in Rubisco specificity factor is restricted to the Mediterranean vegetation or can be generalised when considering species of other biomes would not be elucidated until a deepest analysis on the kinetics of the enzyme is performed in a wide range of species from diverse climates.

In summary, three main implications can be drawn from the high variability found among Mediterranean species in most of the ecophysiological characters studied in this thesis:

- It reflects the effects of the variable micro- and macro-climatic conditions resulting from the diversity of habitats within the Mediterranean region.
- The array of adaptations displayed by the Mediterranean vegetation in response to such diversity may ensure success and competitiveness to such environment.
- Such diversity in the ecophysiological traits and responses to drought found among Mediterranean species has to be considered as a 'resource', in terms of identifying target adaptive traits for breeding plans, but also as a genetic bank to improve crop productivity in a world with increasing water scarce agricultural regions. According to this, it becomes clear the importance of maintenance and conservation of the biological diversity in the region, as well as the sustainable management of their diverse habitats as a main biodiversity driving force.

13.2. SPECIFIC TRAITS DEPENDING ON GROWTH FORMS

As it has been pointed in the previous section, about half of the observed variability was associated with the growth forms of the Mediterranean species analysed. This may be associated with adaptation strategies to Mediterranean stressing conditions, which can provide information on ecophysiological traits and their responses to drought for each specific group.

Regarding to the differences in ecophysiological traits among growth forms, the extent of such differences strongly depended on the traits analysed (Table 13.2). For instance, inter-specific differences in seed germination and its temperature dependence, water relations and stomatal responsiveness to drought presented the lowest dependency on growth forms. On the other hand, all the parameters studied in the experiment of seedling growth resulted strongly depending on the growth form, which was expected

since the differentiation among groups was based precisely on characteristic growth traits, such as the plant architecture, phenology and leaf longevity.

Table 13.2

Relative (high, intermediate or low) values for the ecophysiological parameters that resulted to be growth form-dependent. For Growth Relative Component (GRC) analysis, the main factors scaling *positively* with RGR when comparing different water availabilities were indicated for each growth form. An asterisk in the evergreen shrubs indicates that significant differences were observed between the *Limonium* species and the other evergreen shrubs.

Parameter	Herbaceous	Semi-deciduous shrubs	Evergreen shrubs
%G	Intermediate	Low	High
RGR optimal conditions	High	Intermediate	Low*
RGR drought effects	High	High	Low
NAR optimal conditions	Low	Intermediate	High*
LAR optimal conditions	High	Intermediate	Low*
LMR optimal conditions	High	Intermediate	Low*
SLA optimal conditions	High	Intermediate	Low*
Biomass allocation optimal conditions	Leaves	Intermediate	Roots
GRCs	LAR	SLA	NAR/LMR
$\Psi\pi_{100}$	High	Low	Low
RWC_0	Low	Low	High*
ε	Low	Intermediate	High*
$g_{s\,max}$	High	Intermediate	Low
g_s recovery	High	Intermediate	Low*
Ψ recovery	High	Intermediate	Low*
K_L recovery	High	Intermediate	Low
A_N optimal conditions	High	Intermediate	Low*
$V_{c,max}$ optimal conditions	High	Intermediate	Low*
g_i optimal conditions	High	Intermediate	Low
ETR optimal conditions	High	Low	Intermediate*
A_N recovery	High	Intermediate	Low
τ	Low	Intermediate	High*
R_{Dm} optimal conditions	High	Intermediate	Low
R_{Dm} drought effects	High	Low	Low
A_{Nm} optimal conditions	High	Intermediate	Low*
A_{Nm}/R_{Dm} optimal conditions	High	High	Low
A_{Nm}/R_{Dm} drought effects	High	High	Low
A_{Nm}/R_{Dm} *vs*. A_{Nm} slope	High	Intermediate	Low

With few exceptions, among the growth forms considered, the herbaceous plants and the evergreens shrubs represented the two extremes of the range, with the semi-deciduous in an intermediate position. Generally speaking, under non-stressing conditions herbaceous plants presented a relatively high stomatal conductance, which

allowed high carbon assimilation taxes and high maximum velocity of carboxylation, with also an increased electron transport rates. This, together with a large biomass allocation to photosynthetic tissues and a high specific leaf area, were responsible of the high growth capacity of herbs under well-watered conditions. Their high SLA was mainly due to a low leaf mass density (Castro-Díez *et al*., 2000), and then it could explain the relatively high mesophyll conductance of most herbs (Syversten *et al*., 1995) – although exceptions to this were found –, and their low bulk modulus of elasticity (Salleo & Lo Gullo 1990; Groom & Lamont 1997; Salleo *et al*. 1997). However, since the low CO_2 availability for carboxylation has been hypothesised to induce selection pressure for a better Rubisco, the high stomatal and mesophyll conductances of herbaceous species have lowered such effects, and then the specificity of the enzyme was lower when compared to the other growth forms. The higher growth rates of the herbaceous species were proportionally related to higher absolute respiration rates (Lambers *et al*., 1989; Reich *et al*., 1998c; Poorter *et al*., 1990), although the ratio of assimilation to respiration still remained significantly higher in comparison to evergreen shrubs.

On the other hand, under optimal conditions evergreen shrubs are characterised by low growth rates, induced by low biomass allocation to leaves and low photosynthetic capacity. Leaves with high density, and therefore a low specific leaf area and low mesophyll conductance, are also typical of evergreen shrubs. This low mesophyll conductance and the relatively low stomatal conductance seems to be associated to the improved specificity of Rubisco. The low growth rates are associated with low absolute respiration rates with a lower ratio of carbon assimilation to leaf respiration (Poorter *et al*., 1991; Lambers *et al*., 2005). For most of the parameters considered the semi-deciduous shrubs displayed intermediate values, sharing some characters with herbs and others with the evergreen shrubs.

However, when the effects of drought are taken into account, the characteristics of each growth form differ from that under well-watered conditions (Margaris, 1981; Flexas *et al*., 2003; Gratani & Varone, 2004). For instance, for evergreen shrubs the water stress effects on the growth capacity were lower in comparison with the other growth forms. The different growth forms also differed in the relative contribution of each of the underlying growth parameters to the decrease of RGR due to water deficit. Hence, in semi-deciduous shrubs, SLA decreases mostly explained the decrease in RGR, in evergreen shrubs RGR decreased due to water stress mainly because of

decreases in LMR or NAR, depending on the species, and in perennial herbs LAR component was strongly decreased and therefore responsible for the overall decrease in RGR. Regarding to the effects of drought on leaf respiration rates and carbon balance, the herbs showed the more marked and progressive decreases of respiration rates as drought intensified.

Another important distinctive feature among growth forms is the capacity to recover after a rapid but intense drought stress. Herbaceous species presented a higher capacity to recover its plant water status, stomatal conductance, leaf specific hydraulic conductance, photosynthetic capacity and respiration rates, especially when compared to the evergreen shrubs. This may reflect different adaptations to water stress periods under Mediterranean conditions. For instance, herbs may experience short drought periods during the favourable season, and therefore a capacity for rapid recovery may be of importance to ensure their carbon balance requirements before ending their life cycle in late spring. On the contrary, evergreens suffer less from short dry periods during the favorable season, because of their large root system (Rambal, 1984; Canadell *et al*., 1996), but may have to endure a long drought period in summer during which they may rely on more permanent physiological changes precluding rapid recovery (Mittler *et al*., 2001). From these observations, it can be concluded, at least for the species considered, that the herbaceous species present an array of adaptive traits more suitable to cope with the Mediterranean characteristic short and unpredictable periods of drought when compared to the other growth forms, while evergreen shrubs seem specially adapted to survive the long-term summer drought. Semi-deciduous shrubs share some characteristics with each of the other two groups.

Because of specific and differential morpho-physiological traits of the *Limonium* species when compared to the rest of evergreen shrubs, species of this genus have been eventually considered as a single group (evergreen semi-shrubs). This differentiation allowed getting some amazing insights in the ecophysiology of these species. They resulted, among all the species analysed, those presenting a higher degree of differentiation of ecophysiological traits and their response to drought stress. Therefore, *Limonium* species becomes a powerful potential target for a gene screening devoted to find specific traits for crop improvement in arid and semi-arid areas. Among the most interesting ecophysiological traits of *Limonium* species that may serve this purpose we should mention:

- A high percentage of viable seeds, with high germination capacity, relatively independent of the incubation temperature, and low time response and dormancy period. Therefore, they adapt the strategy of rapid and massive germination, which may constitute, once considered that their seeds are mature in autumn, a positive adaptation to the environment with of the Mediterranean climate, characterised by episodic and unpredictable rainy events (Terradas, 1991).
- A higher growth capacity for any given NAR and LAR, which enables achieving higher growth rates as compared to species with similar morphological leaf traits, such as SLA. In contrast to the rest of evergreen shrubs, for *Limonium* the decrease of RGR under water stress was associated with physiological adjustments. The well-known increased biomass allocation to roots in response to water-stress (Ludlow, 1989; Jackson *et al*., 2000) may not be of adaptive value for *Limonium*, since they inhabit over rocky surfaces with no easy access to deeper water or with access only to marine water intrusions.
- Among the species analysed, only the two *Limonium* presented a clear isohydric behaviour, which becomes surprising when considering that other evergreen shrubs were included in the analysis. This fact allowed maintaining constant leaf water relations under mild and moderate drought, and therefore cell turgor in a narrower range when compared to the remaining species. In addition, their bulk elastic modulus was, surprisingly, the lowest among the species considered, and did not follow the general SLW- ε relationship found for the others. All these considerations point to a drought-avoidant strategy for these species. Other significant traits to be mentioned were a lower stomatal conductance for a given SLW and special characters regarding their stomatal morphology, e.g. low stomatal density and high cell guard size, despite of their highest stomatal responsiveness to water stress. Both *Limonium* species presented the lowest K_L among all the species considered, both at optimal conditions and under drought stress, which implies lower transpiration rates and enhances the water-use efficiency (Schultz, 2003)
- Despite of the low stomatal and mesophyll conductances to CO_2, *Limonium* species displayed relatively high photosynthesis, which could be due to their higher Rubisco specificity factor. In the relationship between the stomatal conductance and several other photosynthetic parameters, such as net photosynthetic rate, electron transport rate, and maximum capacity of carboxylation, these species were found to be somewhat outliers, and in all cases fall above the regression line. From the analysis

of photosynthetic limitation components, both *Limonium* stood out as being among the species with more robust photosynthetic metabolism, being photosynthesis mainly limited by diffusive limitations. Hence, metabolic impairment was negligible under mild and moderate drought, and only appeared when water stress became severe. The photosynthetic limitation components at re-watering showed an almost full recover of the metabolic impairment, with mesophyll conductance as the main component of the limited photosynthetic recovery.

- Finally, the special features with respect to the improved efficiency in the carboxylation process by Rubisco need to be highlighted. As extensively discussed in chapter 10, the Rubisco specificity of *Limonium*, the highest value hitherto reported among higher plant Rubiscos, have to be considered as a potential source for crop Rubisco improvement, especially in environments with limiting water availability. Moreover, the highest activation energies found in the Rubisco of *Limonium* species increase the interest of this source of Rubisco in particular. It would be interesting to search for even higher values within this genus, considering not only *Limonium* species from Mallorca, but also from other stressed habitats.

Chapter 14
CONCLUSIONS

From the above Results and Discussion, a series of conclusions can be drawn which respond to the objectives established in the present thesis.

General objectives

To analyze how biodiversity and adaptation to Mediterranean climate is reflected in a diversity of ecophysiological traits and their responses to drought, and to study whether such diversity was related to growth forms and / or endemicity.

1. A wide range of variation has been observed among the Mediterranean species analysed for most of the parameters measured, confirming the validity of the Mediterranean flora as a genetic background to explore ecophysiological variability.

2. Only about half of this variability in ecophysiological traits was associated to different growth forms. This observation confirms, on one hand, that different growth forms can be characterised on the basis of particular ecophysiological traits and responses and, on the other hand, that species belonging to a single growth form present different adaptations to the Mediterranean environment and vice-versa, i.e., that species belonging to different growth forms can share some similar adaptive traits. This high diversity of responses, particularly those independent of growth forms, likely reflects the fact that any species in this environment has to endure temporary drought periods, which has lead to an array of different adaptive strategies.

3. By contrast, no clear differentiation traits were found when endemic species of the Balearic Islands were compared to other Mediterranean species with wide geographical distribution, in any of the parameters considered for study. Consequently, the early described patterns of ecophysiological disadvantages in endemic species cannot be generalised, and, therefore, other causes not related to physiology may be on the basis of the limited and declining distribution of some endemic taxons.

Specific objectives

Objective 1. To compare plant establishment capacity – from germination to early growth under different water availabilities – in a range of Mediterranean species, belonging to different taxonomic, evolutionary and growth form groups.

4. For most species the optimum temperature range for germination was 10-20ºC, as already described for many Mediterranean species.

5. By contrast, the seedling growth capacity strongly depended on growth forms, from highest values in herbs to lowest in woody perennials, scaling with differences in specific leaf area.

6. The relative contribution of different growth components to water stress induced decrease in growth was also strongly dependent on the growth form. Remarkably, only in evergreen semi-shrubs decreased relative growth rate was mostly explained by large decreases in the physiological component (net assimilation rate), while in the other groups, from herbs to evergreen shrubs, the decrease in growth was mostly associated to different combinations of decreasing morphological components (specific leaf area and leaf mass ratio).

Objective 2. To analyse the diversity in water relations and stomatal regulation among Mediterranean species representative of several functional groups.

7. A large variability was observed in the responses of leaf water relations and stomatal conductance to water stress among Mediterranean plants, which was remarkably independent of the growth forms.

8. The stomatal response to water stress and recovery was mainly related to stomatal density as a constitutive trait, and to leaf hydraulic conductivity as a physiological dynamic trait.

Objective 3. To study the detailed photosynthetic responses to water stress and re-watering in these species, as well as to make a preliminary survey of how respiration – the other component of plant carbon balance – responds to water stress.

9. A quantitative analysis shows that, in spite of slight differences between species, they all follow a general pattern of photosynthetic response to drought when maximum stomatal conductance was used as indicative of stress intensity, consisting in an early phase of drought-induced photosynthesis decline associated to stomatal and

mesophyll conductance reductions, followed by a second phase in which the maximum velocity of carboxylation and electron transport rate were also reduced to some extent. In this sense, Mediterranean plants do not differ from general relationships obtained for C_3 species.

10. However, a low mesophyll conductance was revealed as a specific feature of Mediterranean plants, regardless of their growth form. In all the species analysed, mesophyll conductance was more limiting for photosynthesis than stomatal conductance, and its limited recovery was the most important constrain to photosynthesis recovery after re-watering severely stressed plants.

11. While photosynthesis response to imposing drought did not depend on growth form, leaf carbon balance (i.e. the ration between photosynthesis and respiration) did, as well as the recovery of photosynthesis and carbon balance to re-watering severely stressed plants. In general, herbs showed the highest carbon balance and recovery and evergreen shrubs the lowest, while semi-deciduous shrubs displayed an intermediate behaviour.

Objective 4. To elucidate the diversity among Mediterranean species in the responses of photoinhibition and photoprotection to water stress.

12. All the species analysed proved to be resistant to photoinhibition under drought, although to somewhat different extents.

13. The maintenance of photorespiration and the increase of the thermal energy dissipation were the common responses to water stress in all the species analysed, while adjustments in pigment pool sizes were not an important short-term response to drought.

14. The increase of thermal energy dissipation due to drought mostly depended on the de-expoxidation state of xanthophylls, although the slope and kinetics of such relationship strongly differed among species, suggesting species-dependent additional roles of de-epoxidated xanthophylls.

Objective 5. To survey the variability in the Rubisco specificity factor among Mediterranean species and to discern whether such variability corresponds to evolutionary or adaptive trends.

15. A high variability in Rubisco τ was observed among Mediterranean C_3 plants, with significant differences even between closely related species, which was related to environmental pressure factors as well as to leaf characteristics. Particularly, Rubiscos from species inhabiting drought prone areas and from species with a high degree of sclerophylly showed a catalytic efficiency more suited to the low CO_2 concentrations at the active site imposed by water limitation. These results suggest that if a larger τ is to be found among C_3 terrestrial species, it probably should be found among evergreen woody species from arid environments.

16. The Rubisco τ determined in *Limonium gibertii* (110) is the highest value hitherto reported among higher plant Rubiscos, and must be considered as a potential target for genetic engineering of the efficiency of the enzyme and, therefore, for crop productivity improvement for arid zones.

17. Despite the Rubiscos adaptation to drought stressed conditions, Rubisco τ cannot acclimate to such stress, as proved using tobacco as a model plant. Therefore, the validity of τ estimations by gas-exchange analysis under water stress may be questioned.

Objective 6. To determine whether ecophysiological responses to drought could have been on the basis of the limited success of Lysimachia minoricensis, the only endemic species from the Balearic Islands that is currently extinct in the wild but preserved in nurseries.

18. *L. minoricensis* had a low photosynthetic capacity due to an intrinsically low mesophyll conductance to CO_2 as compared to possible competitors with similar SLA, which could have contributed to its limited success.

19. In contrast, and despite the very low leaf relative water content achieved, the similarity of photosynthesis and photoprotection responses to drought found between *L. minoricensis* and many other C_3 plants suggests that this species presents no particular disadvantage in its response to water stress, which was confirmed by an almost complete recovery 24h after re-watering.

Objective 7. To check whether differences between varieties of Digitalis minor in a specific morphological trait, such as the presence of leaf trichomes, provides some ecophysiological advantage under the Mediterranean climate.

20. The presence of leaf trichomes in *Digitalis minor* var. *minor* seemed an efficient mechanism in preserving leaves from drought-induced photoinhibition when compared to the glabrous var. *palaui*, although other differences in photosynthetic behaviour and leaf morphological traits between both varieties could have contributed to the observed differences in photoinhibition, especially the higher photosynthetic capacity and mesophyll conductance found in var. *minor* as compared to var. *palaui*.

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