

RESEARCH PAPER

Rubisco activity in Mediterranean species is regulated by the chloroplastic CO₂ concentration under water stress

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Abstract

Water stress decreases the availability of the gaseous substrate for ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) by decreasing leaf conductance to CO₂. In spite of limiting photosynthetic carbon assimilation, especially in those environments where drought is the predominant factor affecting plant growth and yield, the effects of water deprivation on the mechanisms that control Rubisco activity are unclear. In the present study, 11 Mediterranean species, representing different growth forms, were subject to increasing levels of drought stress, the most severe one followed by rewatering. The results confirmed species-specific patterns in the decrease in the initial activity and activation state of Rubisco as drought stress and leaf dehydration intensified. Nevertheless, all species followed roughly the same trend when Rubisco activity was related to stomatal conductance (g_s) and chloroplastic CO₂ concentration (C_c), suggesting that deactivation of Rubisco sites could be induced by low C_c , as a result of water stress. The threshold level of C_c that triggered Rubisco deactivation was dependent on leaf characteristics and was related to the maximum attained for each species under non-stressing conditions. Those species adapted to low C_c were more capable of maintaining active Rubisco as drought stress intensified.

Key words: Drought, mesophyll conductance, photosynthesis, stomatal conductance.

Introduction

Worldwide, water availability is considered the environmental factor that most strongly influences plant growth and yield (Boyer, 1982), and global climate change is expected to exacerbate water limitations in semi-arid ecosystems like the Mediterranean (IPCC 2007). Although photosynthesis has frequently been documented among the primary physiological processes affected by water shortage, a controversy still exists on whether drought effects on photosynthesis are due to ‘stomatal’ or ‘non-stomatal’ limitations (Flexas and Medrano, 2002; Lawlor and Cornic, 2002). Stomatal limitations result from the resistance to CO₂ diffusion from the atmosphere to intercellular leaf spaces, while non-stomatal limitations are often assumed to be metabolic

constraints. However, there is increasing evidence that the conductance determining transfer of CO₂ from the intercellular leaf spaces to the vicinity of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), i.e. the mesophyll conductance, g_m , has a finite value and frequently decreases under drought (Flexas *et al.*, 2008; Warren, 2008). Therefore, the controversy of ‘stomatal’ versus ‘non-stomatal’ limitations of photosynthesis has been replaced by ‘diffusive’ versus ‘metabolic’ (or biochemical) limitations (Grassi and Magnani, 2005; Galmés *et al.*, 2007b). While it is well established that under mild to moderate stress, drought-induced reductions in photosynthesis are mainly due to diffusive limitations—i.e. decreased stomatal and mesophyll

Abbreviations: C_c , chloroplastic CO₂ concentration; DTT, dithiothreitol; EGTA, ethyleneglycol-bis(b-aminoethylether)-N,N,N',N'-tetraacetic acid; ETR, electron transfer rate; g_m , mesophyll conductance; g_s , stomatal conductance; LMA, leaf mass per area; MiWS, mild water stress; MoWS, moderate water stress; Na-DIECA, sodium diethyldithiocarbamate; PEG, polyethyleneglycol; PMSF, phenylmethylsulfonyl fluoride; PPFD, photosynthetically active photon flux density; PVP, polyvinylpyrrolidone; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; RW, rewatered; RWC, relative water content; SeWS, severe water stress; SWC, soil water content; WW, well watered.

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conductance—(Chaves *et al.* 2002; Flexas and Medrano, 2002), there is less of a consensus about the limiting process when drought intensity is increased. Similarly, there is much less information on the limitations to photosynthesis recovery by severely stressed plants when water is restored (Galmés *et al.*, 2007b; Gallé *et al.*, 2009).

Current atmospheric CO₂ concentration is well below saturating for photosynthesis, so the metabolic rate that limits photosynthesis in the majority of plants is carboxylation by Rubisco rather than electron transport or RuBP regeneration, although sometimes a co-limitation of both metabolic components has been suggested (Lambers *et al.*, 2008). Under drought, CO₂ availability in leaves is further reduced because of stomatal closure, hence leading plants to operate in a CO₂ range where Rubisco carboxylation is the sole metabolic rate limiting photosynthesis. Therefore, despite claims that drought impairs chloroplast ATP synthesis and RuBP regeneration (Tezara *et al.*, 1999), if any metabolic limitation occurs under drought this is very likely to be mediated by Rubisco activity (Flexas *et al.*, 2004; Grassi and Magnani, 2005). Rubisco activity can be estimated by calculations from *in vitro* and/or *in vivo* measurements (von Caemmerer, 2000). Estimates from *in vivo* measurements are based on the assumptions of the Farquhar model adapted to finite g_m —i.e. calculation of the initial slope of the A_N-C_c curve or $V_{c,max}$ —(Farquhar *et al.*, 1980; Niinemets *et al.*, 2009), and usually show that the carboxylation capacity is preserved until water stress becomes severe (Bota *et al.*, 2004; Grassi and Magnani, 2005; Galmés *et al.*, 2007b; Gallé *et al.*, 2009). On the contrary, inconsistencies arise from *in vitro* measurements concerning the metabolic impairment of photosynthesis, and particularly of Rubisco activity, under drought. The reported effects of water stress on the activity of Rubisco differ between reports, precluding a definitive conclusion. Hence, observed effects range from major reductions of Rubisco activity (Majumdar *et al.*, 1991; Maroco *et al.*, 2002; Parry *et al.*, 2002), to minor decreases (Flexas *et al.*, 2006), and even no effect at all (Giménez *et al.*, 1992; Gunasekera and Berkowitz, 1993; Lal *et al.*, 1996; Pankovic *et al.*, 1999; Delfine *et al.*, 2001; Pelloux *et al.*, 2001). This apparent controversy may be attributed to differences in the intensity of drought (Flexas *et al.*, 2006). Alternatively, it may arise from interspecific differences, but there are studies reporting contradictory effects of water stress on Rubisco activity for the same species. For instance, Parry *et al.* (2002) showed, in tobacco plants, that down-regulation of Rubisco activity occurred concomitantly with decreases in the leaf relative water content (RWC), while Flexas *et al.* (2006) found no correlation between these parameters also in tobacco. In an attempt to explain such discrepancies, Flexas *et al.* (2004) demonstrated that differences between species disappeared when Rubisco activity was plotted against stomatal conductance (g_s) rather than RWC. However, a direct relationship between g_s and Rubisco activity is unlikely. It is possible, though, that this observation might be due to an indirect effect, related to the influence of g_s on the CO₂ concentration in leaves which, in

turn, also depends on the rate of assimilation, controlled by Rubisco activity (Flexas *et al.*, 2004).

Rubisco activity depends on (i) its activation by reaction with CO₂, resulting in the carbamylation of a lysyl residue in its active site, (ii) the concentration of Rubisco, and (iii) the concentration of tight-binding inhibitors (Roy and Andrews, 2000). An inverse correlation between the carbamylation state and Rubisco concentration has been reported (Quick *et al.*, 1991; Ruuska *et al.*, 1998; Eichelmann *et al.*, 2009). Changes in the concentration of Rubisco have been observed under water stress (e.g. Holaday *et al.*, 1992), but even in this situation Rubisco concentration is considered to be present sufficiently in excess not to limit photosynthesis (Pickersgill, 1986; Warren and Adams, 2004). Increases in Rubisco tight-binding inhibitors have also been reported under conditions of water shortage, although this has not been firmly established (Parry *et al.*, 2002). Therefore, adjustments of Rubisco activity under water stress are mainly attributed to changes in the carbamylation and activation state. In situations where light is saturating and leaf conductance does not limit CO₂ supply (i.e. in the absence of stress), the activation state of Rubisco is maximum (Sage *et al.*, 1990). However, water stress, through changes in g_s and g_m , induces a decrease in the concentration of CO₂ in leaves and therefore in the amount of activator CO₂ bound by carbamylation to Rubisco. De-activation of Rubisco by loss of the carbamate complex in the active site has been observed at substomatal CO₂ concentrations (C_i) of <100 $\mu\text{mol mol}^{-1}$ in some species, but not in others (Perchorowicz and Jensen, 1983; von Caemmerer and Edmondson, 1986; Sage *et al.*, 1990). This threshold of internal CO₂ concentration coincides roughly with the threshold of chloroplastic CO₂ concentration (C_c) inducing decreases in *in vivo* $V_{c,max}$ in several species (Galmés *et al.*, 2007b) and *in vitro* Rubisco activity in tobacco and soybean (Flexas *et al.*, 2006). Overall, this evidence suggests that drought-driven effects on Rubisco activity are caused by CO₂ starvation and its consequent de-carbamylation of the Rubisco catalytic sites. Therefore, a survey relating Rubisco activity and C_c for a larger number of species subjected to gradual imposition of water stress and recovery is justified and would help in defining the causes of metabolic limitation of photosynthesis under drought.

Problems exist when directly relating *in vitro* measurements of Rubisco activity to estimates of Rubisco activity derived from gas exchange measurements in leaves. Incomplete extraction of Rubisco, prior to activity analysis, has been shown to be responsible for inadequate activity to support photosynthetic rates measured by gas exchange in loblolly pine (Rogers *et al.*, 2001). Rubisco extractions from leaves are often incomplete even when care is taken to prevent inactivation and precipitation by high contents of phenolic or other secondary compounds (Beadle *et al.*, 1983; Keys and Parry, 1990). There are also inaccuracies in estimating Rubisco activity from *in vivo* measurements of gas exchange in leaves and especially in the determination of g_m needed for the model used. The combined gas

exchange and chlorophyll fluorescence methods for calculating g_m rely on the estimation of parameters requiring a number of assumptions and subject to technical limitations, which become relatively more important in certain groups of species, like sclerophylls, with leaves covered by hairs, and in heterobaric leaves (Flexas *et al.*, 2008; Pons *et al.*, 2009). Despite these difficulties, qualitative relationships of photosynthetic activity can be seen between *in vitro* and *in vivo* estimates.

Here, a set of data showing the effects of water stress on Rubisco activity for 11 Mediterranean species from contrasting environments grown under common conditions is presented. The objectives of the present work were: (i) to test the relationship between *in vitro* measurements and *in vivo* estimations of Rubisco activity; (ii) to check whether there is a general pattern in the response of Rubisco activity to drought stress in different species, and if so, (iii) to discern whether drought-driven changes in Rubisco activity are related to C_c .

Material and methods

Species selection and treatments

Eleven Mediterranean species naturally occurring in the Balearic Islands, six of them endemic to these islands, were selected for this study (see Galmés *et al.*, 2007a for detailed description of the species). The selection of the species included taxons representing different growth forms and leaf habits: two evergreen sclerophyll shrubs (*Pistacia lentiscus* and *Hypericum balearicum*), two evergreen sclerophyll semi-shrubs (*Limonium gibertii* and *Limonium magallufianum*), three summer semi-deciduous shrubs (*Lavatera maritima*, *Phlomis italica*, and *Cistus albidus*), and four herbaceous (*Beta maritima* ssp. *maritima*, *Beta maritima* ssp. *marcosii*, *Diploaxis ibicensis*, and *Lysimachia minoricensis*). *D. ibicensis*, *B. maritima* ssp. *maritima*, *B. maritima* ssp. *marcosii*, *L. gibertii*, *L. magallufianum*, and *L. maritima* are species inhabiting the coastal, driest, and hottest areas with annual precipitation typically $<400 \text{ l m}^{-2}$. *P. lentiscus*, *H. balearicum*, *P. italica*, and *C. albidus* are species typical of Mediterranean macchia with annual precipitation between 400 and 800 l m^{-2} . Finally, *L. minoricensis* lives near open water sources, and probably suffers less from water stress episodes.

The ages of the plants differed because of the different phenology of the species selected. Plants of *P. lentiscus*, *H. balearicum*, *C. albidus*, *P. italica*, and *L. maritima* were 3 years old, plants of *L. magallufianum*, and *L. gibertii* were 18 months old, and plants of *D. ibicensis*, *B. maritima* ssp. *marcosii*, and *B. maritima* ssp. *maritima* were 6 months old at the onset of the experiments. Similarly, the different phenology also influenced the age of leaves used for measurements, which was ~ 1 month for those species with the highest rate of leaf emergence (i.e. herbaceous species), and ~ 2 – 4 months for the remaining species.

Water treatments are explained in detail in Galmés *et al.* (2007a). In brief: 10 adult plants per species were placed in

a controlled growth chamber with a 12 h photoperiod (26° C day , 20° C night) and a photon flux density at the top of the leaves of $\sim 700 \mu\text{mol m}^{-2} \text{ s}^{-1}$. Plants were irrigated daily with 50% Hoagland's solution for 1 month. Measurements corresponding to well-watered treatment (WW) were made during the first day of the experiment and corresponded to plants watered at soil saturation. Thereafter, irrigation was stopped in five plants for each species. Pots were weighed daily to determine the amount of water loss. Plant water availability was referred to the control after measurement of soil dry weight in four samples representative of the substrate mixture used in the experiment. Measurements were performed on days 4, 8, and 13–17 after the last irrigation, when plants were regarded as affected by mild (MiWS), moderate (MoWS), and severe water stress (SeWS), respectively. Plants were considered to be under SeWS when g_s was close to zero, which was achieved between 13 and 17 d after withholding water, depending on the species. At this time, pots were rewatered to field capacity, and recovery treatment (RW) was determined on the next day. Control plants were watered daily throughout the experiment and measured every 5–6 d to ensure they had maintained constant values.

Plant water status

Parameters related to plant water status, leaf relative water content (RWC_{PD}) and water potential (Ψ_{PD}) were measured pre-dawn as indicated in Galmés *et al.* (2007a).

Photosynthetic measurements

Chlorophyll fluorescence parameters were measured on attached leaves using a portable pulse amplitude modulation fluorometer (PAM-2000; Walz, Effeltrich, Germany). At mid-morning, the PSII photochemical efficiency ($\Delta F/F_m'$) was determined by measuring the steady state fluorescence (F_s) and the maximum fluorescence (F_m') during a light-saturating pulse of $\sim 8000 \mu\text{mol m}^{-2} \text{ s}^{-1}$, following Genty *et al.* (1989):

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

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

$$\text{ETR} = \Delta F/F_m' \text{PPFD} \alpha \beta$$

where PPFD is the photosynthetically active photon flux density, α is the leaf absorptance, and β is the distribution of absorbed energy between the two photosystems. β was assumed to be 0.5. Leaf absorptances were determined for all 11 species in 10 replicates on leaves of well-irrigated plants with a spectroradiometer coupled to an integration sphere (UniSpec; PP-Systems, Amesbury, MA, USA). A value of 0.84 was obtained for all species, except for *C. albidus* and *P. italica* (0.74 and 0.77, respectively). Potential changes in leaf absorptance with water stress were not

assessed and assumed to be small and to induce no important biases in the calculations of ETR.

Light-saturated net CO₂ assimilation rates (A_N) and g_s were measured at mid-morning on attached, fully developed young leaves of four to five plants per species and treatment, using a gas exchange system (Li-6400; Li-Cor Inc., Lincoln, NE, USA). Environmental conditions in the leaf chamber consisted of a PPFD of 1500 $\mu\text{mol m}^{-2} \text{s}^{-1}$, a vapour pressure deficit of 1.0–1.5 kPa, an air temperature of 25° C, and an ambient CO₂ concentration (C_a) of 400 $\mu\text{mol mol air}^{-1}$. After inducing steady-state photosynthesis, the photosynthesis response to varying substomatal CO₂ concentration (C_i) was measured as explained in Galmés *et al.* (2007b). To transform A_N-C_i curves to A_N-C_c curves, the CO₂ concentration in the chloroplasts (C_c) was calculated from combined gas exchange and chlorophyll fluorescence measurements according to Epron *et al.* (1995) and using the *in vitro* Rubisco specificity factor values from Galmés *et al.* (2005).

Biochemical measurements

For measurements of Rubisco activity, two leaf discs (1 cm² each) were punched, frozen immediately in liquid N₂, and kept at –80° C until assay. Four to five replicates per species and treatment were taken at midday immediately after steady-state gas exchange measurements. Environmental conditions in the leaf chamber were identical to those explained above in the photosynthetic measurements section.

For Rubisco activity analysis, samples were ground to a fine powder in a mortar, previously chilled with liquid nitrogen, and homogenized in 1 ml of an ice-cold extraction medium. Preliminary tests determined that the most appropriate protein extraction media for Rubisco (i.e. that yielded the maximum activity for each species) were: (A) 0.1 M Bicine, 50 mM β -mercaptoethanol, 11 mM sodium diethyldithiocarbamate (Na-DIECA), 6% (w/v) polyethyleneglycol (PEG) 4000, 1 mM benzamidine, 1 mM *e*-amino-*n*-caproic acid, and 1 mM phenylmethylsulfonyl-fluoride (PMSF), at pH 8, and (B) containing 0.1 M HEPES, 3% (w/v) polyvinylpyrrolidone (PVP) 25, 6% (w/v) PEG 4000, 50 mM β -mercaptoethanol, 2 mM dithiothreitol (DTT), 10% glycerol, 5 mM MgCl₂, 5 mM ethyleneglycol-*bis*(*b*-aminoethylether)-*N,N,N',N'*-tetraacetic acid (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 *P. lentiscus* and *C. albidus*. Extracts were clarified by centrifugation (12 000 rpm at 4° C for 1 min) and the supernatant immediately assayed at 25° C for Rubisco activity.

Initial and total activities were determined according to Keys and Parry (1990). Initial activity was determined by adding 25 μl of supernatant to 475 ml of a CO₂-free assay buffer containing 100 mM Bicine, pH 8.2, and 20 mM MgCl₂, to which NaH¹⁴CO₃ (7.4 kBq μmol^{-1}) and RuBP had been added, to concentrations of 10 and 0.4 mM, respectively, immediately prior to adding the extract. Total

activity was determined by incubating 20 ml of extract for 3 min in 980 ml of the same assay buffer without RuBP and to allow for the carbamylation of all available active sites. The assay was started by adding RuBP to 0.4 mM as above. Rubisco activation state was obtained from the ratio of initial to total activity.

Total soluble protein was determined on the same samples according to the method of Bradford (1976).

Statistical analyses

Regression coefficients and bivariate correlations were calculated with the 11.0 Sigma Plot software package (SPSS, Chicago, IL, USA). A set of simple ANOVAs were performed to compare different treatments. Differences between means were revealed by Duncan analyses ($P < 0.05$) performed with the SPSS 17.0 software package.

Results and discussion

Progressive effects of water stress on Rubisco parameters

The response of pre-dawn leaf water potential (Ψ_{PD}) and relative water content (RWC_{PD}) to water stress and recovery during this experiment has previously been reported (Galmés *et al.*, 2007a). Leaf RWC_{PD} and Ψ_{PD} decreased progressively with soil water content (SWC) in all species (Table 1). The largest decreases in RWC_{PD} were observed at SeWS, and ranged from 38% for *P. italica* to 70% for *L. magallufianum*. Similarly, at SeWS Ψ_{PD} varied from less than –5 MPa for *P. italica* to –1 MPa for *D. ibicensis*. The extent of recovery 24 h after rewatering severely stressed plants depended on the species: all species except *P. lentiscus* and *P. italica* recovered RWC_{PD} and Ψ_{PD} to values similar to those measured under WW conditions.

All species showed a progressive decline in the Rubisco initial activity as water stress intensified, starting at MiWS, except for the two *Beta* spp. (Fig. 1). Rubisco activation state followed a slightly different pattern, maintaining values similar to those in irrigated plants under MiWS to MoWS, depending on the species, and declining under SeWS (Fig. 1). This trend is illustrated by the biphasic relationship between activation state and initial activity (Fig. 2), with an initial phase where activation state remains constant while initial activity decreases followed by a second phase where decreases in both parameters occurred concurrently. This fact suggests that under MiWS to MoWS, decreases in Rubisco activity are mainly due to decreases in the concentration of Rubisco reaction sites (i.e. initial and total Rubisco activity decrease concomitantly), either due to decreases in the enzyme concentration or to increases in the inhibitors bound to the sites of reaction. Early drought-induced losses of Rubisco have been reported by both transcriptomic (Kawaguchi *et al.*, 2003) and proteomic approaches (Holaday *et al.*, 1992), although increases have also been reported in some species (Cramer *et al.*, 2007; Vincent *et al.*, 2007). The species selected in this study,

Table 1. SWC (expressed as a percentage of well-watered treatments), leaf RWC_{PD} , and leaf Ψ_{PD} for the 11 species under the different treatments

 Values are means \pm standard errors of four to five replicates. Data taken from Galmés *et al.* (2007b).

Species		WW	MiWS	MoWS	SeWS	RW
<i>D. ibicensis</i>	SWC (%)	100.0 \pm 0.0	68.6 \pm 3.4	37.5 \pm 5.3	26.7 \pm 5.8	89.7 \pm 4.1
	RWC_{PD}	80.2 \pm 1.3	70.4 \pm 1.4	67.5 \pm 3.2	62.3 \pm 7.2	79.9 \pm 2.8
	Ψ_{PD}	-0.43 \pm -0.03	-0.69 \pm -0.02	-0.94 \pm -0.06	-1.00 \pm -0.11	-0.98 \pm -0.03
<i>L. minoricensis</i>	SWC (%)	100.0 \pm 0.0	65.6 \pm 2.6	58.4 \pm 3.6	49.5 \pm 4.6	90.6 \pm 5.7
	RWC_{PD}	76.1 \pm 0.9	70.6 \pm 6.2	73.5 \pm 4.2	44.7 \pm 3.8	71.8 \pm 3.6
	Ψ_{PD}	-0.57 \pm -0.08	-1.19 \pm -0.31	-1.31 \pm -0.39	-2.79 \pm -0.26	-0.64 \pm -0.07
<i>B. maritima</i> ssp. <i>marcosii</i>	SWC (%)	100.0 \pm 0.0	62.8 \pm 1.5	19.5 \pm 5.0	7.4 \pm 5.0	90.8 \pm 2.1
	RWC_{PD}	80.1 \pm 3.8	87.3 \pm 2.1	78.0 \pm 1.7	51.1 \pm 5.7	86.7 \pm 2.0
	Ψ_{PD}	-0.33 \pm -0.01	-0.45 \pm -0.03	-0.89 \pm -0.04	-3.34 \pm -0.10	-0.52 \pm -0.06
<i>B. maritima</i> ssp. <i>maritima</i>	SWC (%)	100.0 \pm 0.0	56.9 \pm 5.0	26.8 \pm 1.2	12.5 \pm 5.3	91.4 \pm 3.2
	RWC_{PD}	78.0 \pm 0.9	80.2 \pm 4.1	79.0 \pm 2.5	51.0 \pm 3.5	84.7 \pm 1.9
	Ψ_{PD}	-0.33 \pm -0.01	-0.44 \pm -0.05	-0.80 \pm -0.05	-3.74 \pm -0.09	-0.43 \pm -0.01
<i>L. magallufianum</i>	SWC (%)	100.0 \pm 0.0	54.2 \pm 2.3	46.0 \pm 1.5	40.1 \pm 1.3	98.7 \pm 2.8
	RWC_{PD}	88.9 \pm 0.5	85.8 \pm 2.0	80.3 \pm 2.3	69.5 \pm 1.6	87.0 \pm 2.7
	Ψ_{PD}	-0.53 \pm -0.03	-0.55 \pm -0.07	-0.65 \pm -0.07	-1.38 \pm -0.14	-1.02 \pm -0.03
<i>L. gibertii</i>	SWC (%)	100.0 \pm 0.0	56.8 \pm 3.4	49.1 \pm 3.4	44.6 \pm 3.4	92.3 \pm 2.5
	RWC_{PD}	90.6 \pm 0.9	88.3 \pm 1.9	77.6 \pm 0.8	66.8 \pm 5.1	87.7 \pm 4.2
	Ψ_{PD}	-0.55 \pm -0.03	-0.49 \pm -0.04	-0.61 \pm -0.03	-1.05 \pm -0.09	-0.93 \pm -0.07
<i>H. balearicum</i>	SWC (%)	100.0 \pm 0.0	85.9 \pm 7.3	52.7 \pm 7.0	30.7 \pm 5.9	85.4 \pm 4.4
	RWC_{PD}	91.2 \pm 1.3	93.1 \pm 1.1	89.9 \pm 1.3	48.7 \pm 5.2	85.5 \pm 2.4
	Ψ_{PD}	-0.38 \pm -0.00	-0.43 \pm -0.02	-0.66 \pm -0.05	-2.96 \pm -0.16	-0.94 \pm -0.14
<i>P. lentiscus</i>	SWC (%)	100.0 \pm 0.0	61.4 \pm 7.6	25.5 \pm 8.2	14.6 \pm 7.1	88.6 \pm 5.5
	RWC_{PD}	94.8 \pm 0.5	87.5 \pm 3.3	86.1 \pm 3.8	53.7 \pm 4.9	81.2 \pm 5.4
	Ψ_{PD}	-0.30 \pm -0.03	-0.45 \pm -0.05	-1.49 \pm -0.58	-4.55 \pm -0.45	-2.68 \pm -0.28
<i>P. italica</i>	SWC (%)	100.0 \pm 0.0	74.8 \pm 4.6	57.0 \pm 1.8	47.7 \pm 1.8	114.7 \pm 5.4
	RWC_{PD}	83.6 \pm 1.5	80.0 \pm 0.8	75.6 \pm 0.8	37.9 \pm 3.0	74.4 \pm 1.6
	Ψ_{PD}	-0.33 \pm -0.01	-0.42 \pm -0.03	-1.98 \pm -0.11	< -5.00	-0.65 \pm -0.05
<i>C. albidus</i>	SWC (%)	100.0 \pm 0.0	72.7 \pm 4.9	53.4 \pm 1.8	42.7 \pm 2.9	104.4 \pm 1.6
	RWC_{PD}	75.7 \pm 4.3	88.3 \pm 4.3	79.5 \pm 4.0	46.0 \pm 6.1	70.2 \pm 1.5
	Ψ_{PD}	-0.51 \pm -0.04	-0.68 \pm -0.06	-1.14 \pm -0.14	-4.12 \pm -0.56	-2.10 \pm -0.10
<i>L. maritima</i>	SWC (%)	100.0 \pm 0.0	57.7 \pm 6.1	35.9 \pm 6.8	27.0 \pm 6.0	91.1 \pm 3.4
	RWC_{PD}	86.6 \pm 2.3	82.2 \pm 2.5	73.4 \pm 3.5	54.8 \pm 5.5	80.6 \pm 3.2
	Ψ_{PD}	-0.41 \pm -0.05	-0.89 \pm -0.09	-1.53 \pm -0.13	-3.54 \pm -0.25	-0.73 \pm -0.21

although inhabiting contrasting environments, are typically Mediterranean, and therefore adapted to frequent but unpredictable drought events (Galmés *et al.*, 2007a). Moreover, due to their relatively low g_s and g_m (Galmés *et al.*, 2007a), they often present C_c values close to or below the threshold of 100 $\mu\text{mol mol}^{-1}$, i.e. they operate normally close to conditions favourable for Rubisco inactivation (Perchorowicz and Jensen, 1983). Among potentially advantageous adaptations to such stress conditions, interactions of Rubisco with tight-binding inhibitors would prevent Rubisco that is not being used for catalysis from being degraded by proteases (Parry *et al.*, 2008). Thereafter, at MoWS to SeWS, deactivation of Rubisco becomes the predominant cause for decreased Rubisco activity, in agreement with previous reports (Sharkey and Seemann, 1989). When exposed to severe water stress, many plants accumulate osmolytes, such as proline, glycine betaine, and sugar alcohols (Yoshida *et al.*, 1997). *In vitro* analyses demonstrate that high concentrations of these osmolytes have potential to curtail the activity of Rubisco (Sivakumar

et al., 1998; Sivakumar *et al.*, 2002). Therefore, the observed decrease in Rubisco activity at SeWS could be, at least partially, due to the accumulation of osmolytes.

The recovery of Rubisco initial activity and activation state after SeWS was also species dependent. In general, activation state recovered better than initial activity, although with some exceptions, such as the two *Limonium* species (Fig. 1). This pattern is indicative of higher recovery of total activity as compared with initial activity, and consequently that an increase in the concentration of Rubisco active sites occurred after rewatering (Parry *et al.*, 2002).

Relationship between in vitro and in vivo estimates of Rubisco activity

Rapid, one-step homogenization procedures used for Rubisco extraction are required to preserve enzyme activation state (Keys and Parry, 1990). However, Rogers *et al.* (2001) demonstrated that these procedures fail to efficiently

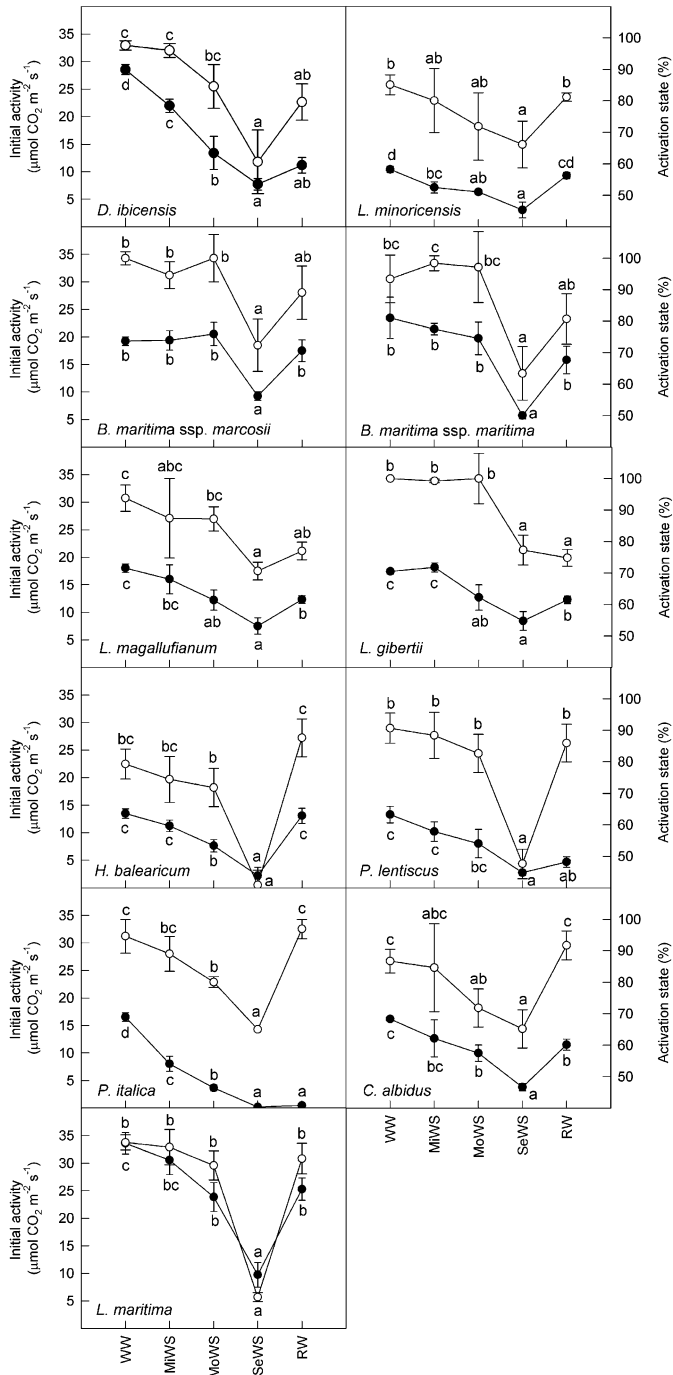


Fig. 1. Rubisco initial activity (filled symbols) and activation state (open symbols) under different treatments: WW, MiWS, MoWS, SeWS, and RW. Values are means \pm SE of four to five replicates per species and treatment. Different letters denote statistical differences by Duncan test ($P < 0.05$) between treatments for each parameter.

extract Rubisco from leaves, and therefore lead to underestimation of Rubisco activity. In the present study, Rubisco activity was $\sim 20\%$ of the estimated maximum velocity of carboxylation ($V_{c,max}$) in all 11 species (data not shown). Fig. 3 shows the relationship between Rubisco initial activity and $V_{c,max}$, both parameters expressed as a percentage of the values at WW, to unify axis scales.

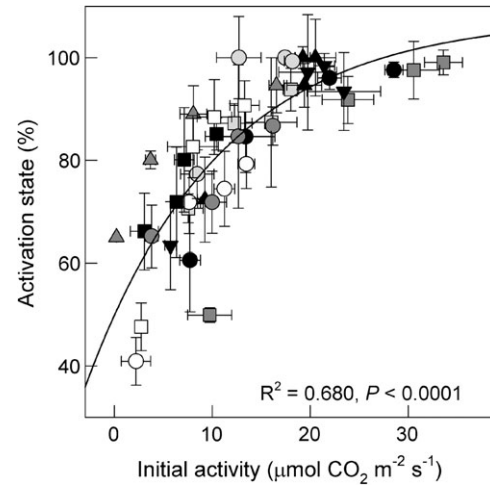


Fig. 2. Relationship between Rubisco initial activity and activation state. Values are means \pm SE of four to five replicates per species and treatment. Values from the RW treatment were excluded from this analysis. Symbols and species as follows: black circles, *D. ibicensis*; black squares, *L. minoricensis*; black triangles, *B. maritima* ssp. *marcosii*; upside down black triangles, *B. maritima* ssp. *maritima*; light-grey squares *L. magallufianum*; light-grey circles, *L. gibertii*; dark-grey circles, *C. albidus*; dark-grey, *L. maritima*; dark-grey triangles, *P. italica*; open circles, *H. balearicum*; and open squares, *P. lentiscus*.

Despite such bias, the relationship between Rubisco initial activity and $V_{c,max}$ was linear and significant for each species (i.e. the lowest R^2 was for *H. balearicum* with 0.522), indicating that *in vitro* measurements of Rubisco activity were qualitatively consistent with *in vivo* values, as previously reported (e.g. Myers et al., 1999; Rogers et al., 2001). On the other hand, it is also documented that the effectiveness of Rubisco extraction and assay procedures is dependent on the presence of phenols and other secondary metabolites (Beadle et al., 1983; Keys and Parry, 1990), which varies depending on the species and stress conditions (Turtola et al., 2003; André et al., 2009). This fact could explain differences in the slope of the relationship between Rubisco initial activity and $V_{c,max}$ between species (Fig. 3). In this sense, *D. ibicensis*, *B. maritima* ssp. *maritima*, *P. italica*, and *L. maritima* were the species presenting the highest slope, while *L. magallufianum*, *L. gibertii*, and *B. maritima* ssp. *marcosii* presented the lowest slope.

Alternatively, it may be argued that the estimation of $V_{c,max}$ might be biased, due to the multiple assumptions included in its calculation. To test this possibility, it may be necessary to compare Rubisco activity values with some *in vivo* parameter, such as the ETR, which is more straightforward to determine. A ‘photosynthetic control’ exists in leaves where the balance between ΔpH , ATP synthesis, and redox state adjust supply to demand so that the [ATP]/[ADP] and [NADPH]/[NADP⁺] ratios are remarkably constant in steady-state conditions (Foyer et al., 1990). Therefore, it is likely that the rate of electron transport in the thylakoids and Rubisco activity in the stroma are co-regulated, and therefore ETR would indirectly reflect

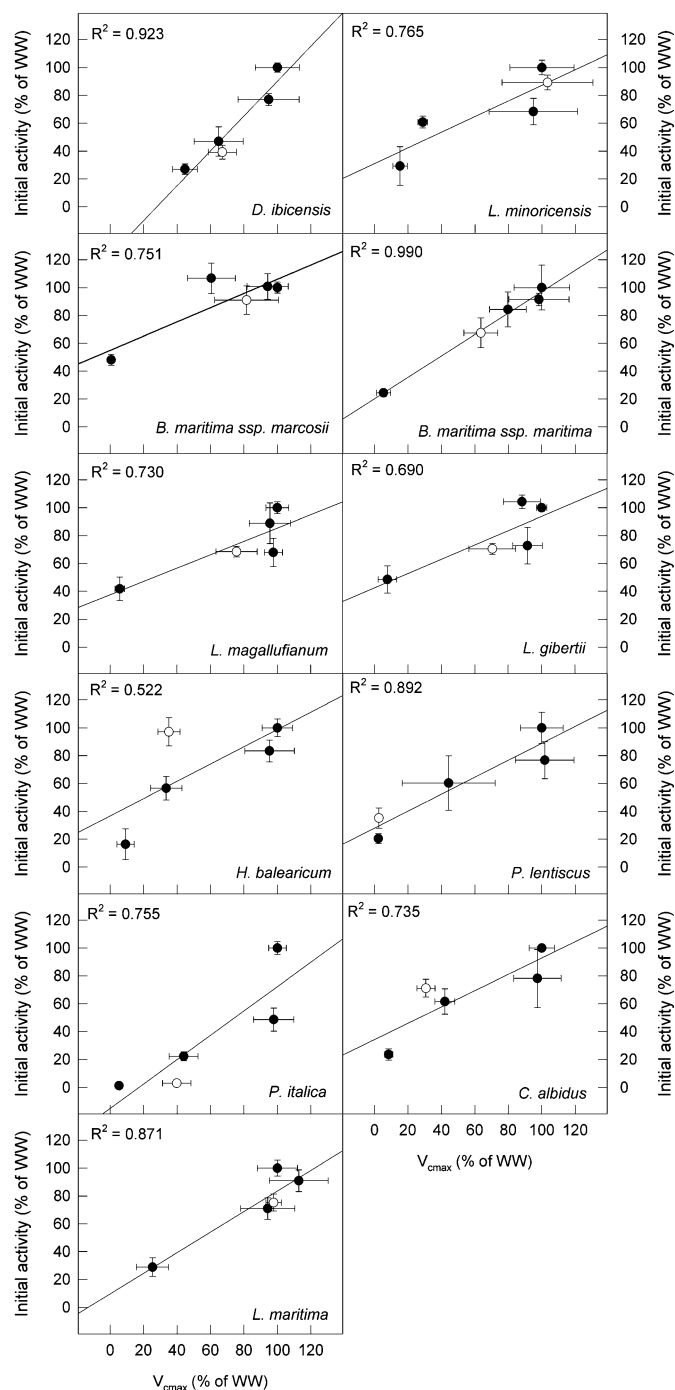


Fig. 3. Relationship between Rubisco initial activity and maximum velocity of carboxylation ($V_{c,max}$) for the 11 species. To unify scales between different species, values are expressed in percentage with respect to WW plants, and represent means \pm SE of four to five replicates per species and treatment. Empty symbols represent values from RW treatments. Regression coefficients for each relationship are shown.

variations in Rubisco activity. ETR is indeed co-regulated with $V_{c,max}$ in Mediterranean species (Galmés *et al.*, 2007a), and its use in place of $V_{c,max}$ as a proxy for metabolic limitation results in very similar estimations of the latter under water stress (Gallé *et al.*, 2009). In the present study, initial activity of Rubisco was highly correlated with ETR

(Fig. 4A), as it was with the CO_2 net assimilation rate (A_N) (Fig. 4B). These results confirm that despite potential activity losses during extraction, *in vitro* measurements of Rubisco activity were proportional to *in vivo* measurements and qualitatively representative of what occurs *in vivo*. Although the relationship between Rubisco initial activity and A_N was linear when plotting all species together (Fig. 4B), it was logarithmic for 6 of the 11 species when considered individually (data not shown). In particular, some of the species with larger maximum A_N and g_s display a logarithmic response, while evergreen sclerophylls spanning a shorter range of values display a linear response. This could be related to the fact that the latter, due to lower maximum g_s and g_m , operate always at C_c below the general inflexion point for Rubisco inactivation (see next section).

Causes for reduced Rubisco activity under water stress

Both Rubisco initial activity and its activation state presented a positive linear relationship when plotted against RWC_{PD} (Fig. 5A, C); however, it turned into a logarithmic biphasic relationship when these two parameters were plotted against g_s (Fig. 5B, D). Although all four correlations were highly significant ($P < 0.0001$), by using g_s the regression coefficients became higher, in agreement with previous surveys suggesting g_s as a reference parameter (Medrano *et al.*, 2002; Flexas *et al.*, 2004). Moreover, when analysed for each individual species, the relationship between Rubisco parameters and RWC_{PD} was significant for only three of the species (Table 2), while that with g_s was generally significant (not shown).

Rubisco initial activity and activation state did not correlate with the substomatal CO_2 concentration (C_i) (Fig. 6A, C). In fact, maximum values for initial activity and activation state were observed at intermediate C_i values, perhaps reflecting the typical response of C_i to drought consisting in decreases at mild to moderate stress followed by increases at more severe stress (Medrano *et al.*, 2002). By contrast, when plotting Rubisco initial activity and activation state against C_c , significant correlations were observed (Fig. 6B, D). As occurred with RWC_{PD} , the relationships between Rubisco initial activity and C_c and between activation state and C_c , presented an important scattering when plotting all species together. However, the regression coefficients generally improved when considering each species individually, being always higher than those with RWC_{PD} (Table 2). By breaking the covariation between g_s and RWC, Flexas *et al.* (2006) observed that down-regulation of Rubisco activity was not dependent on RWC, but occurred at a certain threshold of g_s ($< 0.05 \text{ mol m}^{-2} \text{ s}^{-1}$) and C_c ($< 100 \mu\text{mol mol}^{-1}$), although the relationship between Rubisco activity and C_c was less clear. In the present study, Rubisco initial activity correlated, at $P < 0.05$, with RWC for three species, and with C_c for nine species (Table 2). A similar trend was observed for the activation state (Table 2). It is worth noting that RW values were not consistent with the relationship in several species. Remarkably, the poorest correlations were found in shrubs

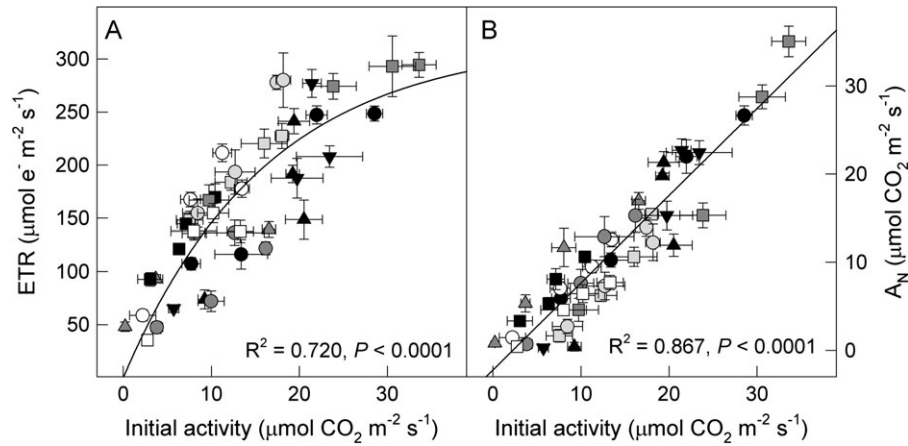


Fig. 4. Relationship between Rubisco initial activity and (A) ETR, and (B) A_N . Values are means \pm standard error of four to five replicates per species and treatment. Values from rewatering treatments were excluded from this analysis. Regression coefficients and significance for each relationship are shown. Symbols and species are as in Fig. 2.

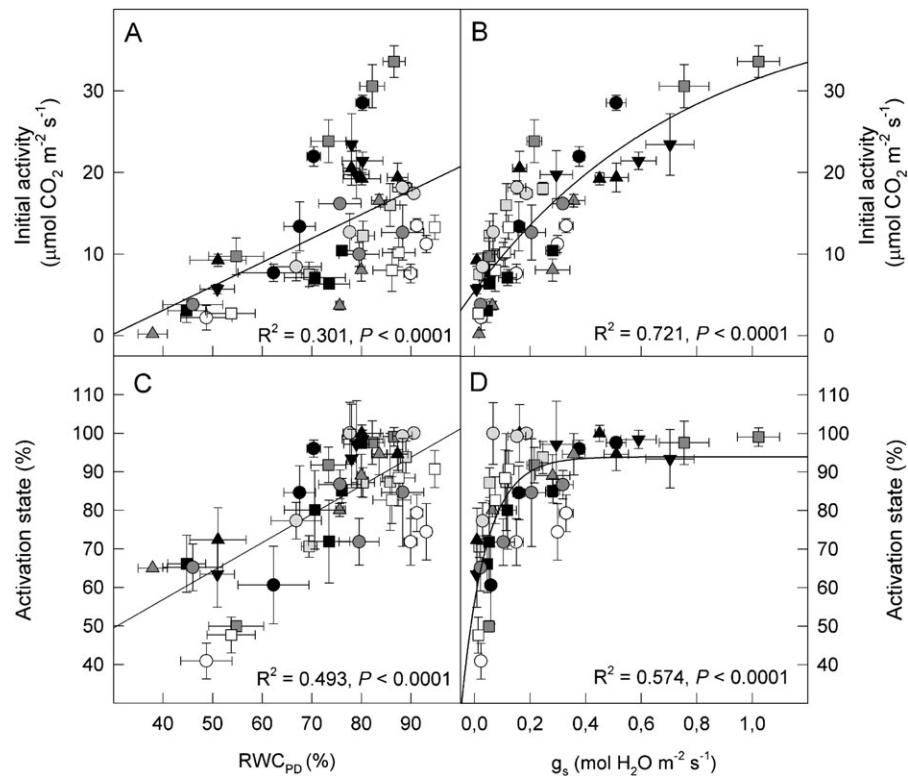


Fig. 5. Relationship between Rubisco initial activity and activation state with the leaf RWC_{PD} and g_s . Values are means \pm SE of four to five replicates per species and treatment. Values from rewatering treatments were excluded from this analysis. Regression coefficients and significance for each relationship are shown. Symbols and species are as in Fig. 2.

(*H. balearicum*, *P. lentiscus*, *C. albidus*, and *P. italica*), with higher leaf mass per area (LMA), while in all herbaceous (*D. ibicensis*, *L. minoricensis*, *B. maritima* ssp. *marcosii*, and *B. maritima* ssp. *maritima*), and semi-shrub (*L. magallufianum* and *L. gibertii*) species both Rubisco initial activity and its activation state significantly correlated with C_c . In general, these data demonstrate that regulation of Rubisco activity is more related to CO_2 availability in the vicinity of the enzyme than to leaf hydration status. Species-dependent patterns of Rubisco activity regulation under drought stress

have previously been documented (Parry et al., 2002; Bota et al., 2004; Flexas et al., 2006).

The present results suggest that low C_c induced by the imposition of water stress could induce deactivation of Rubisco sites. Previously, *in vivo* Rubisco deactivation was observed at $C_i < 100 \mu\text{mol mol}^{-1}$ in several species, e.g. *Chenopodium albus*, *Raphanus sativus*, *Triticum aestivum*, and *Phaseolus vulgaris* (Perchorowicz and Jensen, 1983; von Caemmerer and Edmondson, 1986; Sage et al., 1990; Sage et al., 2002). The $K_{act}[CO_2]$, i.e. the CO_2 concentration that

Table 2. Bivariate correlations between Rubisco initial activity, Rubisco activation state, leaf RWC_{PD} , and C_c for the 11 species included in the study

Rewatering data were included in the analysis. Linear and logarithmic correlations were considered and values correspond to the best fit.

	Initial activity	Activation state
<i>D. ibicensis</i>		
RWC_{PD}	0.314	0.388
C_c	0.700	0.840*
<i>L. minoricensis</i>		
RWC_{PD}	0.725	0.629
C_c	0.862*	0.888**
<i>B. maritima</i> ssp. <i>marcosii</i>		
RWC_{PD}	0.821*	0.673
C_c	0.874**	0.862*
<i>B. maritima</i> ssp. <i>maritima</i>		
RWC_{PD}	0.724	0.653
C_c	0.927**	0.754*
<i>L. magallufianum</i>		
RWC_{PD}	0.785*	0.527
C_c	0.954**	0.890**
<i>L. gibertii</i>		
RWC_{PD}	0.725	0.192
C_c	0.758*	0.992**
<i>H. balearicum</i>		
RWC_{PD}	0.728	0.818*
C_c	0.080	0.008
<i>P. lentiscus</i>		
RWC_{PD}	0.687	0.967**
C_c	0.811*	0.149
<i>C. albidus</i>		
RWC_{PD}	0.651	0.325
C_c	0.800*	0.375
<i>P. italica</i>		
RWC_{PD}	0.334	0.763*
C_c	0.774*	0.661
<i>L. maritime</i>		
RWC_{PD}	0.958**	0.957**
C_c	0.822*	0.780*

* $P < 0.05$; ** $P < 0.01$.

supports half of the maximal activation has been reported to be $120 \mu\text{mol mol}^{-1}$ for *in vitro* systems with activase (Portis *et al.*, 1986). Sage *et al.* (2002) suggested that *in vivo* $K_{act}[\text{CO}_2]$ is probably $<60 \mu\text{mol mol}^{-1}$. In agreement with this suggestion, in the present study Rubisco activation started to decline at C_c below $\sim 120 \mu\text{mol mol}^{-1}$ (Fig. 6D). At this low C_c , the equilibrium between carbamylated and decarbamylated Rubisco will shift towards the latter form. The major aim of the present study was to find global relationships between Rubisco activity and physiology under drought using several species pooled. However, it is clear that species belonging to very different growth forms and leaf habitats, even if they all often experience drought in their natural environments, undergo different average operational and maximum C_c . For instance, evergreen sclerophylls show the lowest stomatal and mesophyll conductance even under irrigation (Galmés *et al.*, 2007b),

and therefore the lowest maximum C_c (Fig. 7, light-grey and empty symbols). Perennial and annual herbs, as well as semi-deciduous species, present much larger g_s and g_m when irrigated, and therefore experience larger C_c (Fig. 7, black and dark-grey symbols). These differential features may induce differences in Rubisco between species. Indeed, Galmés *et al.* (2005) showed that Rubisco specificity factor positively scaled with sclerophylly, i.e. it was larger in species with lower g_s and g_m . It is shown here (Fig. 6D), as in Perchorowitz and Jensen (1983) and Flexas *et al.* (2006) that, globally, inactivation of Rubisco is induced by a C_c of $<100 \mu\text{mol mol}^{-1}$. However, due to their intrinsic leaf morphology and physiology, evergreen species always operate at $C_c < 100 \mu\text{mol mol}^{-1}$, even with stomata fully open (Fig. 7), while herbs and semi-deciduous plants present maximum C_c values of between 130 and $200 \mu\text{mol mol}^{-1}$, operating at lower C_c only when subjected to drought. This seems to have induced evolutionary differences at the level of Rubisco activation sensitivity to C_c . Hence, even when in all species the inflexion point for Rubisco inactivation is at a C_c of $\sim 100 \pm 40 \mu\text{mol mol}^{-1}$ (Fig. 6), there is a linear correlation between the maximum operational C_c and the actual value of C_c at the inflexion point for Rubisco inactivation (Fig. 7).

These results suggest that those species adapted to low $C_{c,max}$ are able to maintain active Rubisco at lower C_c . One possible explanation could be that Rubisco from these species has an intrinsically lower $K_{act}[\text{CO}_2]$. Another possibility is that Rubisco activase is more active or present in higher concentrations in these species. Higher concentration and/or activation of Rubisco activase would improve removal of RuBP and other tightly bound sugar phosphates from the sites of reaction (Portis *et al.*, 2008). Finally, formation of side-products, such as xylulose-1,5-bisphosphate and pentodiulose-1,5-bisphosphate, from the enediol intermediate during Rubisco catalysis can progressively inhibit Rubisco turnover. Pearce (2006) demonstrated that the rate of production of these side-products increased when the $[\text{CO}_2]/[\text{O}_2]$ ratio decreased, and such decreases occur under water stress (Galmés *et al.*, 2006). In consequence, differences in $C_{c,ip}$ may be caused by a lower rate of formation of side-products at a given C_c for species with lower $C_{c,ip}$. In addition, Pearce (2006) also suggested that species with a higher specificity factor produce relatively fewer side-products, and Galmés *et al.* (2005) observed, by studying these same species included in the present work, that Rubisco specificity factor tended to be higher in species with sclerophyll leaves (and therefore lower $C_{c,ip}$). Differences among species and groups in Rubisco responses to drought will deserve better attention in future studies.

Concluding remarks

The present study shows that decreases in the activity of Rubisco in response to drought occur in well-adapted Mediterranean species belonging to different growth forms and leaf habits. Decreases in both Rubisco initial activity and its activation correlated better with changes in stomatal

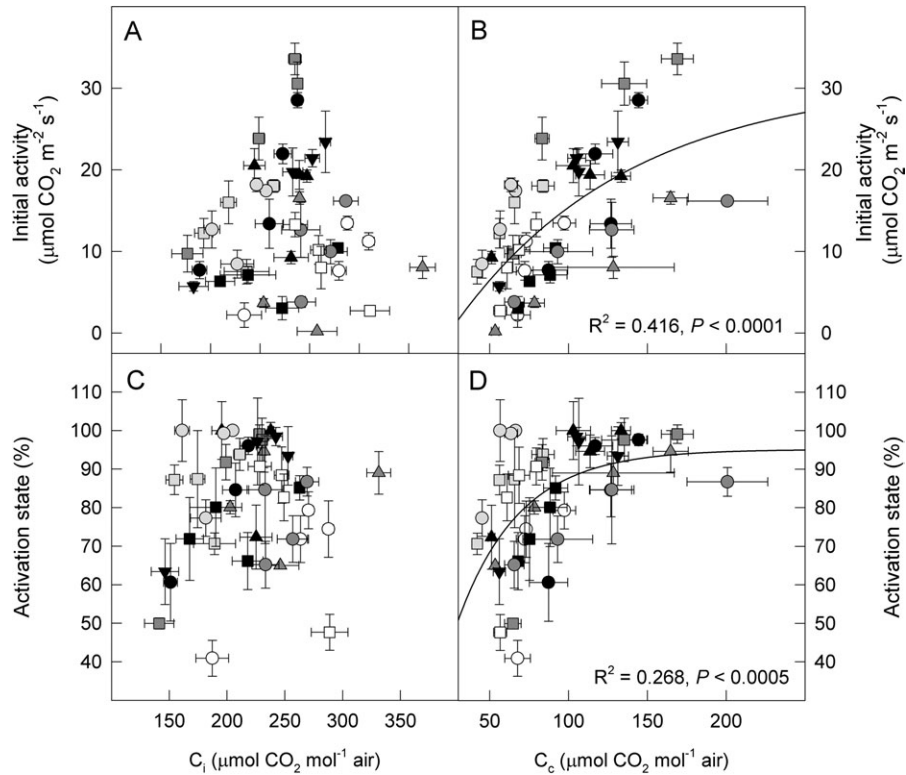


Fig. 6. Relationship between Rubisco initial activity and activation state with substomatal (C_i) and chloroplastic (C_c) CO_2 concentration. Values are means \pm SE of four to five replicates per species and treatment. Values from rewetting treatments were excluded from this analysis. Regression coefficients and significance for each relationship are shown. Symbols and species are as in Fig. 2.

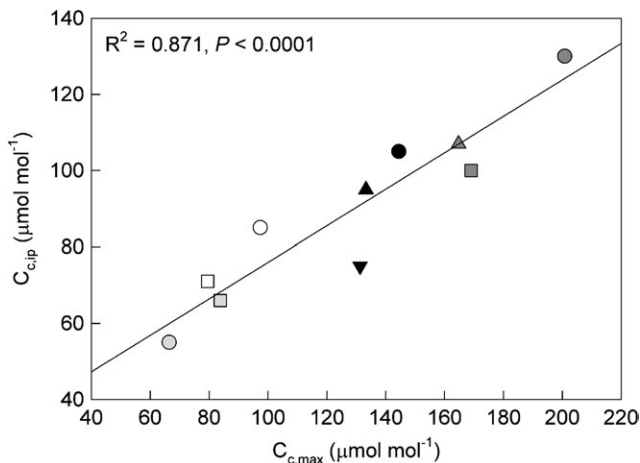


Fig. 7. Relationship between the chloroplastic CO_2 concentration at its maximum ($C_{c,\max}$) and at the inflexion point ($C_{c,\text{ip}}$) in the relationship between Rubisco activation state and C_c . For all species, $C_{c,\max}$ was C_c at WW conditions. For the calculation of $C_{c,\text{ip}}$, RW data were not considered. For all species, except *L. minoricensis*, a logarithmic function was applied in the relationship Rubisco activation state versus C_c , and the lowest R^2 was 0.883. *L. minoricensis* is not included in the analysis because its $C_{c,\text{ip}}$ could not be obtained ($C_{c,\text{ip}} > 100 \mu\text{mol mol}^{-1}$) due to the linear relationship between Rubisco activation state and C_c . Regression coefficient and significance of the relationship are shown. Symbols and species are as in Fig. 2.

conductance than with changes in relative water content. Since a direct effect of stomatal conductance on Rubisco is unlikely, we suggest that the effect is mediated by decreases in CO_2 availability induced by lower stomatal and mesophyll conductance, rather than by increases in leaf dehydration. In fact, both Rubisco initial activity, and especially, Rubisco activation, showed a threshold relationship with *in vivo* estimated C_c that matches theoretical values obtained from *in vitro* studies. While there are some differences between species in the precise C_c threshold value so that species with low g_s and thick leaves are able to maintain carbamylated Rubisco at lower values of C_c , evergreen sclerophyll species operate most of their life below that threshold, due to low maximum g_s and g_m even when water is available. In herbs and semi-deciduous species, the threshold is often reached during the frequent droughts occurring in the Mediterranean.

In conclusion, the results of the present study suggest that drought-driven decreases in C_c may induce deactivation of Rubisco and that photosynthesis in Mediterranean species may be constrained by lower Rubisco activation as a consequence of the need to keep stomata substantially closed in a water-scarce environment.

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