Photosynthesis and photoprotection responses to water stress in the wild-extinct plant *Lysimachia minoricensis*

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Abstract

*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 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. Early stomata closure was observed in response to soil water depletion while leaves desiccated progressively. Although net photosynthesis was low in irrigated plants, due to a remarkably low mesophyll conductance to CO₂, substantial photosynthetic activity was kept at severe drought, where leaf relative water content was as low as 50%, suggesting that *L. minoricensis* is a very drought-tolerant species. 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 was maintained higher than 0.75 through the entire experiment. Moreover, photosynthetic capacity was largely (80%) recovered only 24 h 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.

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1. Introduction

The Balearic Islands, located within the Mediterranean basin, are characterized by the richness of its endemic flora (Cardona and Contandriopoulous, 1979), which contributes to the maintenance of a high biodiversity in the area. However, a high proportion of the endemic species is confined to a few geographically restricted populations, being partially responsible for the high fragility of the 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 in the south of the island of Minorca (Rodriguez, 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 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* to 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 (Ibáñez...
et al., 1999; Calero et al., 1999). The 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 and Elam, 1993; O’Brien, 1994). Therefore, this reason may be argued to explain the unsuccessful re-introduction attempts.

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 of 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 and 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 the growth 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 during summer drought, are considered a limiting factor for the 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_.

In addition to the possible existence of physiologically related negative traits, in many cases the extinction of species derives from a combination of other diverse factors. Among these, habitat disturbance, fluctuating environments, niche competition, pests, predation, introgression and hybridization with relatives, and extensive recollection for economic or museistic purposes, have been proposed responsible for plant extinction. All these factors make resolving the problem of extinction of a given species a complex analysis. However, the study of specific physiological traits could help in such purposes, especially at an evolutionary level. In this sense, 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.

2. Materials and methods

2.1. Plant material and treatments

Seeds of _L. minoricensis_ J.J. Rodr. (Primulaceae) were obtained from a botanical garden (Jardí Botànic de Sóller, Spain) and germinated on filter paper moistened with de-ionized water in a controlled environment (germination chamber, at 18 °C in darkness). After germination and emergence of one true leaf, seedlings were transplanted to 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, 1-year-old plants 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 about 600 μmol m⁻² s⁻¹ (halogen lamps).

Plants were once abundantly supplied with 50% 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 on five plants and measurements made when soil water content reached 65% (mild drought treatment), 58% (moderate drought treatment) and 50% (severe drought treatment) with respect to control. Re-watering was measured 1 day after re-filling pots. Pots were weighted daily to determine soil water content. Five control plants were watered daily throughout the experiment to ensure that they maintained constant values of each parameter during the experiment. The experiment lasted 21 days.

2.2. 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. 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. The turgid leaf weight was determined after keeping the leaf in distilled water in darkness at 4 °C to minimise respiration losses, until they reached a constant weight (full turgor, typically after 12 h). Leaf dry weight was obtained 48 h after keeping the turgid leaf at 70 °C in an oven. Four replicates per treatment were obtained.

2.3. 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 (F₀), the maximum fluores-
cence ($F_m$) 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 1000 μmol m$^{-2}$ s$^{-1}$, obtained using the halogen lamp of the PAM-2000, measuring steady-state fluorescence signal ($F_m$). To obtain the steady-state maximum fluorescence yield ($F_m'$), saturation pulses of about 10,000 μmol photon m$^{-2}$ s$^{-1}$ and 0.8 s duration were applied. PSII photochemical efficiency ($\Delta F/F_m'$, Genty et al., 1989) was calculated as:

$$\frac{\Delta F}{F_m} = \frac{F_m' - F_o}{F_m'},$$

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

$$\text{ETR} = \frac{\Delta F}{F_m} \times PFD \times \alpha \beta,$$

where PFD is the photosynthetically active photon flux density, $\alpha$ the 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 and Loreto, 1996) and $\beta$ is the leaf absorptance. Leaf absorptances were calculated in ten leaves of well-irrigated plants with a spectroradiometer coupled to an absorptance. Leaf absorptances were calculated in ten leaves of well-irrigated plants with a spectroradiometer coupled to an absorptance.

2.4. Gas exchange measurements

Light-saturated net CO$_2$ assimilation rates ($A_N$) and stomatal conductance ($g_s$) were measured at mid-morning with a gas exchange system (Li-6400, Li-Cor Inc., Nebraska, USA) equipped with a light source (6200-02B LED, Li-Cor). Four attached and fully developed young leaves were analysed per treatment. Environmental conditions in the leaf chamber were: photosynthetically active photon flux density = 1500 μmol photon m$^{-2}$ s$^{-1}$; ambient vapour pressure deficit = 1.0–1.5 kPa; leaf temperature = 25 °C; ambient CO$_2$ concentration ($C_a$) = 360 μmol mol air$^{-1}$ and air flux = 300 μmol s$^{-1}$.

Once steady-state was reached, photosynthesis response curves to varying sub-stomatal CO$_2$ concentration ($C_i$) were performed. First, $C_a$ was lowered stepwise from 360 to 50 μmol mol$^{-1}$ and then returned 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 for at least 5 min at the new $C_a$. Measurements consisted in 12–13 measurements for each curve.

Dark respiration measurements ($R_D$) were performed in four leaf samples per treatment 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 and 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.

2.5. 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 ($C_c$) was estimated according to 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 = ETRA + ETRP, where ETRA is the fraction of ETR used for CO$_2$ assimilation, and ETRP is the fraction of ETR used for photorespiration. ETRA and ETRP can be solved from data of $A_N$, $R_D$, and ETR, and from the known stochiometries of electron use in photosynthesis and photorespiration, as follows (Epron et al., 1995; Valentini et al., 1995):

$$\text{ETRA} = \frac{1}{3}[\text{ETR} + 8(A_N + R_D)];$$

$$\text{ETRP} = \frac{2}{3}[\text{ETR} - 4(A_N + R_D)].$$

Dark respiration ($R_D$) was taken as a proxy for $R_D$, following Pinelli and Loreto (2003). From ETRA and ETRP, the apparent Rubisco specificity factor ($\tau^*$) can be calculated according to Laing et al. (1974), as follows:

$$\tau^* = \frac{\text{ETRA}/\text{ETRP}}{(C_c/O)}.$$
and $R_L$ is the rate of non-photorespiratory CO$_2$ evolution in the light. The treatment average of $\Gamma^*$ for the species was obtained, according to Brooks and Farquhar (1985), as:

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

from the specific in vitro specificity factor measured by Galmés et al. (2005) for $L$. minoricensis.

Estimations of $V_{c,max}$ were derived from fitting $A_N$–$C_v$ curves. The $A_N$–$C_v$ curves were obtained from $A_N$–$C_i$ curves using the values of $g_s$, following the method described by Manter and 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 and Magnani (2005). This approach, which requires the measurement of $A_N$, $g_s$, $g_l$ and $V_{c,max}$, makes possible to partition photosynthesis limitations into components related to stomatal conductance ($S_L$), mesophyll conductance ($M_{CL}$) 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, photosynthetic limitations were grouped into non-stomatal and diffusive limitations. Non-stomatal limitations were defined as the sum of the contributions due to mesophyll conductance and leaf biochemistry ($N_{S_L} = M_{CL} + B_l$), while diffusive limitations were the sum of stomatal and mesophyll conductance components ($D_L = S_L + M_{CL}$).

2.7. Pigment analyses

Immediately after chlorophyll fluorescence measurements (at pre-dawn 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 and Abadía (1993) with modifications as described in Larbi et al. (2004).

2.8. Statistical analysis

Statistical analyses of the data were performed with the SPSS 12.0 software package (SPSS, Chicago, IL). One-way ANOVAs, with the treatment as factors, were performed for the studied parameters. Differences between means were revealed by Duncan analyses ($P<0.05$).

3. Results

Soil water depletion from 100 to 50% SWC resulted in large decreases in leaf RWC and $\Psi$ (Table 1). $\text{RWC}_{PD}$ decreased from 91.4% in well-watered plants to approximately 50% under severe drought. Similar decreases in RWC were recorded in leaves sampled at midday. $\text{Ψ}_{PD}$ and $\text{Ψ}_{MD}$ also decreased as water stress increased, ranging from $-0.23$ to $-2.10$ MPa at pre-dawn 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 24 h after re-watering, reaching RWC values of 71.8 and 84.4% at pre-dawn and midday, respectively. Consequently, $\text{Ψ}_{PD}$ and $\text{Ψ}_{MD}$ also recovered to values of $-0.64$ and $-0.70$ MPa, respectively.

Net CO$_2$ assimilation rates were decreased by water stress (Fig. 1A). Plants responded to a 50% reduction in water availability by gradually decreasing their $A_N$ up to approximately a 30% of control plants. Re-watered plants recovered their photosynthetic capacity by 80%. 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. 1B). Only under severe water stress $R_D$ was significantly increased, but 24 h after re-watering plants recovered the initial rates.

Under ambient CO$_2$ concentrations, three factors can limit photosynthesis during drought stress development: stomatal and mesophyll conductances to CO$_2$ and carboxylation capacity (Flexas et al., 2004; Grassi and 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$_2$O m$^{-2}$ s$^{-1}$ (Fig. 2A). Moderate and severe water stress resulted in a further decrease of $g_s$ to values close to 0.05 mol H$_2$O m$^{-2}$ s$^{-1}$. By contrast, mesophyll conductance ($g_i$) and the carboxylation capacity ($V_{c,max}$) were not

<table>
<thead>
<tr>
<th>SWC (% control)</th>
<th>RWC$_{PD}$ (%)</th>
<th>RWC$_{MD}$ (%)</th>
<th>$\Psi_{PD}$ (MPa)</th>
<th>$\Psi_{MD}$ (MPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>100.0 ± 0.0 c</td>
<td>91.4 ± 0.4</td>
<td>76.1 ± 0.9 b</td>
<td>$-0.23 ± 0.01$ c</td>
</tr>
<tr>
<td>MiD</td>
<td>65.6 ± 2.6 b</td>
<td>80.3 ± 3.6 bc</td>
<td>74.5 ± 3.8 b</td>
<td>$-0.78 ± 0.33$ bc</td>
</tr>
<tr>
<td>MoD</td>
<td>58.4 ± 3.6 ab</td>
<td>73.2 ± 4.1 b</td>
<td>61.9 ± 4.0 a</td>
<td>$-1.33 ± 0.17$ b</td>
</tr>
<tr>
<td>SD</td>
<td>49.5 ± 3.6 a</td>
<td>49.8 ± 5.2 a</td>
<td>51.2 ± 5.5 a</td>
<td>$-2.10 ± 0.27$ a</td>
</tr>
<tr>
<td>R</td>
<td>90.6 ± 5.7 c</td>
<td>71.8 ± 3.6 b</td>
<td>84.4 ± 2.0 b</td>
<td>$-0.64 ± 0.08$ c</td>
</tr>
</tbody>
</table>

Values are given in means ± standard deviations. Different letters denote statistical differences by Duncan test ($P<0.05$) among treatments.
affected by mild water stress (Fig. 2B and C), but they were at moderate and severe water stress. Re-watering resulted in a total recovery of \( V_{c,max} \), but only a partial of \( g_s \) and \( g_i \) to 85 and 75%, respectively.

When using these data for a limitation analysis (Table 2), the outcome was that a 21% of the total photosynthetic limitation under mild water stress was due to stomatal limitation (\( S_L \)). Under moderate and severe water stress, mesophyll conductance (\( M_{CL} \)) 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. \( M_{CL} \) 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, total diffusional limitations (i.e. \( S_L \) plus \( M_{CL} \)) were larger than biochemical limitations, accounting from 60 to 100% of the total limitations during the entire experiment. After 24 h re-watering, a total photosynthesis limitation of 18% persisted. This was largely accounted for \( M_{CL} \) (13%) and much less for \( S_L \) (5%), with no contribution of \( B_L \) remaining (Table 2).

### Table 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)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total limitations</th>
<th>Stomatal limitation (( S_L ))</th>
<th>Mesophyll conductance limitation (( M_{CL} ))</th>
<th>Biochemical limitation (( B_L ))</th>
<th>Non-stomatal limitations (( N_{SL} ))</th>
<th>Diffusional limitations (( D_L ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>MiD</td>
<td>24</td>
<td>21</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>22</td>
</tr>
<tr>
<td>MoD</td>
<td>50</td>
<td>25</td>
<td>6</td>
<td>19</td>
<td>25</td>
<td>31</td>
</tr>
<tr>
<td>SD</td>
<td>69</td>
<td>21</td>
<td>25</td>
<td>23</td>
<td>48</td>
<td>46</td>
</tr>
<tr>
<td>R</td>
<td>17</td>
<td>5</td>
<td>13</td>
<td>-1</td>
<td>12</td>
<td>18</td>
</tr>
</tbody>
</table>

### Table 3

Chlorophyll and xanthophyll concentrations at mid-morning and de-epoxidation state at pre-dawn (DPS<sub>PD</sub>) and mid-morning (DPS<sub>MD</sub>), under different irrigation treatments: control (C), mild drought (MiD), moderate drought (MoD), severe drought (SD) and re-watering (R)

<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th>MiD</th>
<th>MoD</th>
<th>SD</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chl a + b</td>
<td>521 ± 46 b</td>
<td>407 ± 40 ab</td>
<td>442 ± 38 ab</td>
<td>395 ± 29 a</td>
<td>436 ± 31 ab</td>
</tr>
<tr>
<td>β-Carotene</td>
<td>85.3 ± 1.8 ab</td>
<td>88.3 ± 2.2 b</td>
<td>81.1 ± 2.5 a</td>
<td>86.4 ± 1.0 ab</td>
<td>87.2 ± 2.0 ab</td>
</tr>
<tr>
<td>Neoxanthin</td>
<td>30.0 ± 0.5 ab</td>
<td>31.2 ± 1.0 abc</td>
<td>34.2 ± 1.5 c</td>
<td>32.3 ± 0.6 bc</td>
<td>28.8 ± 1.1 a</td>
</tr>
<tr>
<td>Violaxanthin</td>
<td>45.7 ± 3.7 bc</td>
<td>48.2 ± 4.7 bc</td>
<td>40.2 ± 3.5 ab</td>
<td>33.2 ± 1.1 a</td>
<td>52.9 ± 4.1 c</td>
</tr>
<tr>
<td>Taraxanthin</td>
<td>2.3 ± 0.5 a</td>
<td>2.4 ± 0.5 a</td>
<td>2.0 ± 0.3 a</td>
<td>1.3 ± 0.3 a</td>
<td>2.3 ± 0.1 a</td>
</tr>
<tr>
<td>Antheraxanthin</td>
<td>6.6 ± 1.3 a</td>
<td>9.5 ± 0.3 ab</td>
<td>14.6 ± 1.9 c</td>
<td>13.4 ± 1.9 bc</td>
<td>7.7 ± 0.6 a</td>
</tr>
<tr>
<td>Lutein</td>
<td>113.2 ± 4.4 a</td>
<td>114.8 ± 3.6 a</td>
<td>117.5 ± 4.8 a</td>
<td>121.9 ± 2.9 a</td>
<td>111.8 ± 2.6 a</td>
</tr>
<tr>
<td>Zeaxanthin</td>
<td>0.9 ± 0.6 a</td>
<td>5.2 ± 1.6 b</td>
<td>11.0 ± 1.8 c</td>
<td>20.2 ± 1.0 d</td>
<td>3.2 ± 0.9 ab</td>
</tr>
<tr>
<td>Σ Xanthophylls</td>
<td>198.6 ± 7.2 a</td>
<td>211.4 ± 7.6 ab</td>
<td>219.5 ± 10.2 ab</td>
<td>222.4 ± 4.8 b</td>
<td>206.8 ± 4.4 ab</td>
</tr>
<tr>
<td>VAZ</td>
<td>53.2 ± 3.4 a</td>
<td>62.9 ± 5.1 a</td>
<td>65.8 ± 4.7 ab</td>
<td>66.8 ± 1.9 b</td>
<td>63.8 ± 3.6 ab</td>
</tr>
<tr>
<td>Z + A</td>
<td>7.5 ± 0.7 a</td>
<td>14.7 ± 1.8 b</td>
<td>25.6 ± 3.2 c</td>
<td>33.6 ± 1.4 d</td>
<td>10.9 ± 1.2 ab</td>
</tr>
<tr>
<td>DPS&lt;sub&gt;PD&lt;/sub&gt;</td>
<td>0.07 ± 0.01 b</td>
<td>0.05 ± 0.01 ab</td>
<td>0.04 ± 0.01 a</td>
<td>0.06 ± 0.01 ab</td>
<td>0.04 ± 0.00 a</td>
</tr>
<tr>
<td>DPS&lt;sub&gt;MD&lt;/sub&gt;</td>
<td>0.08 ± 0.01 a</td>
<td>0.16 ± 0.03 b</td>
<td>0.28 ± 0.03 c</td>
<td>0.40 ± 0.01 d</td>
<td>0.11 ± 0.02 ab</td>
</tr>
</tbody>
</table>

Units for chlorophyll are μmol m<sup>-2</sup> and for xanthophylls, mmol mol<sup>-1</sup> 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 = antheraxanthin and Z = zeaxanthin.
Fig. 2. (A) Stomatal conductance \( (g_s) \), (B) mesophyll conductance \( (g_i) \) and (C) maximum rate of carboxylation \( (V_{\text{c,max}}) \) under different irrigation treatments: control \( (C) \), mild drought \( (\text{MiD}) \), moderate drought \( (\text{MoD}) \), severe drought \( (\text{SD}) \) and re-watering \( (R) \). Values represent means ± standard deviations. Different letters denote statistical differences by Duncan test \( (P<0.05) \) among treatments.

Under water stress conditions, the light which is in excess of what can be used in photosynthesis increases, resulting in photoprotection and/or photoinhibition. Regarding photoprotection, \( \text{ETR}/A_g^* \) (an indicator of photorespiration, see Flexas and Medrano, 2002b) and NPQ (an indicator of thermal dissipation in PSII antennae, see Björkman and Demmig-Adams, 1994) both increased progressively during water stress develop-

Fig. 3. (A) Ratio of electron transport rate to gross \( \text{CO}_2 \) assimilation accounting for dark and photorespiration \( (\text{ETR}/A_g^*) \), (B) non-photochemical quenching (NPQ), and (C) maximum quantum yield of PSII measured at pre-dawn \( (F_v/F_m) \) under different irrigation treatments: control \( (C) \), mild drought \( (\text{MiD}) \), moderate drought \( (\text{MoD}) \), severe drought \( (\text{SD}) \) and re-watering \( (R) \). Values represent means ± standard deviations. Different letters denote statistical differences by Duncan test \( (P<0.05) \) among treatments.
ment (Fig. 3A and B). With 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. 3C).

Area-based total chlorophyll concentration differed among treatments (Table 3), being maximal under non-stressed conditions (521 $\mu$mol m$^{-2}$) and minimal under severe drought treatment (395 $\mu$mol m$^{-2}$). In contrast, $\beta$-carotene was unaffected by drought stress. The total pool of xanthophylls cycle pigments (VAX, 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 VAX 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 did not differ significantly ($P>0.05$) among treatments. 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. 4A). In contrast, DPS$_{MD}$ was highly correlated with NPQ ($r^2=0.845$, $P<0.01$) (Fig. 4B).

4. Discussion

4.1. 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 $\mu$mol 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 sclerophyll 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$_2$O m$^{-2}$ s$^{-1}$) nor to its biochemical capacity to fix CO$_2$ (a $V_{c,\text{max}}$ of ca. 150 $\mu$mol m$^{-2}$ s$^{-1}$ is a very large value for a C$_3$ species, see Wullschleger, 1993; Manter and Kerrigan, 2004). Rather, the low photosynthetic capacity seems to be related to its low mesophyll conductance to CO$_2$, which is remarkably less than 1/3 of $g_s$ (Fig. 2). As a consequence, the estimated CO$_2$ concentration at the Rubisco locus was as low as 91.8 $\mu$mol CO$_2$ mol$^{-1}$. A $g_s/g_l$ ratio below 1 is sometimes found in sclerophyllous species (Niinemets et al., 2005; Peña-Rojas et al., 2005; Galmés, unpublished) and conifers (De Lucía et al., 2003; Warren et al., 2004), i.e. in species with low SLA. This low $g_s/g_l$ ratio in a species with a high SLA adds another milestone to the increasing evidence that the importance of $g_l$ in setting the photosynthetic capacity cannot be neglected (Ethier and Livingston, 2004; Flexas et al., 2004; Manter and Kerrigan, 2004; Warren and 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 large enough 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 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 $\Psi$) as the severity of water stress increased (Table 1). At severe
stress, a very low leaf RWC (50%) was achieved, as compared with values found in other Mediterranean species (Lo Gullo and Salleo, 1988; Gulás 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 24 h 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 C₃ plants (Flexas and Medrano, 2002a; Flexas et al., 2004; Grassi and Magnani, 2005). In response to mild water stress, only gs declined while gᵢ and Vₑ,max were kept at control values. At moderate and severe drought stress, gs and Vₑ,max also declined (Fig. 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 2). The similarity of this pattern to that found in many C₃ 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 CO₂. This fact points out again to the importance of gs in controlling the photosynthetic capacity of plants. Understanding the molecular and physiological mechanisms underlying the observed gᵢ responses may be a research priority for the near future.

Regarding respiration, the other component of plant carbon balance, leaf respiration rates measured in the dark (Rₑ) were quite low in this species (Fig. 1B). Moreover, Rₑ in L. minoricensis was maintained within a narrow range (0.35–0.50 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 Rₑ 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 Rₑ responses, even under severe drought the rates of Rₑ were only 15% of photosynthetic rates. While leaf Rₑ 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.

4.2. Photoprotection responses to water stress

Under water stress conditions, the light in excess 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 and 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 results in important decreases in light absorption by the leaves (Valladares and Pugnaire, 1999). On the other hand, leaves of L. minoricensis showed an early decrease in chlorophyll content in response to mild drought (Table 3), far before any change in the maximum photosynthetic capacity was observed (Fig. 3). In the Mediterranean semi-deciduous shrub Phlomis fruticosa, a chlorophyll loss during summer which is not accompanied by decreased photosynthetic capacity has been suggested as a means of reducing leaf absorbance, therefore contributing to photoprotection (Kyparissis et al., 1995).

In addition to possible reductions in leaf absorbance, increased photosynthesis and thermal dissipation are known to be the major photoprotective responses to avoid photoinhibition under water stress (Flexas and Medrano, 2002b). ETR/Aₑ^ₑ (an indicator of photorespiration, see Flexas and Medrano, 2002b) and NPQ (an indicator of thermal dissipation in PSII antennae, see Björkman and Demmig-Adams, 1994) both increased progressively during water stress development (Fig. 3A and B). As previously described for other Mediterranean species (García-Plazaola et al., 1997; Faria et al., 1998; Martínez-Ferri et al., 2000; Gulás et al., 2002), there was a high correlation between DPS₁ and NPQ (Fig. 4B), suggesting a tight regulation of the xanthophylls cycle in response to drought-induced excess light, resulting in safe energy dissipation (Demmig-Adams and Adams, 1996; Demmig-Adams, 1998).

As a consequence of the activation of these photoprotective mechanisms, while AN was progressively reduced by ca. 70% during water stress, the maximum photochemical efficiency of PSII (Fᵥ/Fₑ) was only depressed by 5% under the most severe water stress situation, which fully recovered after re-watering (Fig. 3C). The fact that this decrease in Fᵥ/Fₑ did not correlate with DPS₁ during the night (Fig. 4A) resulted 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 allows the reduction of photodamage to a minimum, and restricted to severe drought, and therefore helps to preserve the integrity of the photosynthetic apparatus. This may be important for the rapid photosynthetic recovery observed after re-watering.

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