Stomatal and non-stomatal limitations of bell pepper (Capsicum annuum L.) plants under water stress and re-watering: Delayed restoration of photosynthesis during recovery

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A R T I C L E   I N F O
Article history:
Received 29 August 2013
Received in revised form 8 October 2013
Accepted 20 October 2013

Keywords:
Drought stress
Gas exchange
Water relations
Prompt fluorescence
Photosystem I
Photosystem II

A B S T R A C T
Low soil water availability is the major environmental factor limiting plant growth and yield. The objective of this study was to elucidate the mechanisms underlying photosynthesis inhibition during water stress and recovery in Capsicum annuum L. cv. Cannon by evaluating soil and plant water relations, gas exchange and the prompt fluorescence rise OJIP. The soil (Ψs) and leaf (Ψl) water potential decreased from −0.16 and −0.53 to −1.1 and −1.7 MPa, respectively, and recovered after re-watering. The stomatal conductance (gs) decreased to 114 and 13 mmol m−2 s−1 under moderate and severe water stress, respectively. Similarly, the CO2 assimilation (A) and transpiration (Tr) rates decreased during water stress but recovered after re-watering. During severe water stress, photosynthesis decreased due to stomatal closure and to both slower maximum carboxylation rate (Vcmax) and ribulose 1,5-bisphosphate (RuBP) regeneration capacity mediated by maximum electron transport rate (I) in fact, the fluorescence parameters reflecting the electron flow from the inter-system carriers to final reduction of photosystem I (PSI) end electron acceptors declined throughout water deficit development. In conclusion, water stress mainly damaged the electron transfer from the plastoquinone (PQ) pool to the PSI terminal acceptors; this, along with constraints to both stomatal and non-stomatal components of photosynthesis, limited carbon assimilation. Photosynthesis recovery after re-watering was mainly restricted by both stomatal conductance and the gradual recovery of the electron transport chain. Finally, JIP-test parameters that quantifying electron transfer from the PQ pool to the PSI end acceptors are effective for monitoring water stress in crop plants.

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1. Introduction

The majority of climate change scenarios predict an increase in drought incidence throughout different regions of the world (IPCC, 2007). Because agricultural activities are water intensive, the increase in arid and semi-arid cropland, along with increases in population, will produce greater water demands and exploitation that will directly affect crop growth, survival and yield (Chaves et al., 2009). The limitation of plant growth due to low water availability is mainly due to reductions of plant carbon balance, which is largely dependent on photosynthesis (Flexas et al., 2009). However, there is an on-going debate about whether the restrictive factor for photosynthesis during water stress is stomatal closure and diffusional resistance or metabolic uncoupling (Flexas and Medrano, 2002; Lawlor and Cornic, 2002; Flexas et al., 2009; Pinheiro and Chaves, 2011). A decrease in the diffusion of atmospheric CO2 to
the carboxylation site in the leaves is generally considered the main cause of the decrease in photosynthesis under water stress conditions (Flexas et al., 2004a; Grassi and Magnani, 2005; Chaves et al., 2009). Moreover, when a decrease in the stomatal conductance is combined with sustained irradiance, the leaves are subjected to an excess of incident energy relative to the intercellular availability of CO₂ because the reducing power production rate exceeds its consumption rate during the Calvin cycle (Pinheiro and Chaves, 2011). Under these circumstances, down-regulation and even photo-inhibition of photosynthesis can become a potent defence mechanism in C₃ plants. This protection can be afforded by thermoregulated dissipation that occurs in the light harvesting complexes and involves the xanthophyll (Demming-Adams et al., 2006) and lutein cycles (García-Plazaola et al., 2003). These photoprotection mechanisms compete with photochemistry for the absorbed energy, causing down-regulation of photosynthesis, as revealed by the decreased quantum yield of photosystem II (PSII) (Genty et al., 1989). However, reports regarding the effects of water stress on the functionality of PSII are contradictory, and the exact site and the mechanisms for the PSII damage have not yet been elucidated (Georgieva et al., 2005; Sperdouli and Moustakas, 2012). Several in vivo studies have shown that water stress damages the PSII oxygen-evolving core complexes and reaction centers (Scotnica et al., 2000; Comol and Vazana, 2003), while other studies have shown that PSII is not affected at all or is only affected under severe water stress conditions (Massacci et al., 2008; Flexas et al., 2009).

In addition to the plant metabolism response, the carbon balance during water stress and recovery can depend on the velocity and the development of the photosynthetic recovery because the latter depends on the degree and rate of decrease in photosynthesis during water deficit (Flexas et al., 2006). In general, plants subjected to severe water stress exhibit 40–60% of their maximum rate of photosynthesis 1 day after irrigation, and this recovery continues during the following days. However, the maximum photosynthesis rate is not always restored (Sofo et al., 2004; Flexas et al., 2009). The influence of water stress severity on the speed and degree of recovery has been demonstrated in Phaseolus vulgaris (Miyashita et al., 2005) and in Vitis hybrid Richter-110 (Flexas et al., 2009). However, photosynthetic recovery depends not only on the stress severity and the species studied but also on the complex interactions with the plant, leaf age, light intensity and water deficit cycles, among others (Flexas et al., 2004b).

In Mexico, approximately 5800 hectares are planted with bell pepper (Capsicum annum L.), with a production of up to 50 t ha⁻¹ year⁻¹ (Reséndez-Melgar et al., 2010). In 2006, 240,000 tons of bell pepper were exported to the United States and Canada alone (Castellanos and Borbón, 2009), making this a profitable crop. Moreover, bell pepper adds not only flavor and aroma but also medicinal value to foods; it is an excellent source of vitamins A and C, with a low caloric content (Villalón, 1981; Andrews, 1995). However, this crop is especially sensitive to soil water deficit (González-Dugo et al., 2007), and drought stress during the initial developmental stages can reduce the size and number of buds and fruits (Rylski and Spigelman, 1982). Currently, little is known about the physiological limitations of photosynthesis during the acclimation and development of water stress and the recovery after re-watering, but this information is essential to improve the understanding of the plant response to drought and to develop appropriate irrigation practices. Additionally, it would be useful to evaluate the relative importance of the distinct processes that limit photosynthesis at different water stress levels to add to the on-going debate regarding stomatal vs. non-stomatal limitations.

The objective of the present study was to quantify the responses of the stomatal, biochemical and photochemical factors involved in photosynthesis regulation to water deficit and recovery after re-watering in C. annum plants, to better characterize the metabolic changes in the leaves during these processes. We hypothesized that (i) when the excitation pressure exceeds the CO₂ assimilation capacity during water stress it will cause a general decline of the photosynthetic plant performance, with possible damages to PSII functionality, electron transport chain and carboxylation; and (ii) even though the CO₂ assimilation rates are re-established after re-watering, some of the electron transport limitations will persist.

2. Materials and methods

2.1. Plant material and treatments

Seeds of C. annum cv. Cannon (Zeraim Gedera Ltd., Israel) were germinated in germination trays using peat moss as a substrate. Twenty-seven-day-old seedlings were transferred to 250 mL containers filled with soil and grown in a semi-automatic growth chamber (Thermo Scientific, USA) with a 12 h photoperiod and 382 μmol photons m⁻² s⁻¹ of photosynthetically active radiation (PAR) at the plant level. The plants were grown at the Colegio de Postgraduados, México. A nutrient solution (Ultratrol™ Multipurpose 18-18-18 with micro-elements; Chilean Chemical and Mining Society S.A., Chile) was applied directly in the irrigation water. At 23 days after transplanting (DAT), the plants were assigned randomly to two different groups. In the control group, the volumetric water content (θv) was kept between 45% and 30%; in the other group, water was withheld until the θv fell to 20% (medium stress) and 5% (severe stress). Afterwards, the treated plants were watered to field capacity, and their recovery was evaluated.

2.2. Soil and leaf water status

During the experiment, the soil volumetric water content (θv) was measured on a daily basis within the first 8 cm from the substrate surface using a time-domain reflectometer (HH2 hand-held moisture meter meter adapted with a WET sensor; Delta-T Devices, England) for 6 plants for each treatment. At the same time, the soil water potential (ψₛ) values of the different moisture levels were obtained from psychrometer readings from a C-52 sample chamber (Wescor Inc., Utah, USA). To determine the water status of the leaf tissue, 0.5 cm diameter leaf discs were placed into C-52 psychrometric chambers (Wescor Inc., Utah, USA) and equilibrated for 2 h at a constant temperature, with 6 replicates per treatment. Both leaf (ψₛ) and ψₛ were measured using the dew point technique with a HR 33 Dew Point Microvoltmeter (Wescor Inc., Utah, USA).

2.3. Gas exchange and A-C₃ curves

Carbon dioxide (CO₂) assimilation rate (A) in response to the increase of internal CO₂ (Cᵢ) in the leaf were measured in leaves from 5 plants per treatment using an open portable infrared gas analyser system (Ciras-1, PP Systems, UK) and a Parkinson automatic leaf chamber (PLC-B, PP Systems, UK) set at a PAR of 1050 μmol photons m⁻² s⁻¹ at a leaf temperature of 25 °C and relative humidity of 70%. The A-Cᵢ response curves were generated using the protocol described by Long and Bernacchi (2003). The leaf chamber was initially set at a CO₂ concentration of 370 μmol mol⁻¹ for 5 min to ensure the activation of ribulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco) under steady-state conditions. Subsequently, the A-Cᵢ curves were constructed by recording the response of A to different CO₂ concentrations in the chamber (Cᵢ). The CO₂ concentration inside the chamber decreased to 300, 250, 200, 150, 100 and 50 μmol mol⁻¹. Subsequently, 370 μmol mol⁻¹
CO₂ was re-established to verify that the original assimilation was recovered; if that was the case, C₉ was increased stepwise to 450, 550, 650, 800, 1000, and 1200 μmol mol⁻¹. The A-Ci curves were used to estimate the mesophyll conductance (gₘₑₜ), the velocity of the maximum carboxylation of Rubisco (Vₚmax), the electron transport rate (Jₑₚₚₚ), and the CO₂ release rate (Rₑ), using a utility developed by Sharkey et al. (2007) based on an alternative A-Ci curve fitting method (Ethier and Livingston, 2004) that accounts for CO₂ conductance transfer through a non-rectangular hyperbola version of the model of Farquhar et al. (1980). The chloroplast CO₂ concentration (Cₚ) is estimated using the equation [Cₚ] = (A/[(Δgₘₑₜ))] and a reasonable estimate of gₘₑₜ can be made directly from A-Ci data (Sharkey et al., 2007).

2.4. Measurement of leaf JIP transients

The chlorophyll a fluorescence transients were performed using a Plant Efficiency Analyzer (PEA, Hansatech Instruments Ltd., UK) on young fully expanded leaves at room temperature. Leaves were dark adapted for 1 h, and then exposed to a saturating red light pulse (650 nm, 3000 μmol m⁻² s⁻¹) provided by the PEA through an array of six light-emitting diodes. The fluorescence signal was recorded for 1 s, at an acquisition rate of 10 μs for the first 2 ms, and every 1 ms thereafter (Strasser et al., 1995).

The chlorophyll a fluorescence transients were analyzed according to the JIP-test (Strasser et al., 2004, 2010). The following data from the original measurements were used: fluorescence intensity at 50 μs (considered as minimum fluorescence, F₀), 300 μs (F₃₀₀), required for calculation of the initial slope (M₁) of the relative variable fluorescence (V) kinetics, 2 ms (1-step, F₁), 30 ms (1-step, F₃) and P-step (considered as maximum fluorescence, F₉₉₉). The biophysical parameters derived from the OJIP transients were calculated, and the following parameters, which refer to time 0 (onset of fluorescence induction) were used: (a) flux ratios or yields; i.e. maximum quantum yield of primary photochemistry (φp = -TR₀/ABS = F₁/F₉₉₉), quantum yield for electron transport (φe = ET₀/ABS) and quantum yield for the reduction of end acceptors of PSI per photon absorbed (φe = ET₀/ABS); (b) the efficiency with which a trapped exciton can move an electron into the electron transport chain further than Q₋ (φQ₋ = ET₀/FR₀), and the efficiency with which an electron can move from the reduced intersystem electron acceptors to the PSI end electron acceptors (φQ₀ = RE₀/ETO); (c) the fraction of PSI II Chl a molecules that function as reaction centers (RC/ABS); (d) the performance index on an absorption basis (PLₐ₃ₓ) which measures the potential for energy conservation from photons absorbed by PSI to the reduction of the intersystem electron acceptors (PLₐ₃ₓ = (RC/ABS) × (φQ₋/1 - φQ₋)) × (φQ₀/1 - φQ₀)); and (e) the performance index total (PLₐ₃ₓ) which describes the potential for energy conservation from photons absorbed by PSII to the reduction of PSI end acceptors (PLₐ₃ₓ = (PLₐ₃ₓ) × (φQ₀/1 - δQ₀)). The formulae showing how each of the above-mentioned biophysical parameters can be calculated from the original fluorescence measurements have been described previously in detail (Yusuf et al., 2010; Strasser et al., 2010). Additionally, an extended OJIP trace study was performed by estimating fluorescence transients within the individual exchange rate range (i.e., from F₀, measured at 50 μs, to F₉₉₉, measured at 2 ms) and visualizing the L band (between 100 and 200 μs) and the K band (between 200 and 400 μs) by subtracting or estimating the differences between the fluorescence traces for the control and the treated samples (Strasser et al., 2004; Yusuf et al., 2010). Also, the width of the bands presented graphically were calculated as follow (Desotgi et al., 2013): V₀₉₉₉ = (F₃₀₀ - F₀)/(F₀ - F₉₉₉) and V₀₃₀₀ = (F₃₀₀ - F₀)/(F₀ - F₂₀₀) for the L and K band, respectively.

Fig. 1. Effects of the irrigation interruption regime on the soil water potential (A) and leaf water potential (B) in C. annum plants. Data points and error bars represent the means and corresponding ±SE (n = 6).

2.5. Experimental design and statistical analysis

This study was performed using a completely randomized design, and the data were subjected to repeated measures analysis of variance (ANOVA) using the PROC MIXED procedure in SAS (SAS 9.1, SAS Institute Inc., NC) and Tukey’s mean separation test with α = 0.05. Normality and variance homogeneity tests were conducted prior to the analysis of variance using the Anderson–Darling and Levene tests, respectively; for cases in which the hypothesis was rejected, the data set was transformed using the Johnson transformation (Chou et al., 1998).

3. Results

3.1. Soil and plant water status

At the beginning of the water deficit treatment (Fig. 1A), the soil water potential (Ψₛ) was approximately zero; it decreased to −1.1 MPa with the development of the water deficit and remained, on average, at −0.19 MPa in the control. After re-watering and during the recovery period, the Ψₛ reached values similar to those of the control. On the other hand, the leaf water potential (Ψₗ) in the control plants remained, on average, at −0.61 MPa throughout the duration of the experiment (Fig. 1B). In the stressed plants, Ψₗ decreased to −1.7 MPa on day 10 and reached control levels after re-watering.

3.2. Gas exchange responses during water stress and recovery

The water stress caused a significant reduction in A, gₛ, and Tr after 4 and 9 days of water deficit (Fig. 2). The A values of the leaves in the stressed plants were 65% and 11% of that of the control plants after 4 and 9 days, respectively. The gₛ value decreased to 60% and 95% compared to control plants, while the Tr was reduced by 46%.
and 90% during the same experimental period. The gas exchange variables gradually recovered during the first 2 days of irrigation (Fig. 2), with different degrees of recovery observed on days 1 and 2. Leaf A was restored to 54% and 93% of the control values on days 1 and 2, respectively, after re-watering: the $g_s$ value recovered to 29% and 64% and the $Tr$ value recovered to 36% and 77% of the level in the control plants.

3.3. $CO_2$ assimilation rate in response to $CO_2$ concentration at the chloroplast during water stress and recovery

Changes in $A$ as a function of increased $CO_2$ concentrations at the chloroplast were used to determine the biochemical limitations of photosynthesis under stress conditions and after re-watering. Water stress reduced the initial portion of the $A$ vs. $C_i$ curves, as well as $A$ at the highest concentrations of $C_i$ (Fig. 3). The values of $V_{\text{max}}$, $J_{\text{max}}$ and $R_3$ are presented in Table 1. No significant reduction in $V_{\text{max}}$ was observed after 4 days of stress ($\Psi_L$ equal to $-0.85 \text{ MPa}$). However, at day 9 ($\Psi_L$ equal to $-1.24 \text{ MPa}$), $V_{\text{max}}$ was reduced by 52% compared to the control plants. The values of $J_{\text{max}}$ decreased 36% and 57% compared to the control at days 4 and 9, respectively. The $R_3$ variable was significantly higher than that of control plants after 9 days of water stress. After re-watering, $V_{\text{max}}$ and $J_{\text{max}}$ gradually increased to levels equivalent to those of the control plants and even surpassed the controls after day 2 (Table 1). Similarly, the $R_3$ decreased to values similar to those of control plants on day 2 after irrigation (Table 1).

3.4. Changes in chlorophyll $a$ fluorescence during water stress and recovery

The chlorophyll $a$ fluorescence transients showed that the progressive increase of water stress decreased the amplitude of the OJIP curves, mainly that at the P level ($F_P$), with a $\Psi_L$ value of

![Figure 2](image-url)  
Fig. 2. Effect of water stress and recovery on gas exchange variables. (A) $CO_2$ assimilation rate; (B) stomatal conductance; and (C) transpiration rate. The data points and error bars represent the mean ± SE ($n = 10$).

![Figure 3](image-url)  
Fig. 3. Net $CO_2$ assimilation rate ($A$) relative to the internal partial pressure of $CO_2$ in the chloroplast ($C_i$) in $C. anum$ leaves at three water stress levels defined by the leaf water potential: Control at $-0.45 \text{ MPa}$ (closed circles), $-0.85 \text{ MPa}$ (open circles), and $-1.24 \text{ MPa}$ (closed triangles). Data shown are five replicates for each water potential ($n = 5$).

### Table 1

<table>
<thead>
<tr>
<th>Variable</th>
<th>$\Psi_L$ (MPa)</th>
<th>$V_{\text{max}}$ ((\mu\text{mol m}^{-2} \text{s}^{-1}))</th>
<th>$J_{\text{max}}$ ((\mu\text{mol m}^{-2} \text{s}^{-1}))</th>
<th>$R_3$ ((\mu\text{mol m}^{-2} \text{s}^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatments after water withheld</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>$-0.45$</td>
<td>$40.3 ± 3.1ab$</td>
<td>$67.4 ± 3.6ab$</td>
<td>$0.6 ± 0.3bc$</td>
</tr>
<tr>
<td>Stress (4)</td>
<td>$-0.85$</td>
<td>$27.0 ± 2.3b$</td>
<td>$43.1 ± 5.0cde$</td>
<td>$0.4 ± 0.1c$</td>
</tr>
<tr>
<td>Control</td>
<td>$-0.54$</td>
<td>$51.5 ± 15.6a$</td>
<td>$65.8 ± 13.4ab$</td>
<td>$0.7 ± 0.3bc$</td>
</tr>
<tr>
<td>Stress (9)</td>
<td>$-1.24$</td>
<td>$24.6 ± 7.0b$</td>
<td>$28.1 ± 8.1e$</td>
<td>$2.2 ± 0.7a$</td>
</tr>
<tr>
<td>Treatments after re-watering</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>$-0.58$</td>
<td>$32.4 ± 4.2ab$</td>
<td>$55.4 ± 8.0abc$</td>
<td>$0.2 ± 0.1c$</td>
</tr>
<tr>
<td>Day (1)</td>
<td>$-1.08$</td>
<td>$23.4 ± 10.4b$</td>
<td>$45.2 ± 9.8bcd$</td>
<td>$1.5 ± 0.5ab$</td>
</tr>
<tr>
<td>Control</td>
<td>$-0.54$</td>
<td>$33.4 ± 3.1ab$</td>
<td>$57.9 ± 6.2abc$</td>
<td>$0.2 ± 0.1c$</td>
</tr>
<tr>
<td>Day (2)</td>
<td>$-0.67$</td>
<td>$48.4 ± 4.5ab$</td>
<td>$78.7 ± 8.2a$</td>
<td>$0.6 ± 0.2bc$</td>
</tr>
</tbody>
</table>

Different letters within the same column represent significant differences at $P < 0.05$ by Tukey test. Values are means with ±SE, $n = 5$. 
Table 2

Leaf water potential ($\Psi_w$) and JIP-test parameters of dark-adapted leaves of C. annuum plants under water stress and after re-watering. For parameter description, see Section 2.4. Numbers in brackets on the first part show elapsed days after stress imposition whereas in the second part indicates elapsed days after re-watering.

<table>
<thead>
<tr>
<th>Variable</th>
<th>$\Psi_w$</th>
<th>TR0/ABS</th>
<th>ET0/ABS</th>
<th>ET0/ABS</th>
<th>ET0/ETR</th>
<th>BC/ABS</th>
<th>Pm</th>
<th>Pnmax</th>
<th>L-bend</th>
<th>K-bend</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.57</td>
<td>0.30</td>
<td>0.00</td>
<td>0.53</td>
<td>0.00</td>
<td>0.19</td>
<td>0.635</td>
<td>0.21</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Stress (1)</td>
<td>0.00</td>
<td>0.53</td>
<td>0.00</td>
<td>0.50</td>
<td>0.00</td>
<td>0.19</td>
<td>0.635</td>
<td>0.21</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Stress (2)</td>
<td>0.00</td>
<td>0.53</td>
<td>0.00</td>
<td>0.50</td>
<td>0.00</td>
<td>0.19</td>
<td>0.635</td>
<td>0.21</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Stress (3)</td>
<td>0.00</td>
<td>0.53</td>
<td>0.00</td>
<td>0.50</td>
<td>0.00</td>
<td>0.19</td>
<td>0.635</td>
<td>0.21</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Stress (4)</td>
<td>0.00</td>
<td>0.53</td>
<td>0.00</td>
<td>0.50</td>
<td>0.00</td>
<td>0.19</td>
<td>0.635</td>
<td>0.21</td>
<td>0.00</td>
<td>0.00</td>
</tr>
</tbody>
</table>

Quantities are expressed as dimensionless ratios (Pm and Pnmax is expressed as arbitrary units) except $\Psi_w$ expressed in MPa. Different letters within the same column represent significant differences at $P < 0.05$ by Tukey test. Values are means with ±SE, n = 8.
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(2002). The decrease in Vmax values may result from a reduction in the amount of active Rubisco (Peña-Rojas et al., 2004). The reduction in Jmax is associated with a limited regeneration of RuBP due to an inadequate supply of ATP or NADPH or to a low enzymatic activity during the photosynthetic carbon reduction cycle, such as the sedoheptulose-1,7-biphosphatase and fructose-1,6-biphosphatase (Flexas et al., 2004b; Lawlor, 2002; Parry et al., 2002). The results also indicate that plants under severe water stress exhibited a greater R0 (Table 1) and therefore can contribute with more CO2 to that needed during photosynthesis, maintaining a positive carbon balance behind closed stomata (Hu et al., 2010). The above suggests that stomatal limitations in A were crucial during the initial stress stages, while non-stomatal mechanisms become limiting over prolonged periods of time. This response pattern is consistent with that described by Hu et al. (2010) under controlled experimental conditions. However, the reduction of A in stressed plants may also be the result of different effects on the efficiency of light capture and on the photosynthetic electron transfer system (Tezara et al., 2003).

4.2. Chlorophyll a fluorescence and leaf photochemistry

In the present study, the fluorescence induction trace shape was sensitive to the development of water stress in the leaf tissue of the plants (Fig. 4). The analysis of several parameters of the JIP-test allowed us to locate the modifications induced by water stress on the electron transport chain (Table 2). The redox reactions were strongly affected by water stress; mainly the quantum yields of the reactions after QA− were less tolerant to water deficit (Table 2). In fact, the three JIP-parameters which reflect the quantum yields of photo-induced electron transfer from P680 to QA (ψp0 = TR0/ABS), from QA− to PQ (ψp0 = ET0/ABS) and from PQ to the PSI electron acceptors (ψp0 = RE0/ABS), can be ranked according to their sensitivity to water stress in the sequence ψp0 > ψp0 > ψp0. Moreover, the efficiency or probability of electron transport beyond QA (ψp0 = ET0/TR0) and that of the intersystem electron carriers will reduce end electron acceptors at the PSI acceptor side (ψp0 = RE0/ET0) also decreased in plants under water stress (Table 2). The reduction of ψp0 and ψp0 along with the maintenance of ψp0 indicate that the water deficit inhibits not the primary light reactions but rather the redox reactions that follow QA− (Chen et al., 2011). This indicates that the water deficit causes the slowdown of the electron transfer and the reduction of end electron acceptors at the PSI acceptor side (Chen et al., 2011). Under normal conditions, the transfer of energy to the reaction centers of PSI and PSII results in the transport of the electron to ferredoxin (Fd) and the reduction of NADP+. However, as A decreases gradually with the water deficit, the consumption of NADPH decreases as well, causing a decrease in the linear electron transport as the sinking capacity is reduced (Lawlor and Tezara, 2009). This leads to an impairment of the electron transport and a decline in the requirement of the reduction of end electron acceptors such as NADP+ and Fd, that in turn limits

−1.72 MPa, while F0 (step O) remained unchanged (Fig. 4). To further investigate the mechanisms underlying the observed changes, the JIP-test was applied to the fluorescence induction transients. The effects of water stress on several parameters of the JIP-test are presented in Table 2. The parameters reflecting the quantum yields for electron transport in PSI and between both photosystems (RE0/ABS and ET0/ABS, respectively), were sensitive to water stress and decreased as the Vt became more negative. In contrast, water stress had a little effect on the maximum quantum yield of the primary photochemistry of PSII (TR0/ABS = F0/Fo), as confirmed by the high stability of this parameter during water deficit (Table 2). Similarly, the composed parameters PABS and Ptotal changed significantly during water stress (Table 2). The rewatering of C. annuum plants at the end of the stress treatment gradually restored the Vt (Fig. 1). With the complete rehydration of leaves, all the fluorescence signals were recovered, i.e., the OJIP transient both as amplitude and shape, and hence all the JIP-test parameters (Table 2).

Additionally, the fluorescence traces of Chl a of the different treatments and the control samples were double normalized at F0 (0.05 ms) and Fk (0.3 ms), and expressed as W0k = (Fk − F0)/(Fk − Fo). Subsequently, the traces of the control samples were subtracted from the traces of the treated leaves (ΔW0k). This difference makes the L band visible and its response was distinct at the different Vt and after re-watering (Fig. 5A and B), showing a high amplitude in days 1–4 after re-watering (Table 2), and similar to control on days 7 and 10 (Table 2). In Fig. 5C and D, the Chl a fluorescence transients were double normalized between F0 and F1 (=1 ms), and expressed as W01 = (F1 − F0)/(F1 − Fo), and the difference among different levels of stress and the control were determined (ΔW01). This allowed the visualization of the K band with a peak between 0.25 and 0.30 ms. An increasing positive deviation was also observed, indicating that the K band becomes more pronounced with more severe water stress (Fig. 5C). This limitation lasted from days 1 to 4 after re-watering (Table 2 and Fig. 5D).

4. Discussion

4.1. CO2 assimilation, biochemical and stomatal limitations to photosynthesis

In plants under water stress, a rapid increase in A after re-watering indicates that the biochemical mechanisms were not affected by water deficit, which suggests that the decrease in A is the result of stomatal closure (Cornic, 2000). The results showed that gS did not fully recover after 2 days of deficit irrigation, despite the almost complete recovery of A (Fig. 2). Under stress conditions, a reduction in stomatal conductance can have protective effects because it allows the plant to save water and to improve the water use efficiency (Chaves et al., 2009). However, although stomatal closure appears to be the main limiting factor of A in C. annuum plants subjected to water deficit, non-stomatal factors were also important for the regulation of photosynthesis during severe water stress (Ψt > −1.24 MPa) (Fig. 3 and Table 1). In fact, the reduction of A at high Cc values (Fig. 3) was accompanied by reductions in Vmax and Jmax (Table 1), which indicates that a decrease in CO2 assimilation occurs to adjust the mesophyll capacity to the lower CO2 supply caused by stomatal closure (Chaves et al., 2002; Lawlor and Cornic, 2002).
Fig. 5. Change in the shape of the Chl a fluorescence transient curves under waters stress (panels A and C) and recovery (panels B and D) revealing the L-band and K-band. Each result is average of eight repetitions. Panels A and B: $W_{exc} = (F_{t} - F_{o}) / (F_{m} - F_{o})$; panels C and D: $W_{exc} = (F_{t} - F_{o}) / (F_{m} - F_{o})$. In panel A and C the difference kinetics $\Delta W_{exc} = W_{exc(treatment)} - W_{exc(control)}$ are shown where treatment is control $\Psi_{L}$ (dash line), −0.60 (closed circle), −0.77 (open circle), −1.27 (closed triangle), and −1.72 MPa (open triangle). In panels B and D the difference kinetics $\Delta W_{exc} = W_{exc(treatment)} - W_{exc(control)}$ are shown where treatment is control (dash line), day 1 (closed circle), day 2 (open circle), day 3 (closed triangle), day 4 (open triangle), day 7 (closed square) and day 10 (open square) after re-watering.

the synthesis of ATP and the regeneration of RuBP (Lin et al., 2009). The shortage of ATP causes the incomplete activation of Rubisco (Strueand and Portis, 1987), which partially explains why plants under water stress exhibit a lower $V_{c,max}$ (Table 1). Thus, the limitations in photosynthetic electron transport capacity, along with the ATP shortage, limit the regeneration of RuBP ($J_{max}$) (Table 1) and are the main non-stomatal factors that contribute to the decreased CO2 assimilation under water stress conditions.

The results showed that moderate $\Psi_{L}$ had little effect on the maximum quantum yield of primary photochemistry (TR0/ABS = $F_{v}/F_{m}$) (Table 2) which reflects the efficiency of the light reactions (Strasser et al., 2004), consistent with previous reports (Lu and Zhang, 1999). The same observation has been reported by other researchers regarding the use of the $F_{v}/F_{m}$ ratio to study the fluorescence in stressed plants (Oukarroum et al., 2007; Van Heerden et al., 2007). In contrast, a marked reduction in the performance index $\Pi_{ABS}$ was observed along with the sudden decrease of $\Psi_{L}$ in the leaf (Table 2). The reduction in $\Pi_{ABS}$ as a property of the antenna (RC/ABS) and to the decrease in the photosynthetic efficiency in the transport of photosynthetic electrons beyond $Q_{a}$ (ET0/TR0) (Table 2), which indicates a reduction in the efficiency of the redox reactions in the electron transport chain (Chen et al., 2011). The composite parameter $\Pi_{total}$ combines $\psi_{PSO}, \psi_{ETO}, \delta_{ETO}$, and RC/ABS and therefore summarizes all the partial conductive forces and their individual effects (Strasser et al., 2010). The $\Pi_{total}$ was notably reduced as the $\Psi_{L}$ value decreased due to the cessation of watering (Table 2); therefore, the reduction of this parameter during water deficit was mainly due to the added slowdown of the reduction of PSI final acceptors. This finding indicates that $\Pi_{total}$ is sufficiently sensitive to characterize, quantify and detect water stress, even prior to the appearance of visible symptoms on the leaves of C. amrum.

A more exhaustive analysis of the OJIP transients revealed more information about the effects of water stress on the photosynthetic units of C. amrum plants. The increase in the positive amplitude of the L band at $\Psi_{L} = −1.72$ MPa (Fig. 5A) and during the first 4 days of recovery (Table 2), implies that the PSI units are less grouped together, i.e., less energy is being exchanged among the independent PSI units (Yang et al., 2012), as a response to water stress. Grouping is sensitive to the unstacking of the thylakoid membranes, which can be induced by conditions such as high or low salinity (Strasser and Greppin, 1981; Strasser, 1981) and has been observed in Chlamydomonas reinhardtii in response to hyperosmotic stress (Cruz et al., 2001). The reduction in the amplitude of the L band during days 7 and 10 after re-watering (Fig. 5B) suggests that the grouping of the units of PSI is restored, resulting in a greater stability of the samples after stress (Yang et al., 2012). Moreover, the K step is exhibited in the fluorescence traces of several higher plants that are native to hot and dry climates i.e., Cyclus revoluta, Permelia sp. and Juniperus sp., as reported by Srivastava et al. (1997). It is normally hidden by the O–J increase because the limitation of the electron transport through PSI is rarely strong enough to render the K step visible. However, as shown in Fig. 5C, water stress induces the positive appearance of the K band, and its amplitude increases as the $\Psi_{L}$ value decreases (Table 2). The appearance of the K band is explained by an imbalance within PSI, among the electrons that leave the reaction centers in the electron acceptor side and the electrons donated by the donor side (Strasser, 1997),
and it has been related to a dissociation of the oxygen-evolving complex (OEC) (Guisard et al., 1995; De Ronde et al., 2004). We also observed the persistence of the K band during the first 4 days after re-watering and its disappearance after 10 days (Table 2), possibly co-occurring with the restoration and normal functioning of OEC.

4.3. Photosynthetic limitations during recovery

In contrast with the photosynthetic responses during the development of stress, the limitations after re-watering and recovery have received considerably less attention (Galmés et al., 2007a). Normally, the recovery of photosynthesis after a moderate water stress (g > 0.15 mol m⁻² s⁻¹) is rapid (1 d) and almost complete (Flexas et al., 2006). In contrast, after severe water stress, the recovery of photosynthesis is progressive, slow (can take several days to weeks) and usually incomplete (De Souza et al., 2004; Miyashita et al., 2005). Our results showed that gₚ remained below the values of the control plants 2 days after re-watering, but with similar CO₂ assimilation rates (Fig. 2), and that the Vₖ was slowly restored 10 days after re-watering (Fig. 1B). The limited recovery of hydraulic conductivity in the leaf is the apparent cause of the abatement of stomatal conductance after re-watering (Galmés et al., 2007b), and it was recently shown that the aquaporins play a determinant role in the regulation of hydraulic conductivity with the available hydraulic conductance in the leaves (Cochard et al., 2007). Metabolic damage and a corresponding restriction in the photosynthetic recovery of the leaves after irrigation is commonly assumed to occur (Flexas et al., 2004b); however, the values of Vₘₚ and Jₘₚ were similar to those of control plants 2 days after re-watering (Table 1), consistent with reports by Galmés et al. (2007a) in 10 Mediterranean species that exhibited excellent recovery and few biochemical limitations after severe water stress. In addition, the efficiency of the photosynthetic electron transport system (Table 2), the connectivity among the units of PSII (Fig. 5B) and the normal functioning of the donor side of PSII (Fig. 5D) recovered gradually after 10 days of re-watering. The recovery of such parameters suggests that the water stress did not irreversibly damage the light reactions and that the damage to the photosynthetic electron transport chain was gradually repaired during re-watering, promoting the recovery of photosynthesis.

5. Conclusion

In conclusion, the results indicate that photosynthesis in C. annuum plants during water stress is inhibited by stomatal closure, which limits the diffusion of CO₂ to the chloroplast and causes a reduction of the internal CO₂ concentration, leading to a decrease in the enzymatic activity of Rubisco. At the same time, water stress reduces the efficiency of photosynthetic electron transport by uncoupling the electron transport chain mainly from the PSI acceptor side to the PSI end electron acceptors, limiting the regeneration of RuBP and the CO₂ assimilation rate. Importantly, the decreased electron transfer efficiency from the plastocyanine pool to the PSI terminal acceptors showed to be a key regulatory mechanism underlying the photochemical limitations of C. annuum plants under water stress. These results emphasize that, early under water stress, the diffusive limitations are the main factor for the observed depression of photosynthesis and the photosynthetic metabolism is progressively down-regulated as water stress intensifies, whereas during recovery, photosynthesis is limited by stomatal closure and the gradual recovery of the electron transport chain activity. Our results also show that the JIP-test parameters (Pₑ/ABS, REₑ/ET₀ and Pₑ/total) referring to the electron flow from the reduced inter-system electron acceptors to the PSI end-electron acceptors may represent good parameters for monitoring drought stress effects on photosynthesis.

Acknowledgement

This work was supported by a scholarship (166340) from Consejo Nacional de Ciencia y Tecnología (CONACyT), Mexico.

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