DOI: 10.1007/s11099-011-0001-7 PHOTOSYNTHETICA 49 (1): 3-12, 2011

Influence of enhanced temperature on photosynthesis, photooxidative damage, and antioxidant strategies in *Ceratonia siliqua* L. seedlings subjected to water deficit and rewatering

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Abstract

Predicted future climatic changes for the Mediterranean region give additional importance to the study of photooxidative stress in local economic species subjected to combined drought and high-temperature conditions. Under this context, the impact of these stresses on photosynthesis, energy partitioning, and membrane lipids, as well as the potential ability to attenuate oxidative damage, were investigated in *Ceratonia siliqua* L. Two thermal regimes (LT: 25/18ºC; HT: 32/21ºC) and three soil water conditions (control, water stress, and rewetting) were considered. HT exacerbated the adverse effects of water shortage on photosynthetic rates (P_N) and PSII function. The decrease in P_N was 33% at LT whereas at HT it was 84%. In spite of this, the electron transport rate (ETR) was not affected, which points to an increased allocation of reductants to sinks other than CO_2 assimilation. Under LT conditions, water stress had no significant effects on yield of PSII photochemistry (Φ_{PSII}) and yields of regulated (Φ_{NPQ}) and nonregulated (Φ_{NO}) energy dissipation. Conversely, drought induced a significant decrease of Φ_{PSII} and a concomitant increase of Φ_{NO} in HT plants, thereby favouring the overproduction of reactive oxygen species (ROS). Moreover, signs of lipid peroxidation damage were detected in HT plants, in which drought caused an increase of 40% in malondialdehyde (MDA) content. Concurrently, a marked increase in proline content was observed, while the activities of catalase (CAT) and ascorbate peroxidase (APX) were unaffected. Despite the generation of a moderate oxidative stress response, *C. siliqua* revealed a great capability for photosynthetic recovery 36 h after rewatering, which suggests that the species can cope with predicted climate change.

Additional key words: antioxidative protection; carob tree; energy partitioning; lipid peroxidation; proline.

Introduction

Water deficits and high temperature are major abiotic stress factors restricting plant growth and productivity in many regions, and they often occur simultaneously (Boyer 1982). Models of global climate change predict a further 1.8–4.0ºC warming until 2100 (Mc Arthy *et al*. 2001) and changing patterns of rainfall (Houghton *et al*. 2001) as a result of rising atmospheric carbon dioxide concentration (CO_2) and other greenhouse gases. Although the increase in CO_2 concentration can directly affect plant productivity, their indirect effects on climate can be more important in determining the response of plants than the enhancement of CO_2 *per se*. Therefore, the impact of climate change on temperature and rainfall patterns is of great importance in determining the future

Received 8 June 2010, *accepted* 20 August 2010.
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Abbreviations: APX – ascorbate peroxidase; C_i – substomatal CO_2 concentration; Cars – carotenoids; CAT – catalase; Chl – chlorophyll; ETR – apparent linear electron transport rate; F_v/F_m – maximum PSII photochemical efficiency; g_s – stomatal conductance; HT – 32/21ºC; LT – 25/18ºC; MDA – malondialdehyde; NPQ – nonphotochemical quenching; P_N – net photosynthetic rate; PPFD – photosynthetic photon flux density; PSII – photosystem II; ROS – reactive oxygen species; RWC – relative water content; WS – water-stressed plants; WW – well watered plants; Ψ_w – leaf water potential; Φ_{NO} – quantum yield of nonregulated energy dissipation of PSII; Φ_{NPQ} – quantum yield of regulated energy dissipation of PSII; Φ_{PSII} – actual PSII quantum yield.
Acknowledgements: This work was supported by a grant from Portuguese Foundation for Science and Technology (FCT) to MLO: SFRH/BPD/35410/2007.

response of tree crops to the new environmental conditions (Chartzoulakis and Psarras 2005). According to this climate-change scenario, water stress will be the most important factor limiting plant growth in the Mediterranean area, but the exposure of plants to warmer conditions can alter the photosynthetic response to water stress (Chaves *et al*. 2002). Stomatal limitation is generally accepted to be the main cause of reduced photosynthesis under water deficit, particularly if it is moderate, by leading to decreases in substomatal CO_2 concentration (Cornic 2000). This limitation to CO_2 assimilation may promote an imbalance between photochemical activity in photosystem II (PSII) and electron requirement for photosynthesis, thus leading to photodamage of PSII centers. Although being very resilient to drought (Petsas and Grammatikopoulos 2009), the primary events of photosynthesis (*e.g*. the electron transport capacity) proved very sensible to temperature stress (Berry and Björkman 1980). However, when heat and drought stresses co-occur, water stress exerts some of its effect through oxidative damage (Chaves *et al*. 2002). In this way, photoinhibition of photosynthesis and photodamage may be observed when these two stresses are imposed simultaneously, even at low light intensities. Photooxidative damage can be prevented by dissipation of excess excitation energy through carotenoids (Cars) or by detoxification of ROS (Lawlor 1995). If photochemical and nonphotochemical capacities are exceeded, the surplus of energy is transferred to O_2, and ROS are produced (Wilson *et al*. 2006). So, under water stress and high temperature the production of ROS, such as hydrogen peroxide (H_2O_2), superoxide ($O_2^{\bullet-}$) and hydroxyl (OH•) radicals and the singlet oxygen ($^1O_2$) may be exacerbated. Plants have developed enzymatic and nonenzymatic scavenging systems to quench active oxygen, and to eliminate the harmful effects of active oxygen. When the accumulation of ROS under water stress conditions exceeds the removing capacity of the antioxidant system, the effects of oxidative damage arise, including oxidation of cellular lipids and proteins, destruction of photosynthetic pigments and inactivation of photosynthetic enzymes (Smirnoff 1993, Yordanov *et al.* 2000). However, according to Ashraf and Foolad (2007) some osmolytes such as glycine betaine (GB) and proline contribute to stabilizing subcellular structures (*e.g*. membranes and proteins), scavenging free radicals, and buffering cellular redox potential under stress conditions. The concentration of MDA is used as indicator of oxidative stress as it is a by-product of lipid peroxidation. In addition, chlorophyll (Chl) fluorescence provides a rapid, efficient and nonintrusive tool to identify abiotic stress effects on the photosynthetic apparatus. A few years ago, based on a Stern–Volmer approach using a lake model, Kramer *et al*. (2004) developed new fluorescence parameters, able to provide a better understanding of the photosynthetic processes under stress conditions. In the Kramer model, Φ_{PSII}, Φ_{NPQ}, and Φ_{NO} in PSII satisfy the condition $\Phi_{PSII} + \Phi_{NPQ} + \Phi_{NO} = 1$. These quantum yields describe the energy partitioning in PSII and allow deep insights into the plant's capacity to cope with excess excitation energy. The Φ_{PSII} values indicate the fraction of absorbed quanta that are converted into chemical energy by the photochemical charge separation in PSII reaction centres. High Φ_{NPQ} values testify not only that a plant is dealing with an excessive photon flux density but also that it is trying to protect itself by a regulation process, in this case the dissipation of excessive excitation energy into harmless heat. Without such dissipation there would be formation of ROS, which cause irreversible damage. On the other hand, high Φ_{NO} values indicate that both photochemical energy conversion and protective regulatory mechanisms are inefficient, and plant has serious problems to cope with the incident radiation. Either it is already damaged or it will be photodamaged upon further irradiation.

C. siliqua is a Mediterranean sclerophyllous evergreen tree that behaves as a drought-avoider species with a water-spender strategy in field conditions (Lo Gullo and Salleo 1988). The research about the responses of *C. siliqua* to the typical stresses of the Mediterranean region assumes an increased importance in the context of the predicted future climatic changes. Although a few studies concerning the effects of high temperature, high light, or drought on gas exchanges and photosynthetic characteristics of the species have been conducted with potted plants or in field conditions (Nunes *et al*. 1992, Ramalho *et al*. 2000), there is still a considerable lack of knowledge about the interactive effects of these stressors and their impacts on the extent of leaf oxidative damage and the subsequent recovery after water-stress relief. In an attempt to fill this lacuna, the present work focuses on the link between oxidative stress and key processes like photochemistry and overall photosynthesis. To achieve this goal, we analysed the effect of gradual water availability decrease and subsequent rewatering in *C. siliqua* seedlings growing in two contrasting temperatures, through gas exchange, Chl fluorescence, proline, leaf pigments and lipid peroxidation analysis, and antioxidant activity of catalase and ascorbate peroxidase.

Materials and methods

Plant material and growth conditions: One-year-old seedlings of *C. siliqua* cv. Mulata growing in 3 dm^3 pots filled with a mixture of a fertilized substrate (*SIRO Plant*) and natural soil from the local area (2:1, v/v) were placed in an environment-controlled chamber (*Fitoclima 16.000 EHVP*, Aralab, Portugal). The set conditions were: day/night air temperature 25/18ºC (LT) or 32/21ºC (HT), relative humidity 60%, CO_2 concentration 350–360 ppm

and photoperiod 12/12 h, with light provided by incandescent and fluorescent lamps, supplying a photosynthetic photon flux density (PPFD) of about 450 μmol m^{-2} s^{-1} at the top of the plants. Plants were watered until field capacity each two days and fertilized weekly with a commercial nutrient solution (*Complesal 12-4-6*, *Bayer*, Carnaxide, Portugal). To avoid bias caused by microenvironmental variation within the cabinet (light, temperature, and humidity gradients), the pots were rotated on the benches every watering day. The duration of acclimation to thermal regime was 20 d. Then water stress (WS) was imposed by stopping irrigation. In the case of the plants referred as well watered (WW), all the water lost by evapotranspiration (determined gravimetrically) was replaced throughout the entire experimental period. Measurements and sampling of plants subjected to water deficit took place 15 d after the onset of soil drying. Afterwards, in order to study recovery from drought at HT, 5 water-stressed pots were rewatered to field capacity (RW) and plants were sampled 36 h following rewatering. A different group of plants was sampled on each day (five plants per treatment), and all measurements were undertaken on fully expanded, nonsenescent leaves. Unless otherwise stated, all measurements were carried out 4 h after the lights were turned-on (midday).

Soil and plant water measurements: Soil water depletion was evaluated in pots used for plant water measurements immediately before the controls were watered. It was calculated as $W_{CC} - W_R$, where W_{CC} is the mass of pots at a field capacity and W_R at a day of measurements. Leaf water status was assessed by measuring leaf water potential (Ψ_w) at the end of the dark period (predawn) and leaf relative water content (RWC) at midday. Water potential was measured using a Scholander type pressure chamber model 600 (*PMS Instruments*, *Corvallis*, OR, USA). RWC was determined in leaf discs, and calculated as (FM − DM)/(TM − DM) × 100, where FM is a fresh mass, TM is a turgid mass (determined after floating discs for 3 h on distilled water at 5ºC) and DM is a dry mass (determined after drying at 80ºC for 48 h).

Gas-exchange rates and Chl fluorescence: Stomatal conductance for water vapour diffusion (g_s), net photosynthetic rate (P_N) and substomatal CO_2 concentration (C_i) were determined using a portable gas-exchange measuring system (*HCM-1000, H. Walz,* Effeltrich, Germany). Measurements were made under environmental conditions inside the growth chamber.

Chl fluorescence was imaged using a mini blue version of *Imaging-PAM* Chl fluorometer (*IMAG-MIN/B, Walz*, Effeltrich, Germany) in entire leaves after 20 min of darkness. Pixel value images of the fluorescence parameters were displayed with the help of a false colour code ranging from black (0.000) through red, yellow, green, and blue to pink (ending at 1.000). In order to evaluate spatial and temporal heterogeneity four areas of interest (AOIs) were selected. Images of F_0 were obtained by applying measuring light pulses modulated at 1 Hz, while images of the maximal fluorescence yield (F_m) were obtained with the help of a saturating blue pulse (10,000 μmol m^{-2} s^{-1} PPFD) at 10 Hz and the images of F_v/F_m were derived from that. Then, actinic illumination [249 μmol(photon) m^{-2} s^{-1}] was switched on and saturating pulses were applied at 20-s intervals for 5 min in order to determine the maximum fluorescence yield (F_m') and the Chl fluorescence yield (F_s) during illumination. On light-adapted state, ϕ_{PSII} was calculated according to Genty *et al*. (1989). The coefficient of photochemical quenching, q_L, was defined and calculated as in Kramer *et al*. (2004) as $(F_m' - F_s)/(F_m' - F_0') \times F_0'/F_s = q_p \times F_0'/F_s$. The value of F_0' was estimated using the approximation of Oxborough and Baker (1997): $F_0' = F_0/(F_v/F_m + F_0/F_m')$. Φ_{NPQ} and Φ_{NO} in PSII were calculated according to Kramer *et al*. (2004) by the equations $\Phi_{NPQ} = 1 - \Phi_{PSII} - 1/[NPQ + 1 + q_L (F_m/F_0 - 1)]$ and $\Phi_{NO} = 1/[(NPQ + 1 + q_L) (F_m/F_0 - 1)]$, respectively.

Leaf pigments (Chls, Cars) analysis: Photosynthetic pigment analysis was performed on leaf samples (2.0 cm^2) of freeze-dried tissue that was extracted in 100% acetone. The extracts were measured in a spectrophotometer (*Shimadzu UV-160*, Kyoto, Japan) and pigment contents were estimated according to Lichtenthaler (1987).

Lipid peroxidation and proline content: Leaf oxidative damage was estimated in terms of lipid peroxidation by determining the concentration of MDA, a product of the oxidation of polyunsaturated fatty acids. Measurements of MDA were performed as in Hodges *et al*. (1999), taking into account the possible influence of interfering compounds in the assay for the 2-thiobarbituric acid-reactive substances. The MDA concentration was expressed in terms of mg g^{-1}(FM), using an extinction coefficient of 155 mM^{-1} cm^{-1} at 532 nm. Absorbances measured at 600 and 440 nm allowed to take into account interference due to nonspecific turbidity and carbohydrates, respectively.

Proline was determined following the ninhydrin method as described by Bates *et al*. (1973) with some modifications according Magné and Larher (1992). Briefly, fresh leaf tissue was extracted in 1.5 ml of 80% ethanol. After centrifugation at 10,000 × *g* for 5 min, 100 μl of the supernatant was added to 400 μl of a mixture of 1% ninhydrin and glacial acetic acid in a 60:40 (v/v) ratio. The reaction mixture was incubated in a water bath at 100ºC for 1 h, then rapidly cooled and portioned against 1 ml of toluene. After centrifugation at 3,000 × *g* for 5 min the organic phase was collected and absorbance was read at 520 nm using toluene as a blank. Proline concentration was determined against a standard

curve (0 to 750 μmol ml^{-1}) with L-proline (*Sigma-Aldrich Chemie GmbH*, Steinheim, Germany) dissolved in 80% ethanol.

Antioxidant enzymes activities and total soluble proteins content: Extracts for determination of antioxidant enzymes catalase (CAT, EC 1.11.1.6) and ascorbate peroxidase (APX, EC 1.11.1.11), activities were prepared from frozen leaves (100 mg FM) homogenised with a mortar and pestle in 1.0 ml of ice-cold 50 mM sodium-potassium-phosphate buffer (pH = 7.0), containing 2% Triton X-100, 0.2 mM EDTA, 1.5% PVP (w/v) and 5 μl DTT. The same extraction medium supplemented with 2% ascorbic acid (5 mM) was used for APX.

CAT activity was determined by H_2O_2 consumption measured as the decrease in absorbance at 240 nm, according to the method described by Aebi (1983). The assay medium contained 50 mM sodium-potassium-phosphate buffer (pH = 7.0), 40 mM H_2O_2 and 20 μl of the extract. Catalase activity was calculated based on an extinction coefficient of 39.4 mM^{-1} cm^{-1}.

APX activity was measured by ascorbate consumption measured as the decrease in absorbance at 240 nm, according to the method described by Nakano and Asada (1981). The assay medium contained 50 mM sodium-potassium-phosphate buffer (pH = 7.0), 0.25 mM H_2O_2, 0.25 mM ascorbate and 20 μl extract. APX activity was calculated based on an extinction coefficient of 2.8 mM^{-1} cm^{-1}.

Soluble protein content was measured according Bradford (1976) in the same extracts used for the determination of enzyme activities.

Statistical analysis and graphic display were performed with *SPSS*® (*Release 16.0, SPSS Inc*., Chicago, IL) and *SigmaPlot*® (*Version 10.00, Systat Software, Inc*., San Jose, CA, USA) software packages, respectively. All the determinations were obtained with randomly chosen plants. Comparisons among groups were performed by analysis of variance or Student's *t*-test for unpaired data. For additional pairwise comparisons, Student-Newmans-Keul test (SNK) was used. Differences were considered significant at $P<0.05$).

Results

Soil and plant water measurements: Soil water depletion was not appreciably altered by temperature in well watered pots. In this case, total water loss between watering events was 219.0 ± 10.4 g H_2O and 221.0 ± 22.5 g H_2O in LT and HT plants, respectively. In contrast, in water-stressed pots the total water loss by the end of stress exposure was significantly higher in HT than in LT plants (943 ± 69.6 *vs*. 720 ± 40.8 g H_2O). Predawn leaf water potential (Ψ_w) decreased in both thermal regimes (Fig. 1*A*), but Ψ_w of plants kept at HT was 68% lower than that of plants kept at LT. Despite the significant depression of Ψ_w observed in plants grown at both temperatures, RWC did not change significantly in the LT group (Fig. 1*B*), which displayed a value of about 90%, pointing to a mild water stress. On the contrary, at HT the RWC fell to 63.8 ± 4.29% after 15 days of water withholding, indicating that the level of water stress to which these plants were subjected was more severe. Both Ψ_w and RWC had fully recovered to prestress levels 36 h following rewatering.

Gas-exchange rates and Chl fluorescence: Photosynthetic rates were significantly depressed by water deficit in both thermal regimes but the effects were much more pronounced at high temperature than the low one (Fig. 2*A*). Decreases in P_N were of about 33% and 84%

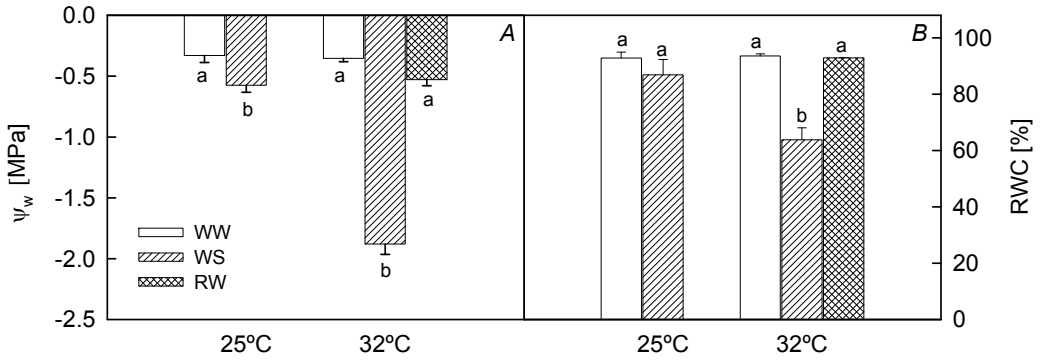

Fig. 1. (*A*) Predawn water potential (Ψ_w) and (*B*) relative water content (RWC) at midday determined in leaves of *C. siliqua* well watered (WW), water-stressed (WS), and rewatered (RW) under low- (25ºC) and high temperature (32ºC). *Different letters* indicate significant differences between water treatments at the same temperature ($P<0.05$) according SNK test. Values shown are means ± standard error of five replicates.

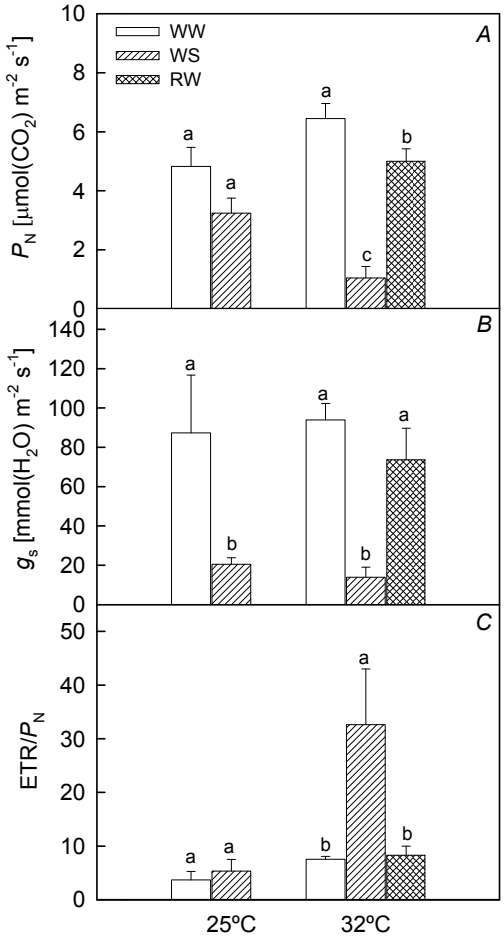

Fig. 2. (*A*) Photosynthetic rate (P_N), (*B*) stomatal conductance (g_s) and (*C*) ETR/P_N ratio in leaves of *C. siliqua* well watered (WW), water-stressed (WS), and rewatered (RW) under low- (25ºC) and high temperature (32ºC). *Different letters* indicate significant differences between water treatments at the same temperature (P<0.05) according SNK test. Values shown are means ± standard error of five replicates.

in LT and HT plants, respectively, even though the decline in g_s has been similar (*ca*. 85%) in both groups (Fig. 2*B*). The negative effects of soil drying on P_N and g_s of HT plants were no longer detected 36 h following rewatering. No statistically significant changes due to drought were observed in C_i of plants subjected to both thermal regimes (data not shown). Moreover, decreases in g_s and in P_N observed in water-stressed plants were not accompanied by significant changes in the apparent linear electron transport rate (ETR), thus leading to a noteworthy rise in the ETR/P_N ratio in WS/32ºC plants relative to WW/32ºC plants (Fig. 2*C*).

The fluorescence imaging technique was used to assess the heterogeneity of photosynthetic performance and the extent to which performance is limited by regulated or nonregulated energy dissipation under water stress at HT and LT. As can be seen in Figs. 3 and 4*A*,*B*; following drought at HT leaves had both a slight heterogeneity and a lower average value of PSII efficiency (F_v/F_m and Φ_{PSII}) compared with leaves grown at LT. However, leaves of well watered plants grown under both thermal regimes displayed values F_v/F_m close to 0.8, indicating that photosynthetic acclimation had occurred in response to HT. Plants kept at HT displayed significant decreases in F_v/F_m and Φ_{PSII} 15 days after water deprivation, 26% and 36% respectively, while in plants kept at LT values of F_v/F_m and Φ_{PSII} were maintained fairly well throughout water deprivation. Both F_v/F_m and Φ_{PSII} had fully recovered to prestress levels 36 h following rewatering.

The decline of Φ_{PSII} in plants grown at HT caused by water stress was accompanied by a clear increase of Φ_{NO} without appreciable change of Φ_{NPQ} (Fig. 4*B,C,D*). On the contrary, at LT no significant effects were observed in these parameters. Furthermore, as shown in Fig. 5, a strong and negative correlation was found between Φ_{NO} and Φ_{PSII} (R^2 = 0.892; P<0.001), whereas no correlation between Φ_{NPQ} and Φ_{PSII} was evident (data not shown).

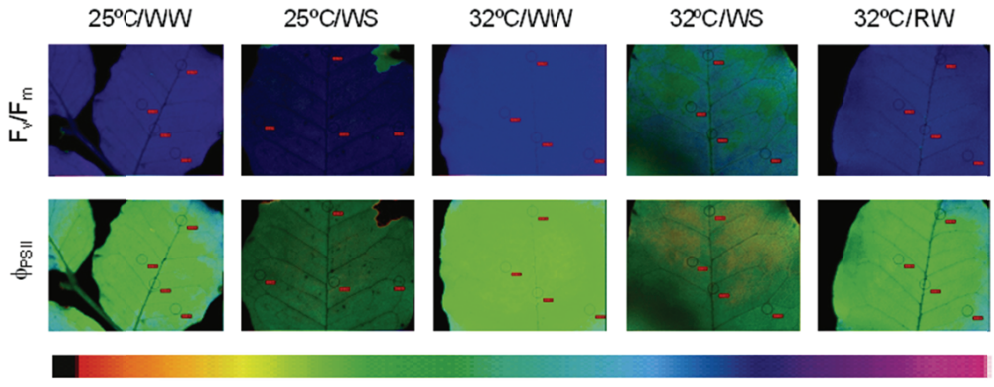

Fig. 3. Chlorophyll fluorescence images of maximum PSII photochemical efficiency (F_v/F_m) in dark-adapted leaf and actual PSII quantum yield (Φ_{PSII}) at steady state measured in leaves of *C. siliqua* well watered (WW), water-stressed (WS), and rewatered (RW) under low- (25ºC) and high temperature (32ºC). The false colour code depicted at the bottom of images ranges from 0.000 (black) to 1.000 (pink). The false colour code depicted at the bottom of images ranges from 0.000 (black) to 1.000 (pink). The four small circles in each image are the AOIs which are accompanied by a little red box displaying the averaged values of the selected fluorescence parameters (not visible in Fig. 3) displayed in a report file used to calculated means showed in Fig. 4.

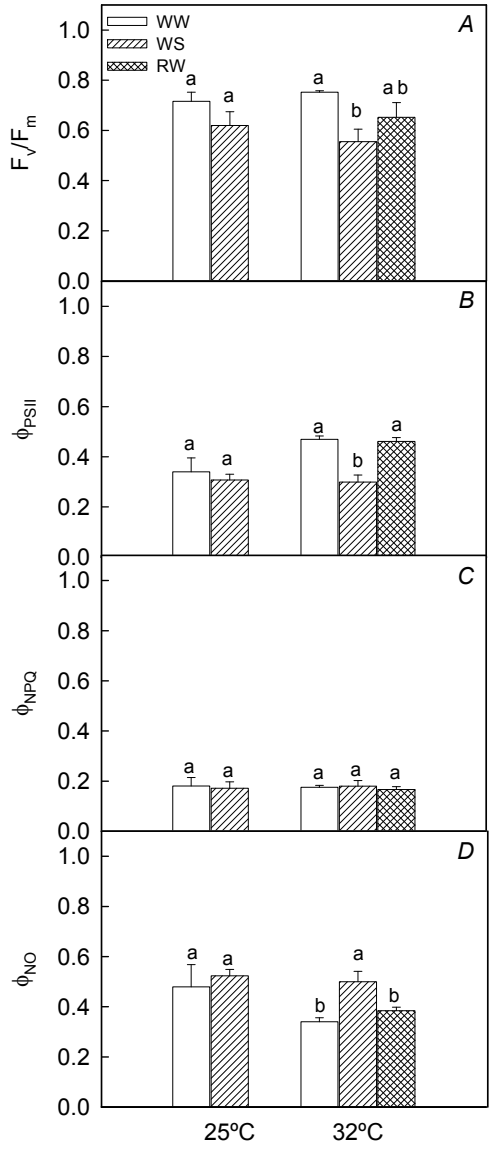

Fig. 4. (*A*) Maximum PSII photochemical efficiency (F_v/F_m) in dark-adapted leaf, (*B*) actual PSII quantum yield (Φ_{PSII}), (*C*) quantum yield of regulated energy dissipation of PSII (Φ_{NPQ}), and (*D*) quantum yield of nonregulated energy dissipation of PSII (Φ_{NO}) at steady state measured in leaves of *C. siliqua* well watered (WW), water-stressed (WS), and re-watered (RW) under low- (25ºC) and high temperature (32ºC). *Different letters* indicate significant differences between water treatments at the same temperature (P<0.05) according SNK test. Values shown are means ± standard error of five replicates.

Leaf pigments (Chls, Cars): The contents of Chl *a*, Chl *b,* and total carotenoids did not differ between treatments, and consequently also the Chl *a*/*b* and Chl *a*/Car ratios remained unchanged (data not shown).

Lipid peroxidation and proline content: In the case of plants kept under high temperature, drought induced increases in lipid peroxidation, as estimated through malondialdehyde (MDA) production (Fig. 6*A*). By the end of the soil drying period the foliar concentration of MDA increased by 40% above values found in leaves of well watered plants, but drought-induced increase in lipid peroxidation was fully reversed following rewatering. In contrast, under low-temperature conditions, the concentrations of MDA in leaves of well watered and water-stressed plants were not significantly different. As shown in Fig. 6*B*, water deficit under 32ºC resulted in a marked increase in proline content (*ca*. 2-fold relative to WW) and the trend of variation with treatments was very similar to that of MDA.

Antioxidant enzymes activities: The activities of CAT and APX are presented in Fig. 7. Data on leaf protein basis reflect the relative proportion of the enzyme in the total protein content. CAT and APX activities were unaffected by water status in both temperatures, but CAT activity at HT was significantly depressed as compared with that of LT.

Discussion

As expected, high temperature accelerated depletion of soil water in pots subjected to water stress, an effect attributed to increases in both evaporation and evapotranspiration (Machado and Paulsen 2001). As a result, HT reinforced the negative impact of water shortage on Ψ_w and RWC and one can conclude that the aggravated effects of HT on photosynthesis are mainly a consequence of the higher level of drought reached. According to this, plants under LT suffered a mild stress (slight decreases in Ψ_w and RWC) whereas those under HT experienced a severe water stress (considerable decreases in Ψ_w and RWC). The decline of plant water status induced an increased stomatal closure and reductions in stomatal conductance in both LT and HT plants, which in turn negatively impacted photosynthetic rates. Although the decline observed in g_s has been similar for both temperatures, the reduction of P_N was considerably more drastic at HT (Fig. 2), a finding suggesting that under HT the decline in P_N was not due merely to stomatal limitation. Such a view is supported by the similar values of C_i found in WS/32ºC and WW/32ºC plants. This type of response has classically been interpreted as evidencing a gradual increase in nonstomatal limitation of photosynthesis during drought (Flexas and Medrano 2002, Cechin *et al.* 2006).

Evidence gathered from fluorescence measurements gives also support to the idea that the reduction of P_N in WS/32ºC plants was partially due to nonstomatal limitation, inasmuch as they displayed a marked decline in the F_v/F_m ratio (Fig. 4*A*). In fact, declines in the F_v/F_m ratio reflect a reduction in light energy utilization by chloroplasts in the photosynthesis. Furthermore, the

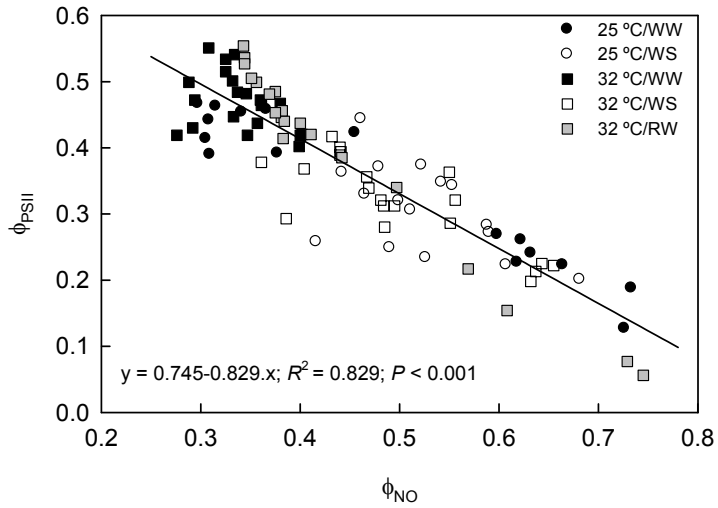

Fig. 5. Relationship between quantum yields of PSII photochemistry (Φ_{PSII}) and quantum yields of nonregulated energy dissipation (Φ_{NO}). Values shown are individual data points determined in leaves of *C. siliqua* well watered (WW), water-stressed (WS), and rewatered (RW) under low- (25ºC) and high temperature (32ºC).

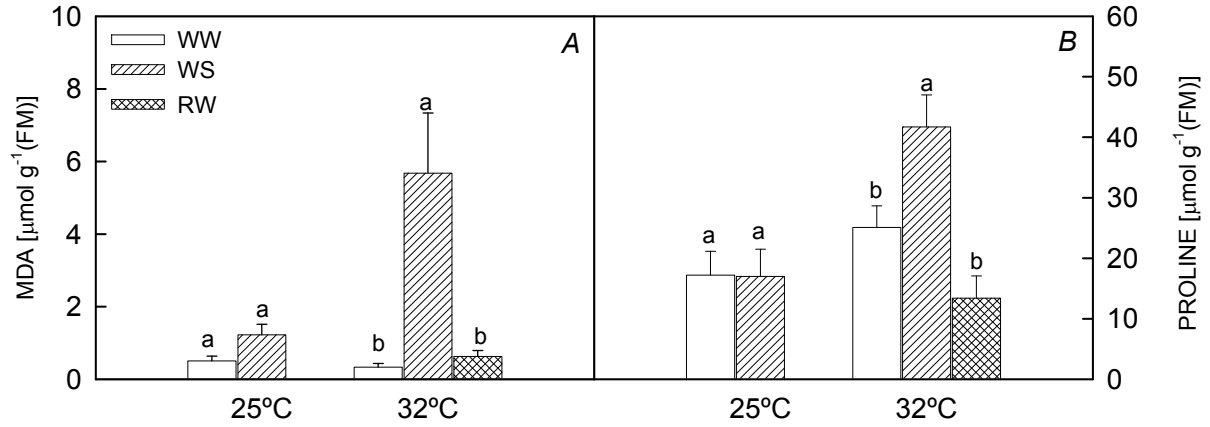

Fig. 6. (*A*) Concentration of malondialdehyde (MDA) and (*B*) proline determined in leaves of *C. siliqua* well watered (WW), water-stressed (WS), and rewatered (RW) under low- (25ºC) and high temperature (32ºC). *Different letters* indicate significant differences between water treatments at the same temperature (P<0.05) according SNK test. Values shown are means ± standard error of five replicates.

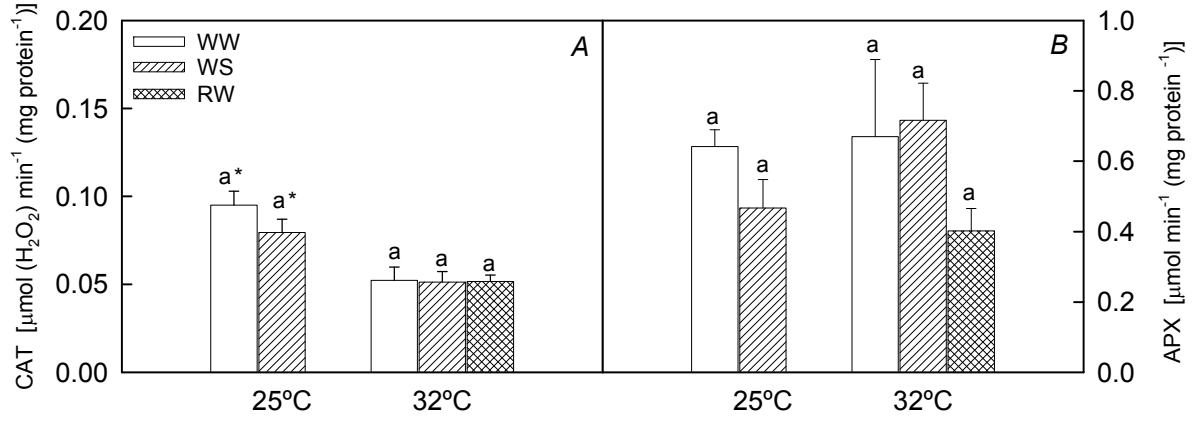

Fig. 7. (*A*) Activities of catalase and (*B*) ascorbate peroxidase determined in leaves of *C. siliqua* well watered (WW), water-stressed (WS), and rewatered (RW) under low- (25ºC) and high temperature (32ºC). *Letters* indicate no significant differences between water treatments at the same temperature (P<0.05) according SNK test and (*) indicates significant differences between LT and HT (P<0.05) according Student's *t*-test. Values shown are means ± standard error of five replicates.

significant increase of the ETR/P_N ratio suggests that in WS/32ºC plants the excitation energy usually utilized for photochemistry is being partially diverted to the photosynthetic reduction of O_2, *via* photorespiration and/or Mehler-peroxidase reaction. Similar results were obtained in other studies and were taken as indicative of an increased activity of nonassimilatory electron transport processes (Flexas *et al*. 1998, Osório *et al*. 2006). In addition, as usually, drought stress results in heterogeneity of stomatal conductance and photosynthesis (Downton *et al*. 1988, Mott and Buckley 2000), a considerably heterogeneity of Φ_{PSII} and F_v/F_m in WS/32ºC carob tree plants would be expectable. However, Chl fluorescence images revealed a quite homogeneous pattern of distribution of F_v/F_m and Φ_{PSII} over the screened leaf area (Fig. 3). Similar results were reported by Massacci *et al*. (2008) which suggested that photorespiration could act as a buffer that keeps spatial heterogeneity of Φ_{PSII} low. Although such processes may play a protective role by decreasing the degree of reduction of the PSII acceptor side and the risk of photoinhibition, it is well known that they are prone to lead to overproduction of ROS. Reactive oxygen species are continuously produced and removed during normal physiologic events, and in a way that the two processes accomplish a dynamic equilibrium (Baker 1991). However, when plant experiences a stress situation, more O_2 molecules are expected to be used as electron acceptors, potentiating in this way the disruption of the production-removal balance and the accumulation of ROS. As direct measurement of ROS production is not currently feasible in plants, monitoring of the changes in absorbed energy distribution related to their production could be a useful tool to develop a better understanding of the mechanisms of heat- or drought damage (Wang *et al.* 2009). In this study, the evidence collected from fluorescence measurements (Fig. 4) showed that drought under high temperature reduces the amount of energy absorbed by photochemistry (decrease in Φ_{PSII}), diverting a large part of it to nonregulated energy conversion processes (increase in Φ_{NO}), a circumstance that favours the production of ROS. Moreover, a strong overall relationship (R = 0.904, P<0.001) was found between Φ_{PSII} and Φ_{NO}, whereas no correlation was evident between ϕ_{PSII} and ϕ_{NPQ}. These results point out that energy dissipation by nonregulated quenching mechanisms (*e.g.* photorespiration and/or Mehler reaction) tend to dominate PSII photoprotection under drought and high temperature, with the xanthophyll cycle-mediated thermal dissipation playing possibly a much less important role. This is in agreement with early findings of other authors which have collected important data using the fluorescence parameters Φ_{PSII}, Φ_{NPQ}, and Φ_{NO} to study plant responses to heat or cold stress (Szyszka *et al*. 2007, Savitch *et al.* 2009, Wang *et al*. 2009).

Data on cell membrane peroxidation also support the above interpretations. The peroxidation of membrane lipids is often generated by an attack of ROS to cell membranes, being MDA the major secondary product of the resulting lipoperoxide hydrolysis (Hodges *et al*. 1999, Yordanov *et al*. 2000). Thus, MDA can be regarded as a marker for lipid peroxidation, and therefore used as an indicator of oxidative damage. In our study, a significant rise in MDA (*ca*. 40%) was observed in WS/32ºC plants, suggesting that the combination of high temperature and drought resulted in a moderate oxidative stress at the whole-leaf level, similarly to what has been reported for other species and stresses (Jiang and Zhang 2002, Correia *et al*. 2006, Costa *et al*. 2010). In order to prevent oxidative damage, plants are equipped with complex enzymatic antioxidant defence systems (Lawlor 1995). Catalase and APX are primary H_2O_2-scavenging enzymes, generally playing an important role in protection of plants against oxidative damage. However, in *C. siliqua* CAT and APX activities were unaffected by water stress at both temperatures (Fig. 7). This explains, at least in part, the increased lipid peroxidation observed in WS/32ºC plants (Fig. 6*A*), which was coincident to unchanged activity (in comparison to control plants) of these enzymes. Contrasting trends have been reported in the literature about changes in antioxidative enzyme activities with drought, depending on the mode of imposition, duration and severity of stress. In general, increased APX and CAT activities were reported for a mild water deficit (Feng *et al*. 2004), whereas severe or prolonged drought stress caused a decline in activities of these enzymes (Guo *et al*. 2006). Costa *et al*. (2010) reported results similar to ours in *Carapa guianensis* submitted to water stress for 15 days. Interestingly, *C. guianensis* showed higher enzyme activities coincident with higher levels of glycine betaine (GB) when the stress period was extended to 27 days, whereas in *C. siliqua*, a significant accumulation of proline was found in water-stressed plants grown at high temperature (Fig. 6*B*), which was reversed after soil rehydration. Both GB and proline are compatible solutes that accumulate in plants under diverse stress conditions, and that have the potential to play an important role in protecting membranes and proteins from the deleterious effects of water deficits (Ashraf and Foolad 2007). Moreover, proline may stabilize antioxidant enzymes and it may directly stimulate the ROS production in the mitochondria due to its effect on the electron transport processes in Pro-pyrroline-5-carboxylate cycle (Szabados and Savouré 2010). Therefore, we can speculate that in *C. siliqua* the anti-ROS enzymatic system is triggered by the accumulation of an appropriate amount of proline when water stress is more severe. But we cannot discard other possible explanations for proline accumulation, inasmuch as its concentration decreased upon relief from water stress. The accumulated pool of proline can also serve as a carbon and nitrogen reserve for growth after stress relief, or it can provide the reducing agents to support the levels of mitochondrial oxidative phospho-

rylation and ATP generation needed for fast recovery from stress and repairing of stress-induced damages (Hare and Cress 1997).

As a final conclusion of this study, we can state that although *C. siliqua* seedlings exhibit clear signs of oxidative stress under drought and high temperature, they retain a remarkable ability to quickly restore normal physiological activity upon rehydration, which let us believe that they can satisfactorily deal with predicted climate warming and increased soil drying in the Mediterranean area**.**

References

Aebi, H.: Catalase *in vitro*: – In: H. Bergmeyer (ed.): Methods of Enzymatic Analysis. Pp. 273-277. Verlag Chemie, Weinheim 1983.

Ashraf, M., Foolad M.R.: Roles of glycine betaine and proline in improving plant abiotic stress resistance. – Environ. Exp. Bot. **59**: 206-216, 2007.

Baker, N.R.: A possible role for photosystem II in environmental perturbations of photosynthesis. – Physiol. Plant. **81**: 563-570, 1991.

Bates, L.S., Waldren, R.P., Tear, I.D.: Rapid determination of free proline for water stress studies. – Plant Soil **39**: 205-207, 1973.

Berry, J., Björkman, O.: Photosynthetic response and adaptation to temperature in higher plants. – Annu. Rev. Plant Physiol. **31**: 491-543, 1980.

Boyer, J.S.: Plant productivity and environment. – Science **218**: 443-448, 1982.

Bradford, M.M.: A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. – Anal. Biochem. **72**: 248-254, 1976.

Cechin, I., Rossi, S.C., Oliveira, V.C., Fumis, T.F.: Photosynthetic responses and proline content of mature and young leaves of sunflower plants under water deficit. – Photosynthetica **44**: 143-146, 2006.

Chartzoulakis, K., Psarras, G.: Global change effects on crop photosynthesis and production in Mediterranean: the case of Crete, Greece. – Agr. Ecosyst. Environ. **106**: 147-157, 2005.

Chaves, M.M., Pereira, J.S., Maroco, J., Rodrigues, M.L., Ricardo, C.P.P., Osório, M.L., Carvalho, I., Faria, T., Pinheiro, C.: How plants cope with water stress in the field: photosynthesis and growth. – Ann. Bot. **89**: 907-916, 2002.

Cornic, G.: Drought stress inhibits photosynthesis by decreasing stomatal aperture – not by affecting ATP synthesis. – Trends Plant Sci. **5**: 187-188, 2000.

Correia, M.J., Osório M.L., Osório J., Barrote I., Martins M., David M.M.: Influence of transient shade periods on the effects of drought on photosynthesis, carbohydrate accumulation and lipid peroxidation in sunflower leaves. – Environ. Exp. Bot. **58**: 75-84, 2006.

Costa, A.C., Pinheiro, B.G, Cordeiro, E.S., Shimizu, F.T., Santos-Filho, B.,G., Moraes, F.K.C., Figueiredo, D.M.: Lipid peroxidation, chloroplastic pigments and antioxidant strategies in *Carapa guianensis* (Aubl.) subjected to water-deficit and short-term rewetting. – Trees **24**: 275-283, 2010.

Downton, W.J.S., Loveys, B.R., Grant, W.J.R.: Non-uniform stomatal closure induced by water stress causes putative non-stomatal inhibition of photosynthesis. – New Phytol. **110**: 503-509, 1988.

Feng, Z., Jin-Kui, G., Ying-Li, Y., Wen-Liang, H., Li-Xin, Z.: Changes in the pattern of antioxidant enzymes in wheat exposed to water deficit and rewatering. – Acta Physiol. Plant. **26**: 345-352, 2004.

Flexas, J., Escalona, J.M., Medrano, H.: Down-regulation of photosynthesis by drought under field conditions in grapevine leaves. – Aust. J. Plant Physiol. **25**: 893-900, 1998.

Flexas, J., Medrano, H.: Drought-inhibition of photosynthesis in C_3 plants: stomatal and non-stomatal limitations revisited. – Ann. Bot. **89**: 183-189, 2002.

Genty, B., Briantais, J.M., Baker, N.R.: The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. – Biochim. Biophys. Acta **990**: 87-92, 1989.

Guo, Z., Ou, W., Lu, S., Zhong, Q.: Differential responses of antioxidative system to chilling and drought in four rice cultivars differing in sensitivity. – Plant Physiol. Biochem. **44**: 828-836, 2006.

Hare, P.D., Cress, W.A.: Metabolic implications of stress induced proline accumulation in plants. – Plant Growth Regul. **21**: 79-102, 1997.

Hodges, D., DeLong, J.M., Forney, C.F., Prange, R.K.: Improving the thiobarbituric acid-reactive-substances assay for estimating lipid peroxidationin plant tissues containing anthocyanin and other interfering compounds. – Planta **207**: 604-611, 1999.

Houghton, J.T, Ding, Y., Griggs, D.J, Noguer, M., van der Linden, P.J., Dai, X., Maskell, K., Johnson, C.A.: IPCC Report on Climate Change 2001: The Scientific Basis. – Cambridge University Press, New York 2001.

Mc Arthy, J.J., Canziani, O.F., Leary, N.A., Dokken, D.J., White, K.S. (ed.): IPCC: Climate Change 2001: Impacts, Adaptation and Vulnerability. – Cambridge University Press, Cambridge 2001.

Jiang, M.Y., Zhang, J.H.: Water stress-induced abscisic acid accumulation triggers the increased generation of reactive-oxygen species and up-regulates the activities of antioxidant enzymes in maize leaves. – J. Exp. Bot. **53**: 2401-2410, 2002.

Kramer, D.M., Johnson, G., Kiirats, O., Edwards, G.E.: New fluorescence parameters for the determination of Q_A redox state and excitation energy fluxes. – Photosynth. Res. **79**: 209-218, 2004.

Lawlor, D.W.: The effects of water deficit on photosynthesis. – In: Smirnoff, N. (ed.): Environment and Plant Metabolism, Flexibility and Acclimation. Pp. 129-160. BIOS Scientific Publishers, Oxford 1995.

Lichtenthaler, H.K.: Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. – Meth. Enzymol. **148**: 350-382, 1987.

Lo Gullo, M.A., Salleo, S.: Different strategies of drought resistance in three Mediterranean sclerophyllous trees growing in the same environmental conditions. – New Phytol. **108**: 267-276, 1988.

Machado, S., Paulsen, G.M.: Combined effects of drought and high temperature on water relations of wheat and sorghum. –

Plant Soil **233**: 179-187, 2001.

Magné, C., Larher, F.: Higher sugar content of extracts interferes with the colorimetric determination of amino acids and free proline. – Anal. Biochem. **200**: 115-118, 1992.

Massacci, A., Nabiev, S.M., Pietrosanti, L., Nematov, S.K., Chernikova, T.N., Thor, K., Leipner, J.: Response of the photosynthetic apparatus of cotton (*Gossypium hirsutum*) to the onset of drought stress under field conditions studied by gas-exchange analysis and chlorophyll fluorescence imaging. – Plant Physiol. Biochem. **46**: 189-195, 2008.

Mott, K.A., Buckley, T.N.: Patchy stomatal conductance: emergent collective behaviour of stomata. – Trends Plant Sci. **5**: 258-262, 2000.

Nakano, Y., Asada, K.: Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. – Plant Cell Physiol. **22**: 867-880, 1981.

Nunes, M.A., Ramalho, J.D.C., Rijo, P.S.: Seasonal changes in some photosynthetic properties of *Ceratonia siliqua* (carob tree) leaves under natural conditions. – Physiol. Plant. **86**: 381-387, 1992.

Osório, M.L., Breia, E., Rodrigues, A., Osório, J., Le Roux, X., Daudet, F.A., Ferreira, I., Chaves, M.M.: Limitations to carbon assimilation by mild drought in nectarine trees growing under field conditions. – Environ. Exp. Bot. **55**: 235-247, 2006.

Oxborough, K., Baker, N.R.: Resolving chlorophyll *a* fluorescence images of photosynthetic efficiency into photochemical and non-photochemical components – calculation of q*P* and *F*v'/*F*m' without measuring *F*o'. – Photosynth. Res. **54**: 135-142, 1997.

Petsas, A., Grammatikopoulos, G.: Drought resistance and recovery of photosystem II activity in a Mediterranean semi-deciduous shrub at the seedling stage. – Photosynthetica **47**: 284-292, 2009.

Ramalho, J.C., Lauriano, J.A., Nunes, M.A.: Changes in photosynthetic performance of *Ceratonia siliqua* in summer. – Photosynthetica **38**: 393-396, 2000.

Savitch, L.V., Ivanov, A.G., Gudynaite-Savitch, L., Huner, N.P.A., Simmonds, J.: Effect of low temperature stress on excitation energy partitioning and photoprotection in *Zea Mays*. – Funct. Plant Biol. **36**: 37-49, 2009.

Smirnoff, N.: Tansley review .52.The role of active oxygen in the response of plants to water deficit and desiccation. – New Phytol. **125**: 27-58, 1993.

Szabados, L., Savouré, A.: Proline: a multifunctional amino acid. – Trends Plant Sci. **15**: 89-97, 2010.

Szyszka, B., Ivanov, A.G., Huner, N.P.A.: Psychrophily is associated with differential energy partitioning, photosystem stoichiometry and polypeptide phosphorylation in *Chlamydomonas raudensis*. – Biochim. Biophys. Acta – Bioenergetics **1767**: 789-800, 2007.

Wang, L-J., Loescher, W., Duan, W., Li, W-D., Yang, S-H., Li, S-H.: Heat acclimation induced acquired heat tolerance and cross adaptation in different grape cultivars: relationships to photosynthetic energy partitioning. – Funct. Plant Biol. **36**: 516-526, 2009.

Wilson, K.E., Ivanov, A.G., Öquist, G., Grodzinski, B., Sarhan, F., Huner, N.P.A: Energy balance, organellar redox status and acclimation to environmental stress. – Can. J. Bot. **84**: 1355-1370, 2006.

Yordanov, I., Velikova, V., Tsonev, T.: Plant responses to drought, acclimation, and stress tolerance. – Photosynthetica **38**: 171-186, 2000.