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Photosynthesis, energy partitioning, and metabolic adjustments of the endangered Cistaceae species *Tuberaria major* under high temperature and drought

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Abstract

In view of predicted climatic changes for the Mediterranean region, study of high temperature and drought impacts on physiological responses of endangered species regains relevance. In this context, micropropagated plants of *Tuberaria major*, a critically endangered species, endemic of Algarve, were transferred to a controlled-environment cabinet with day/night temperatures set at 25/18ºC (Reference) or 32/21ºC (HT). After 15 days of HT acclimation, some plants were subjected to progressive drought followed by rewatering. The enhancement of temperature alone did not affect water relations and photosynthetic rates (P_N) but the stomatal conductance (g_s) exhibited a 3-fold increase in comparison with reference plants. The maximum quantum yield of photosystem (PS) II (F_v/F_m), the effective quantum yield of PSII photochemistry (Φ_{PSII}), carotenoid (Car) and anthocyanin content enhanced, whereas the quantum yields of regulated (Φ_{NPQ}) and nonregulated (Φ_{NO}) energy dissipation decreased. Drought combined with HT reduced predawn leaf water potential to values of about –1.3 MPa, which had adverse effects on gas exchange and PSII activity. Values of P_N and g_s were 71 and 79% lower than those of HT plants. An impairment of photochemical activity was also observed: the decrease in Φ_{PSII} and the increase of Φ_{NPQ}. However, an irreversible photoinhibitory damage had not occurred. Carotenoid and anthocyanin content remained elevated and soluble sugars (SS) increased twice, whereas proline and MDA accumulation was not detected. On the first 24 h after water-stress relief, g_s, P_N, Φ_{PSII}, and Φ_{NPQ} did not recover, but SS returned to the reference level. Overall, *T. major* acquired an adequate capacity for a protection against the development of oxidative stress during drought and water recovery under HT. These findings suggest that *T. major* is prepared to deal with predicted climate changes.

Additional key words: anthocyanins; chlorophyll fluorescence imaging; energy partitioning; malondialdehyde; proline; soluble sugars.

Introduction

Tuberaria major (Willk.) P. Silva & Rozeira is a Cistaceae species endemic of Algarve (South of Portugal) that needs to be preserved. It is legally protected under the European Habitats Directive 92/43/CEE and the Portuguese law (reference 140/99 from April 24). In the past, its distribution included a greater part of the coast of the Algarve, but nowadays it is reduced to small nuclei located in the center of the region among xerophilous bushes. The decline of the species can be attributed to several causes, such as a destruction, degradation, and fragmentation of habitats, human population expansion, low genetic variability and climate change. Judging from

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Abbreviations: AOI – area of interest; C_i − intercellular CO_2 concentration; Car – carotenoid; Chl – chlorophyll; E – transpiration rate; ETR − apparent linear electron transport rate; F_v/F_m − maximum PSII photochemical efficiency; F_v'/F_m' − efficiency of excitation energy capture by open PSII reaction centers; g_s − stomatal conductance; HT − 32/21ºC well-watered plants; HT+WS − 32/21ºC water-stressed plants; HT+RW − 32/21ºC rewatered plants; MDA − malondialdehyde; P_N − net photosynthetic rate; PPFD − photosynthetically active photon flux density; PS − photosystem; q_p − coefficient of photochemical quenching; ROS – reactive oxygen species; RWC − relative water content; TBARS – thiobarbituric acid-reactive substances; WUE – water-use efficiency (P_N/E); Φ_{PSII} – effective quantum yield of PSII photochemistry; Φ_{NPQ} − quantum yield of regulated energy dissipation of PSII; Φ_{NO} − quantum yield of nonregulated energy dissipation of PSII; Ψ_w − leaf water potential.
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the declining distribution of *T. major* even in protected habitats, it seems that the species may have not adequate plasticity to adapt to changing climatic and environmental conditions. Since climate change models predict that more arid conditions will prevail in the Mediterranean Basin (Osborne *et al*. 2000), *T. major* could face an extinction. An improved knowledge of the physiological traits of endemic species could help to solve the problem of its extinction and to be used as a tool for a correct management of biodiversity in the Mediterranean area (Galmés *et al*. 2007). Limitations in the capacity to adjust photosynthesis to the Mediterranean environmental conditions, as well as, a weak development of photoprotection mechanisms have been suggested to be among the underlying causes of the geographically limited distribution of endemic species (Gulías *et al*. 2002). However, Galmés *et al*. (2007) reported that either nonendemic or endemic Mediterranean species naturally occurring in the Balearic Islands are resistant to water-stress-induced photoinhibition.

Photosynthesis is a highly regulated process, which is extremely sensitive to any changes in environmental conditions, because it needs to balance the light energy absorbed by the two photosystems with the energy consumed by metabolic sinks of the plant (Ensminger *et al*. 2006). Despite the primary events of photosynthesis, such as the electron transport capacity, being very resilient to drought (Epron and Dreyer 1992, Petsas and Grammatikopoulos 2009), in a combination with a high temperature, plants under drought conditions tend to down-regulate photosynthesis or they are even subject to photoinhibition (Chaves *et al*. 1992, Osório *et al*. 2011). However, an irreversible damage to the photosynthetic apparatus may be avoided, or at least alleviated, by an array of photoprotective mechanisms that involve adjustments in a leaf morphology, leaf biochemistry and photochemistry (Ort 2001, Galmés *et al*. 2007). Protection mechanisms that prevent the production of excessive reducing power are important strategies under the water stress and high temperature. Such protection may be achieved by the regulated thermal energy dissipation occurring in the light-harvesting complexes, involving the xanthophyll cycle (Demmig-Adams and Adams 1996) or by nonregulated thermal energy dissipation (Kramer *et al*. 2004). Alternatively, the energy absorbed can be diverted to other sinks, for example, photorespiration (Genty *et al*. 1990, Wingler *et al*. 1999) or cyclic electron transport and the Mehler-peroxidase reaction (Biehler and Fock 1996). Furthermore, changes in levels of both primary (proline, soluble sugars) and secondary (chlorophylls, carotenoids, anthocyanins) metabolites are believed to protect the cellular structures from the oxidative damage and they are relevant in improving net assimilation and heat tolerance (Wahid 2007). On the other hand, an osmotic adjustment has been considered one of the most important processes in a plant adaptation to drought, proline and soluble sugars being the most studied osmoprotectants (Sperdouli and Moustakas 2012). In addition, an elevated proline and soluble sugar content has been associated with a protection of membranes from damage by reactive oxygen species (ROS) produced under drought (Ashraf and Foolad 2007, Chaves *et al*. 2009, Szabados and Savouré 2010, Sperdouli and Moustakas 2012). Anthocyanins may also act as osmotic adjusters offering plants a resistance to drought stress (Chalker-Scott 1999) and they have an inherent potential to protect cell membranes from the effects of an oxidative damage (Gould *et al*. 2002). Reduced light absorption through an accumulation of red carotenoids in leaf surfaces has been also described as a particular photoprotective mechanism (Hormaetxe *et al*. 2005). It was also reported that the accumulation of antioxidant compounds and increased thermal dissipation in the antennae associated with the xanthophyll cycle are among the physiological mechanisms that contribute to a photoprotection in Cistaceae throughout the season (Kyparissis *et al*. 2000, García-Plazaola *et al*. 2003, Munné-Bosch *et al*. 2003). Despite episodic drought periods in the Mediterranean region having a great importance, the short-term response (days to weeks) and a recovery after rewatering have been both evaluated less than long-term response (Galmés et *al*. 2007). Furthermore, nearly all reports describe the effect of a water deficit plus a heat shock. Much less is known about mechanisms how plants respond to a water deficit combined with a continuous and moderately high temperature. However, for perennial plants, the most important strategy is not the maintenance of growth or production during stress, but the ability to survive and recover rapidly after stress relief (Wang and Huang 2004).

In relation to *T. major*, despite it being a particularly rare and endangered species, only little work has been carried out on it and scientific papers published are restricted to its requirements for seed germination and to a protocol for its *in vitro* propagation (Gonçalves *et al*. 2009, 2010). In consequence, there is still a considerable lack of knowledge concerning the impact of changing environmental conditions on physiological responses of *T. major*. In an attempt to fill this lacuna, we evaluated the response of photosynthesis of *T. major* to water stress and recovery under an enhanced temperature, both in terms of CO_2 assimilation, as measured by a leaf gas exchange, and of the functionality of the photosynthetic apparatus, as assessed by chlorophyll *a* fluorescence measurements. Additionally, photosynthetic pigments, anthocyanins, proline, nonstructural carbohydrates and thiobarbituric acid-reactive substances (TBARS) content were simultaneously examined in order to detect possible metabolic alterations and to identify potential mechanisms of a protection against photooxidative damage. The main goal of this study was to test whether a weak development of photoprotection mechanisms is one of the underlying causes of the decline of the species responsible for its undergoing extinction due to the predicted

climatic changes for the Mediterranean region. The contribution of this work can be relevant for physiological understanding and development of conservation options of Mediterranean endangered or threatened species.

Materials and methods

Plant material and growth conditions: Plants of *T. major* were produced by *in vitro* germinating seeds collected in two populations from *Campus* of Gambelas-Faro (Algarve region, southern Portugal) and subsequently micropropagated (Gonçalves *et al*. 2010). After the acclimatization, micropropagated plants were transferred to 3-L pots filled with a mixture of a fertilized substrate (*SIRO Plant*, Mira, Portugal) and natural soil from the local area (2:1, v/v). Pots were placed in a controlled-environment cabinet (*Fitoclima 16.000 EHVP*, Aralab, Portugal) under the following conditions: photoperiod 12 h, relative humidity 60% and photosynthetic photon flux density (PPFD) *ca*. 450 μmol m^{-2} s^{-1} at the top of the plants. A set of plants, identified as the reference, were grown under a thermal regime similar to that of spring at Algarve: 25/18ºC (day/night) and they were maintained always well watered. Another set of similar plants were placed under the same conditions but the air temperature was adjusted to 32/21ºC, (2ºC above the summer average temperature at Algarve). Throughout the experiment, each pot was daily brought to field capacity replacing all the water lost by the evapotranspiration (HT). To avoid bias caused by a microenvironmental variation within the cabinet (light, temperature, and humidity gradients), the pots were rotated on the benches every day. Following 15 d of acclimation, a group of 10 plants from thermal regime of 32/21ºC were subjected to progressive drought by stopping irrigation for 10 days (HT+WS). At this time, 5 plants were analysed for water stress effects and, in order to study the recovery from drought, 5 water-stressed pots were rewatered to a field capacity (HT+RW) and plants were sampled 24 h after rewatering. 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 (mid-morning).

Soil and plant water status: 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 the field capacity and W_R at the day of measurements. A plant water status was assessed by measuring both leaf water potential (Ψ_w) at the end of the dark period and a leaf relative water content (RWC). Ψ_w was measured using a *Scholander* type pressure chamber model *600* (*PMS Instruments*, Corvallis, OR, USA). RWC was determined in leaf discs, and calculated as 100 × (FM − DM)/(TM − DM), where FM is the fresh mass, TM is turgid mass (determined after floating discs for 3 h on distilled water) and DM is the dry mass (determined after drying discs at 80ºC until they reached a constant mass).

Gas-exchange measurements: g_s, P_N, transpiration rate (E) and substomatal CO_2 concentration (C_i) were determined using a portable gas exchange measuring system (*HCM-1000, H. Walz*, Effeltrich, Germany). The ratio P_N/E (water-use efficiency, WUE), P_N/C_i (carboxylation efficiency), and ETR/P_N (an indicator of electron transport to acceptors different than those used in CO_2 assimilation), were also calculated. Measurements were made under environmental conditions inside the growth chamber: 360 μmol(CO_2) mol^{-1}(air), relative air humidity 50–60%, air temperature 25/32ºC, PPFD 250–300 μmol m^{-2} s^{-1} and a flow rate 800 cm^3 min^{-1}.

Chlorophyll (Chl) fluorescence was imaged using a mini blue version of *Imaging-PAM* Chl fluorometer (*IMAG-MIN/B*, *Walz*, Effeltrich, Germany) on the adaxial side of leaves after 20 min of darkness. In order to evaluate spatial and temporal heterogeneity, four areas of interest (AOIs), circle diameter of 3 mm, 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 using a saturating blue pulse (2,700 μmol m^{-2} s^{-1} PPFD and 800 ms duration) at 10 Hz and the images of F_v/F_m were derived from that. Then, the actinic illumination [250 μmol(photon) m^{-2} s^{-1}] was switched on and saturating pulses were applied in order to determine the maximum fluorescence yield (F_m') and the Chl fluorescence yield (F_s) during illumination. In the light-adapted state, the effective quantum yield of PSII photochemistry, 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): $(F_m' - F_s)/(F_m' - F_0') \times F_0'/F_s = q_p \times F_0'/F_s$. The value of F_0' was estimated using the approximation of Oxborough and Baker (1997): $F_0' = F_0/(F_v/F_m + F_0/F_m')$. Quantum yields of regulated energy dissipation (Φ_{NPQ}) and of nonregulated energy dissipation (Φ_{NO}) in PSII were calculated according to Kramer *et al*. (2004).

Analysis of leaf pigments (Chl, Car, and anthocyanin) 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 total Chl and Car contents were estimated according to Lichtenthaler (1987).

Anthocyanin was extracted from leaf samples (1.5 cm^2) in 1 mL of methanol-HCl solution (0.1% HCl, v/v) and kept at 4ºC in the dark during 4 h. After centrifugation at 10,687 × g for 20 min, 1 ml of the supernatant was transferred to glass cuvette and measured in a spectrophotometer (*Shimadzu UV-160*, Kyoto, Japan).

Anthocyanin concentration was calculated according to Mancinelli (1984) using the formula $A_{530} - 0.25\ A_{657}$, to account for interference from chlorophylls. Anthocyanin content was calculated as cyanidin-3-glucoside using an extinction coefficient 30 mL μmol^{-1} and 445 as the molecular mass.

Lipid peroxidation, proline and soluble sugar contents: The level of the oxidative damage to lipids was estimated as described in Hodges *et al*. (1999), with some modifications according Correia *et al*. (2006). Lipid peroxidation was estimated as the accumulation of thiobarbituric acid-reactive substances (TBARS) and expressed as equivalents of MDA, calculated from their extinction coefficient of 155 $mM^{-1}\ cm^{-1}$. Absorbance 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 described by Bates *et al*. (1973) omitting phosphoric acid to avoid interference with concentrated sugars (Magné and Larher 1992). The reaction mixture containing the plant extract and 1% ninhydrin (w/v, in 60% acetic acid) was boiled for 1 h. After cooling, the resulting chromogen was extracted with toluene and the free proline in the organic phase was quantified by spectrophotometry at 520 nm against a set of proline standards (0–750 μM), as explained in Osório *et al*. (2011).

Nonstructural carbohydrates (soluble sugars) were extracted from intact leaf discs, with 80% (v/v) ethanol, at 80ºC, for 20 min. Sucrose, fructose, and glucose were then measured in the ethanol extract using a spectrophotometric enzyme-coupled assay, as described by David *et al*. (1998) and the results were expressed as glucose equivalents.

Statistical analysis: All experiments were carried out using randomly-selected plants. *SPSS® release 16.0.1* (*SPSS*, Chicago, IL, USA) was used for statistical analysis, and *SigmaPlot® version 10.00* (*Systat Software*, San Jose, CA, USA) for data presentation. Intergroup comparisons were carried out by analysis of variance. For additional pairwise comparisons, the *Student-Newmans-Keul* test was used. Differences were considered significant at $P \leq 0.05$. Values are presented as mean ± standard error for 5 replicates.

Results

Soil and plant water measurements: The enhancement of the temperature did not cause a soil water depletion and changes in water relations in well-watered plants. Indeed, the high temperature, *per se*, did not change the soil water content, Ψ_w or RWC in comparison with the reference (Fig. 1). However, by the end of the water deprivation period (HT+WS), the soil water content dropped to 40% of maximal capacity and Ψ_w attained a mean value about 3-fold lower (−1.28 ± 0.28 MPa) than that of the well watered plants (reference or HT). Despite the marked depression observed in Ψ_w, the RWC was kept considerably high (~88%), the lower leaves begin to wilt and show symptoms of senescence. Plants exhibited a great ability for regaining the optimal water status, as recovery was complete 24 h after rewatering.

Gas exchange and Chl fluorescence: P_N was not affected by HT alone. On the contrary, it was strongly inhibited (~ 71%) by a combination of HT and water stress in comparison with the reference plants (Fig. 2*A*). g_s increased considerably due to HT to values approximately 256 mmol $m^{-2}\ s^{-1}$, which were three times superior to those of the reference plants (Fig. 2*B*). However, g_s decreased considerably due to water stress (~79%) achieving values similar to those of the reference plants. The effects on the transpiration (E) were quite similar to those of g_s (Fig. 2*C*). After rewatering, both P_N and g_s remained low for at least 24 h. As shown in Fig. 2*D*, the instantaneous carboxylation efficiency (P_N/C_i) was significantly depressed by all treatments relative to the reference, but the lowest value was

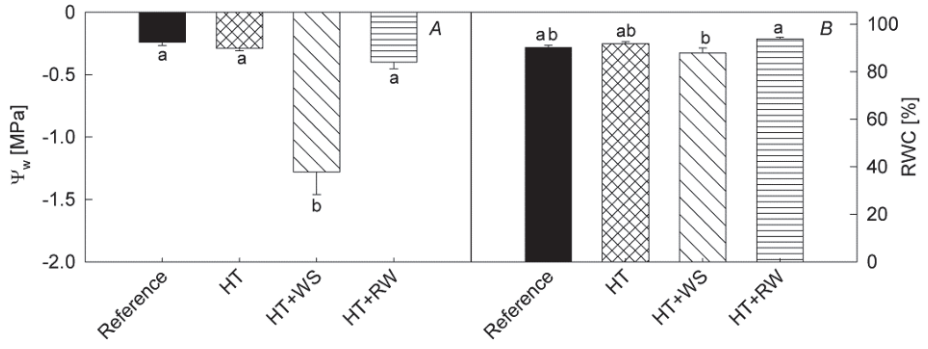

Fig. 1. (*A*) Predawn water potential (Ψ_w) and (*B*) relative water content (RWC) determined in leaves of *T. major* grown at 25ºC and well watered (Reference), and grown at 32ºC and well watered (HT), water-stressed (HT+WS) or rewatered (HT+RW). *Different letters* indicate significant differences between treatments ($P<0.05$) according SNK test. Values shown are means ± standard error of 5 replicates.

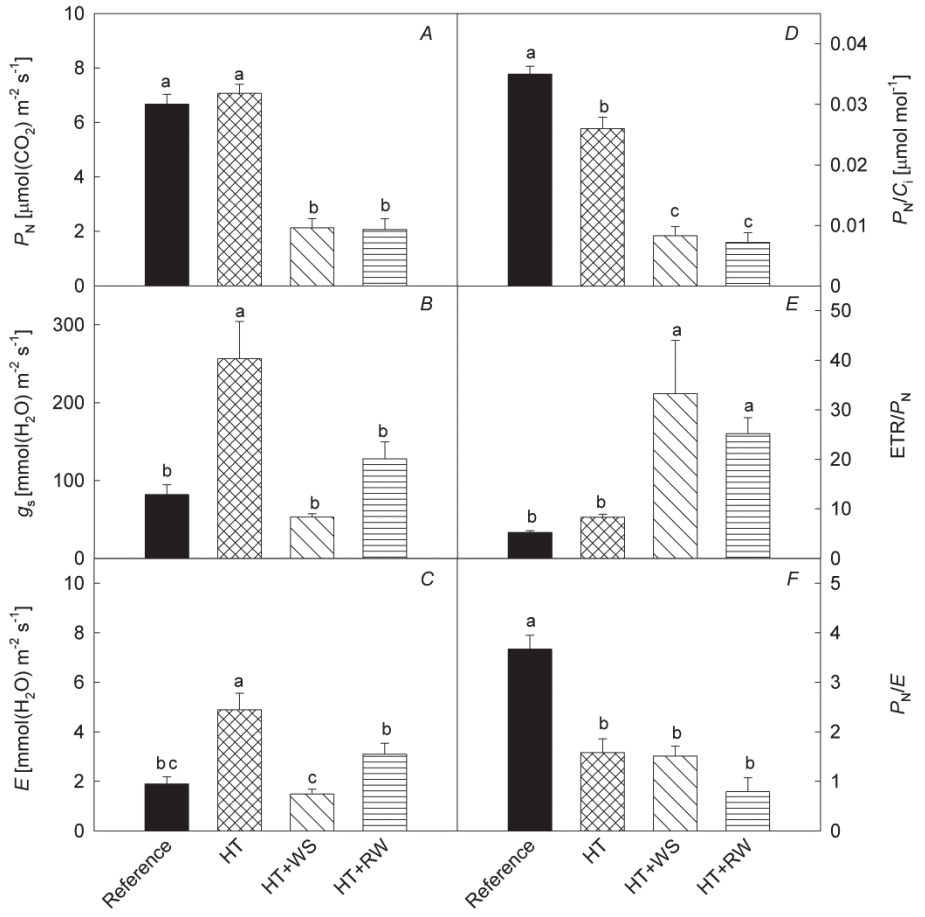

Fig. 2. (*A*) Photosynthetic rate (P_N), (*B*) stomatal conductance (g_s), and (*C*) transpiration (*E*), (*D*) P_N/C_i ratio, (*E*) ETR/P_N ratio, and (*F*) water-use efficiency (P_N/E) determined in leaves of *T. major* grown at25ºC and well watered (Reference), and grown at 32ºC and well watered (HT), water-stressed (HT+WS) or rewatered (HT+RW). *Different letters* indicate significant differences between treatments ($P<0.05$) according to SNK test. Values shown are means ± standard error of 5 replicates.

observed in the plants exposed to the combined stress (HT+WS). On the contrary, the ETR/P_N ratio (Fig. 2*E*) increased significantly in those plants. WUE was reduced by the HT and HT+WS treatments in comparison with the reference plants (Fig 2*F*). The alterations in all of these parameters persisted twenty four hours after rewatering.

The Chl fluorescence images revealed almost a homogeneous pattern of the distribution of F_v/F_m and Φ_{PSII}, Φ_{NPQ}, and Φ_{NO} (Fig. 3). Since no significant differences were found between the values of the four selected areas of interest (AOIs), the statistical analysis was performed on the pooled data of all AOIs. The reference plants exhibited lower F_v/F_m values (9%) than HT plants (Fig. 4*A*), but despite being significantly different, they were roughly close to that usually observed in healthy leaves (around 0.8). The data plotted in Fig. 4*A*, show also that the water-stress-induced depression in photosynthesis was not accompanied by irreversible photoinhibitory damage to PSII reaction centres. In fact, the maximum quantum efficiency of PSII, estimated by the fluorescence ratio, F_v/F_m, of dark-adapted leaves, did not change under water stress relative to the well watered plants at HT. However, the effective quantum yield of PSII photochemistry (Φ_{PSII}) of light-adapted leaves, declined significantly in HT+WS plants and, here again, no recovery was observed (Fig. 4*B*). Such a change resulted solely from a significant decrease in the efficiency of excitation energy capture (F_v'/F_m'), given that the fraction of open PSII centers (q_L) was not affected (data not shown). Interestingly, in HT plants, Φ_{PSII} was significantly higher than in the reference plants (70%).

Photosynthetic energy partitioning: The model proposed by Kramer *et al*. (2004) was used to examine the partitioning of the absorbed light energy into three fractions which add up to unity: (*1*) utilised by PSII photochemistry (Φ_{PSII}), (*2*) thermally dissipated *via* ΔpH and xanthophyll-dependent energy quenching (Φ_{NPQ}), and (*3*) absorbed light going neither to Φ_{PSII} nor Φ_{NPQ}, *i.e.* nonregulated energy dissipation (Φ_{NO}). The results obtained are shown in Fig. 4 (*B*,*C*,*D*). The pattern of energy partitioning changed when the growth temperature was enhanced. The significant increase observed in Φ_{PSII}

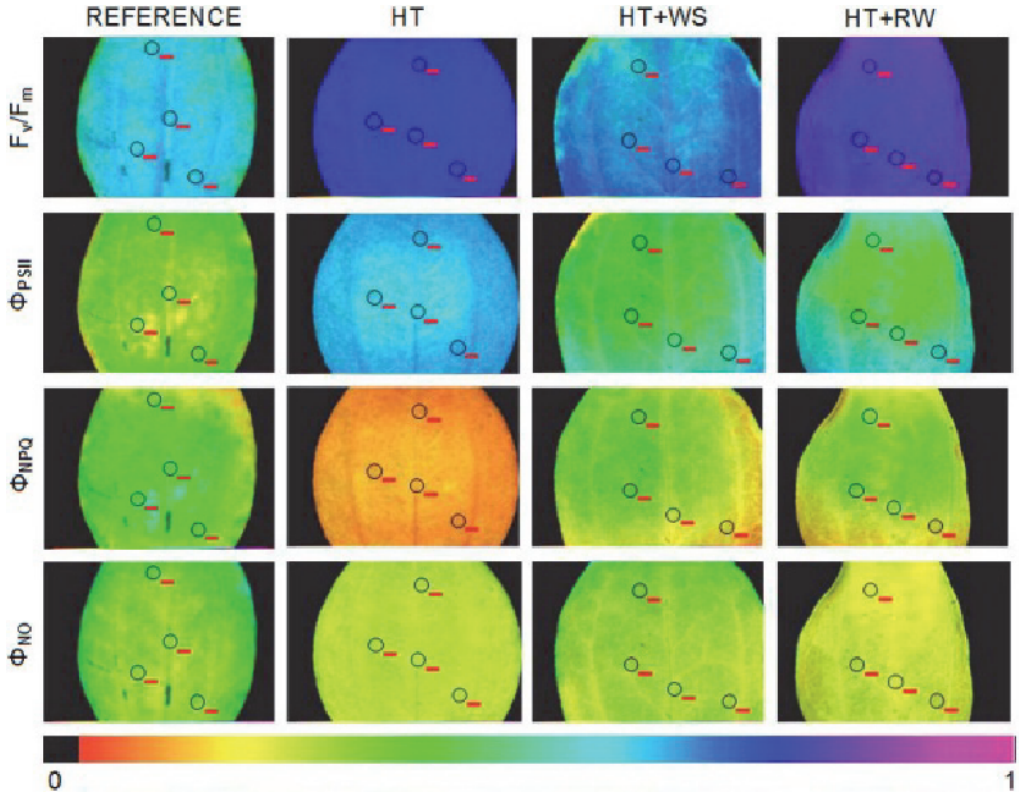

Fig. 3. Chlorophyll fluorescence images of maximum PSII photochemical efficiency (F_v/F_m) in the dark-adapted leaf, actual PSII quantum yield (Φ_{PSII}), quantum yield of regulated energy dissipation of PSII (Φ_{NPQ}) and quantum yield of nonregulated energy dissipation of PSII (Φ_{NO}) at steady-state measured in leaves of *T. major* grown at 25ºC and well watered (Reference), and grown at 32ºC and well watered (HT), water-stressed (HT+WS) or rewatered (HT+RW). The false colour code depicted at the bottom of images ranges from 0.000 (black) to 1.000 (pink). For each selected sample leaf, 4 areas of interest (AOIs) were defined and displayed as small circles in each image, accompanied by a little red box with the averaged values of fluorescence parameters used for quantitative analysis showed in Fig. 4.

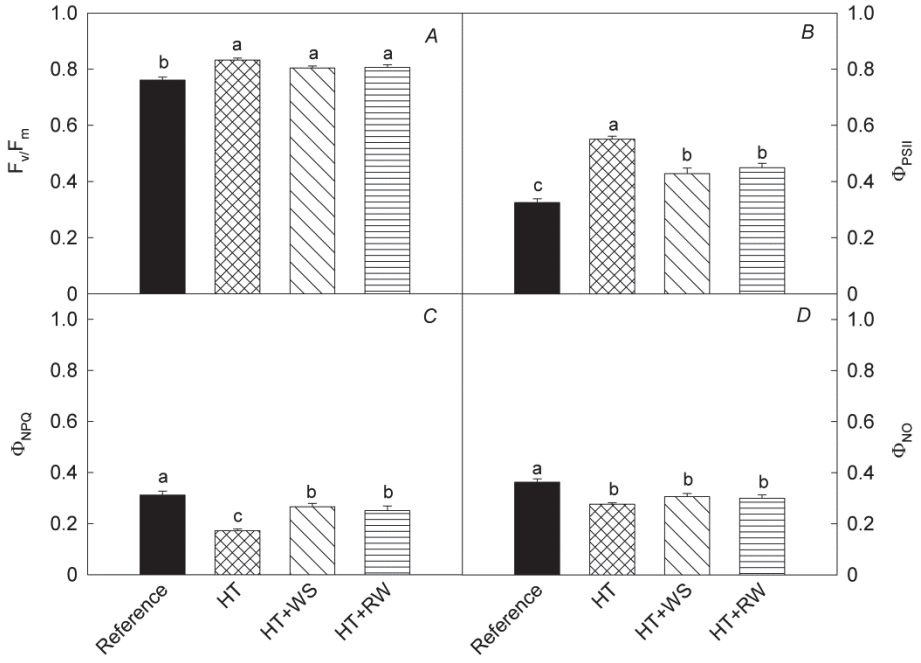

Fig. 4. Quantitative analysis for imaged chlorophyll fluorescence parameters: (*A*) maximum PSII photochemical efficiency (F_v/F_m), (*B*) actual PSII quantum yield (Φ_{PSII}), (*C*) quantum yield of regulated energy dissipation of PSII (Φ_{NPQ}) and (*D*) quantum yield of nonregulated energy dissipation of PSII (Φ_{NO}) at steady-state of *T. major* grown at 25ºC and well watered (Reference), grown at 32ºC and well watered (HT), water-stressed (HT+WS) and/or rewatered (HT+RW). *Different letters* indicate significant differences between treatments (P<0.05) according SNK test. Values shown are means ± standard error of 5 replicates.

Table 1. Changes in total chlorophyll (Chl), total carotenoids (Car), anthocyanins, malondialdehyde (MDA), proline, and total soluble sugars (SS) determined in *T. major* leaves of reference, HT, HT+WS and recovery (HT+RW) treatments. *Different letters* indicate significant differences of those parameters between treatments (P<0.05) according SNK test. Values shown are means ± standard error of 5 replicates.

Treatment	Chl [g m^{-2}]	Car [g m^{-2}]	Anthocyanins [mg m^{-2}]	MDA [nmol g^{-1}(FM)]	Proline [μmol g^{-1}(FM)]	SS [μmol g^{-1}(FM)]
Reference	0.472 ± 0.019^{a}	0.047 ± 0.005^{b}	0.699 ± 0.810^{b}	20.3 ± 9.5^{a}	0.073 ± 0.0003^{a}	6.73 ± 0.61^{b}
HT	0.499 ± 0.027^{a}	0.072 ± 0.003^{a}	1.354 ± 0.224^{a}	23.9 ± 5.1^{a}	0.107 ± 0.064^{a}	8.15 ± 0.90^{ab}
HT+WS	0.444 ± 0.037^{a}	0.077 ± 0.003^{a}	1.471 ± 0.186^{a}	30.8 ± 10.0^{a}	0.119 ± 0.088^{a}	11.03 ± 1.38^{a}
HT + RW	0.447 ± 0.014^{a}	0.079 ± 0.002^{a}	1.163 ± 0.143^{ab}	16.1 ± 4.8^{a}	0.335 ± 0.273^{a}	6.66 ± 0.82^{b}

(~70%) of HT plants was accompanied by a significant decrease in Φ_{NPQ} (~45%) and a lesser decline in Φ_{NO} (~24%) relative to reference plants. When drought was applied, the decline in Φ_{PSII} (~22%) relative to well-watered plants (HT) was balanced by a redistribution of heat dissipation, with a significant increase in Φ_{NPQ} and a slight change in Φ_{NO}. The observed differences in Φ_{PSII} were tightly correlated with the variation in Φ_{NPQ} (Φ_{PSII} = 0.696 – 1.029 Φ_{NPQ}; R = 0.779**) and less strongly with Φ_{NO} (Φ_{PSII} = 0.766 – 1.038 Φ_{NO}; R = 0.426*). Likewise, Φ_{PSII}, Φ_{NPQ} and Φ_{NO} images (Fig. 3) showed clearly that high temperature modified the pattern of energy partitioning with little spatial heterogeneity.

Leaf pigments: As shown in Table 1, total Chl content did not change when plants were subjected to HT alone or in a combination with water stress. In contrast, Car content was significantly higher in HT, HT+WS and HT+RW plants in comparison with the reference ones. Anthocyanin content was also significantly higher in HT and HT+WS plants, but following rewatering, although remaining elevated, there was a small, but statistically insignificant decrease relative to the reference plants.

Lipid peroxidation, proline, and soluble sugar contents: HT individually or in a combination with water stress did not affect significantly TBARS content expressed as equivalents of MDA (malondialdehyde), as well as the proline content (Table 1). In contrast, total soluble sugar (glucose, fructose, sucrose) content was increased by HT and significantly enhanced (twice) in leaves of HT+WS plants, returning to the reference level after rewatering.

Discussion

Heat stress often inhibits photosynthesis in higher plants even at temperatures only slightly greater than those optimal for a growth (Yamamoto *et al*. 2008). In contrast, a high temperature stimulates stomata opening, regardless of CO_2 assimilation status and there is a trade-off between leaf cooling and limiting a water loss during drought periods (Feller 2006). Our results showed that the temperature rise to 32ºC in the absence of water stress was not sufficient to affect the leaf CO_2 assimilation rate of *T. major*. This condition markedly enhanced stomatal conductance and consequently increased evaporative cooling, which in turn led to a leaf temperature decline. In fact, *T. major* plants submitted to 32ºC underwent a leaf temperature drop of *ca*. 5ºC, therefore exhibiting values close to those of the reference plants (26.9 ± 0.84ºC), which could explain why P_N was not depressed in HT plants. Several reports ascribed an important role to a high stomatal conductance in heat avoidance mechanisms of plants. For example, in cotton species both higher yield and heat resistance were attributed to higher stomatal conductance and photosynthetic rate (Lu *et al*. 1997). In our study, despite an increase in g_s and Φ_{PSII}, P_N was not improved, which could result from a loss of CO_2 by an enhanced photorespiration and respiration at a high temperature (Rizhsky 2004). The high temperature alone seemed not to be sufficient to cause the photoinhibition in *T. major*, as it can be inferred from photochemical efficiency data. On the contrary, the photochemical efficiency increased due to HT, as indicated by the observed increase in F_v/F_m and Φ_{PSII} relative to the reference (Fig. 4*A*,*B*), which indicated that photosynthetic apparatus of *T. major* was well adapted to the higher temperature (32ºC). The unexpected increase in Φ_{PSII} by HT alone could be partially explained by different photoprotective mechanisms at different growth temperatures: at 25ºC, such photoprotection may be achieved by regulated thermal dissipation in light-harvesting complexes, which compete with photochemistry for the absorbed energy leading to a decrease in Φ_{PSII} (Demmig-Adams and Adams 1996); at 32ºC, photoinhibition is mitigated by alternative electron sinks for the absorbed energy such as oxygen, mediated by two photochemical mechanisms stimulated by HT, photorespiration (Genty *et al*. 1990, Wingler *et al*. 1999) and the Mehler-peroxidase reaction (Biehler and Fock 1996), leading to an increase of Φ_{PSII}.

However, in the Mediterranean conditions, plants are normally exposed to a high temperature combined with a

low water availability. It has been proven that combined abiotic stresses often generate responses antagonistic or synergistic to those triggered by each individual stress acting alone (Mittler 2006).This is the case of the heat stress, which alone increases the stomatal conductance in order to cool leaves by a transpiration, but when combined with drought, it prevents stomata opening and it may cause a leaf temperature rise of about 2–5ºC (Rizhsky *et al*. 2004). Our results are consistent with this expectation, since leaves of HT+WS plants showed higher temperature sensitivity than HT plants, with oscillations in the range 29–32ºC. Yet despite this, the RWC was kept quite high in order to avoid excessive cell dehydration. It seems that the strategy of the water-stress resistance of this species was to diminish the foliar area, thus maintaining the new leaves in a turgid state. Regardless of high RWC, both CO_2 assimilation rate and photochemical activity were depressed by water stress. Reductions in P_N could be partially attributed to stomatal (low g_s) and to metabolic limitations (low P_N/C_i) (Fig. 2). Additionally, ETR/P_N ratio increased substantially, which suggests that in water-stressed *T. major* energy dissipation from PSII involved also other photochemical processes including electron flux to CO_2 or O_2 *via* photorespiration or the Mehler-peroxidase reaction exacerbating the effects of HT. However, as can be seen in Fig. 4, the decline in actual photochemical efficiency (Φ_{PSII}) in HT+WS plants was mainly counterbalanced by an increase in regulated energy dissipation (increase in Φ_{NPQ}), which requires capability of nonphotochemical quenching, such as has already been reported for other members of the Cistaceae (Kyparissis *et al*. 2000, García-Plazaola *et al*. 2003, Munné-Bosch *et al*. 2003). In our study, both the observed carotenoid content rise at 32ºC (Table 1) and Chl/Car decrease with drought testified the presence of this ability in *T. major*.

Besides being accessory photosynthetic pigments participating in energy capture, Cars also play an important role in preventing oxidative damage, namely by quenching Chl triplets and singlet oxygen ($^1O_2$) and destroying other harmful products of oxidation caused by $^1O_2$ (Frackowiak and Smyk 2007). Anthocyanins are also considered potential protectors of cell membranes against an oxidative damage (Gould *et al*. 2002). Therefore, the elevated content of carotenoid, as well as of anthocyanin, observed in *T. major* might indicate that this species had a large capacity to avoid the oxidative stress. This hypothesis was supported by the low levels of MDA (Table 1), a product of lipid peroxidation and an indicator of an oxidative stress (Weber *et al*. 2004). An accumulation of proline is also often a sign of disturbed physiological conditions triggered by abiotic stress. However, in our case, free proline levels seemed to remain unaffected throughout the experiment, suggesting that other osmolytes, such as nonstructural carbohydrates, were accumulating to provide *T. major* with a good tolerance to combined drought and elevated temperature stress. Although the accumulation of proline, appears to play usually an important role in protecting plants during drought stress (Cushman and Bohnert 2000, Sperdouli and Moustakas 2012), it was reported that it is strongly suppressed during a combination of drought and heat stress (Rizhsky *et al* 2004). It was found that high temperatures amplify the toxicity of proline to cells and it was suggested that during a combination of drought and heat stress, sucrose replaces proline in plants as the major osmoprotectant (Shulaev *et al*. 2008), which is in agreement with our results. Thus, *T. major* achieved photoprotection by a combination of physiological and metabolic responses throughout drought and high temperature exposure periods. These findings reinforce those postulated by Galmés *et al*. (2007) that the Mediterranean species (endemics or not endemics) are resistant to the water-stress-induced photoinhibition and photooxidation. Although in the Mediterranean conditions other factors, such as a high irradiance, may interact with HT and WS affecting physiological behavior of the species, the present work did not allow the assessment of the relative contribution of other factors to the possibility of photoinhibition by high PPFD. However, in the field we verified that F_v/F_m in *T. maj*or was close to the maximum value during midsummer drought (data not published). This finding suggests that *T. major* was able to prevent irreversible photo-oxidative damage to PSII under high PPFD. Additional studies would be required to determine the interaction between water stress, high temperature, and high irradiance.

Conclusion: Although *T. major* exhibited a reduction in photosynthetic capacity in response to drought under the high temperature, its ability to dissipate energy through downregulatory processes and metabolic adjustments were adequate to protect plants from oxidative stress damage during drought and recovery. The species revealed adequate antioxidant protection to cope with episodic water-stress periods under the high temperature similar to that which could occur in the near future in the Mediterranean areas. This leads us to believe that *T. major* could deal satisfactorily with predicted climate warming and increased soil drying without danger of extinction.

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