Manuscript Details

Manuscript number	UFUG_2019_183_R1
Title	An integrated overview of physiological and biochemical responses of Celtis australis to drought stress
Article type	Research Paper

Abstract

Trees in Mediterranean areas frequently face severe drought stress events, due to sudden decreases in soil water availability associated to intense heat waves. The knowledge of strategies adopted by plants to cope with the environmental pressures associated to Mediterranean climate is crucial for reforestation strategies and planning future urban greening. Here we investigated the physiological and biochemical adjustments activated by Celtis australis in response to dought stress during summer. Despite widely used for reforestation in Southern Mediterranean, how C. australis responds to the severe challenges imposed by Mediterranean climate has not investigated yet. In our study, we performed analyses of water relations, gas exchange and PSII performance, the concentration of photosynthetic pigments, the activity and the concentration of primary antioxidants in plants exposed to drought stress of increasing severity. Data of our study reveal that C. australis displays both conservative water use and isohydric behavior in response to drought, and diffusive resistance mostly limits photosynthesis even at severe drought. Our study also reveals an effective down-regulation rather than permanent impairment of PSII photochemistry in response to drought stress of increasing severity, since excess electron transport due to declines in photosynthesis was matched by an increase in nonphotochemical quenching. However, our study highlights that under severe drought, zeaxanthin (and neoxanthin), likely served an important function as chloroplast antioxidant, other than sustaining nonphotochemical quenching. Antioxidant enzymes and ascorbate also contributed in countering oxidative stress in severely droughted plants. Large adjustments in the suite of physiological and biochemical traits may effectively enable C. australis to gain carbon at appreciable rates while avoiding irreversible damage to the photosynthetic apparatus even when challenged by severe drought stress, thereby making this species an excellent candidate for forest and urban plantings in sites experiencing extended periods of drought stress.

Keywords	antioxidant enzymes; gas exchange and PSII performance; isohydry; Mediterranean climate; photo-oxidative stress; xanthophylls

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Response to reviewers

Authors would like to thank reviewers for their comments and suggestions. We edited the manuscript accordingly.

Reviewer 1

Abstract: The second part of the abstract relative to the results should be reported the differences in percentage in order to clearer for the readers.

Percentages have been added in the second part of the abstract

Line 6: drought

Added

Line 83: et al it must not be in italics. Review all the text.

Done

Line 175: How did you determine the proteins?

Protein content was determined using the Protein Assay Kit (Bio Rad®, Hercules, CA, USA). This information has been added in the text.

Line 209-218: Add the differences in percentage in order to clearer for the readers.

Percentages have been added for Jmax, Fv/Fm, F0/Fm, and NPQ as requested

Line 232: also the Apx did not differe between WW and WS at moderate drought. Rewrite.

The sentence has been rewritten as requested: "The activities of antioxidant enzymes, with the exception of CAT, varied significantly early during drought stress imposition (on average by 45% between WW and WS leaves at moderate drought, Fig.6 a-c). In contrast, the activities of all antioxidant enzymes greatly increased at severe drought (on average + 130%)".

Report the unit of measure in the figure 6.

Unit of measure have been added to figure 6 except for ASA/DHA which is a pure number

-Reviewer 2

-Line 81-83. This sentence seems to be extremely speculative, I recommend avoid this sentence. It is not contributing to the surrounding information.

The sentence and the references cited therein have been removed

Line 179: Carbonyl content is measured at 360/370 nm and the protein absorbance is measured at 280 nm.

The sentence was rephrased: "The lipid peroxidation was determined spectrophotometrically based on the formation of malondialdehyde (MDA) using the thiobarbituric acid (TBA) reaction, whereas protein-carbonyl content was determined by the reaction with 2,4-dinitrophenylhydrazine, following the protocols reported in Tattini et al., 2015."

Line 215-216: No statistical differences were observed between moderate and severe drought stress.

The sentence has been rephrased to highlight that Fv/Fm did not show progressive decline during drought: "Maximum efficiency of PSII photochemistry (Fv/Fm, Fig. 4a) decreased similarly at moderate stress and severe stress (-5% and -10%, compared to well-watered trees)"

Line 232: SOD and APX specific activities were statistically increased at moderate stress.

SOD was erroneously indicated as not changing at moderate drought. Actually this was a mistake, because the only antioxidant enzyme not affected at moderate drought was CAT. The text was edited accordingly.

Line 254-255: This sentence seems highly speculative. Could the authors measure those variables? Or has been described as a characteristic for C. australis?

We did not measure stomatal frequency and anatomy in this study. However, previous research (Abrams, 1994) compared these traits in 17 species, including Celtis, and we used these findings to interpret our data. We are aware this might be somehow speculative, so we rephrased the sentence to highlight that we did not measure this traits directly: "Though we have not measured morphological traits of leaf surface, the dense indumentum of non-glandular trichomes coupled with a low frequency of paracytic and small-sized stomata previously reported in Celtis (Abrams et al., 1994) might have contributed to high stomatal limitations to photosynthesis observed in our study"

Line 252-256: please revise the coherence of this sentence. It seems a redundant or cyclic thought, mixing causes and consequences.

The sentences have been revised (see comment above).

Line 282: Is the reference related to the experimental system or is it a possible explanation of the results? Please rewrite the sentence.

The sentence has been rephrased: "The carotenoid reduction in WW leaves was possibly due to the concomitant effect of high both solar irradiance and air temperature to which leaves were exposed in our study, as also recently reported by (Tattini et al. (2015)."

Line 283: please provide the meaning of "MEP".

MEP has been spelled out

Line 326-327: ASA decreased in WS plants, as revealed by the reduced ASA/DHA ratio.

ASA does not change significantly between WW and WS plants at both moderate and severe stress. This is clear in figure 6d. The change in ASA/DHA observed at severe stress was due to a slight increase in DHA. The sentence was changed, however, to improve clarity and readability: "Since drought stress did not affect the ASA concentration, whereas the ASA/DHA ratio decreased relatively little (-18%) even in severely droughted WS leaves, it is suggested that the chloroplasts were equipped with an efficient system for the removal of H2O2 (Jubani-Mari et al., 2010)."

Line 328-331: Please clarify this sentence meaning. Are the authors aiming to correlate CAT specific activity with photorespiration? Or are independent mechanisms that could contribute to C. austrails drought-response?

CAT and photorespiration are independent mechanisms that contribute to drought response. However, because CAT is mainly located in peroxisomes, enhanced activity of this enzyme may be particularly important to metabolize photorespiratory H2O2, which, as noted above, can be produced at higher rates when water becomes limiting. The sentence has been rephrased to improve clarity

Lines 467, 471, 486: Please revise the reference style.

Done

Line 493: Please provide the year of publication.

Done

Highlights

An avoidance mechanism (isohydry) governs the responses of *C. australis* to drought

Diffusional limitations mostly constrain photosynthesis even at severe drought

De-epoxided xanthophylls mostly sustain NPQ at moderate, but not at severe drought

Zeaxanthin and neoxanthin likely serve relevant antioxidant functions at severe drought

Antioxidant enzymes and ASA constitute an effective ROS detoxification system

An integrated overview of physiological and biochemical responses of *Celtis australis* to drought stress

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ABSTRACT

Trees in Mediterranean areas frequently face severe drought stress events, due to sudden decreases in soil water availability associated to intense heat waves. The knowledge of strategies adopted by plants to cope with the environmental pressures associated to Mediterranean climate is crucial for reforestation strategies and planning future urban greening. Here we investigated the physiological and biochemical adjustments activated by *Celtis australis* in response to drought stress during summer. Despite widely used for reforestation in Southern Mediterranean, how *C. australis* responds to the severe challenges imposed by Mediterranean climate has not investigated yet. In our study, we performed analyses of water relations, gas exchange and PSII performance, the concentration of photosynthetic pigments, the activity and the concentration of primary antioxidants in plants exposed to drought stress of increasing severity. Data of our study reveal that *C. australis* displays both conservative water use and isohydric behavior in response to drought, and diffusive resistance mostly limits photosynthesis even at severe drought. Our study also reveals an effective down-regulation rather than permanent impairment of PSII photochemistry in response to drought stress of increasing severity, since excess electron transport due to declines in photosynthesis (-61% at severe stress, compared to control) was matched by an increase in nonphotochemical quenching (+71% at severe stress, compared to control). However, our study highlights that under severe drought, zeaxanthin (and neoxanthin) increased by 75% (and 25%), likely served an important function as chloroplast antioxidant, other than sustaining nonphotochemical quenching. Antioxidant enzymes and ascorbate also increased (+132% on average for superoxide dismutase, ascorbate peroxidase, and catalase) and contributed in countering oxidative stress in severely droughted plants. Large adjustments in the suite of physiological and biochemical traits may effectively enable *C. australis* to gain carbon at appreciable rates while avoiding irreversible damage to the photosynthetic apparatus even when challenged by severe drought stress, thereby making this species an excellent candidate for forest and urban plantings in sites experiencing extended periods of drought stress.

Key words: antioxidant enzymes, gas exchange and PSII performance, isohydry, Mediterranean climate, photo-oxidative stress, xanthophylls

1. Introduction

There is strong interest in understanding plant responses to drought since the frequency of intense dry periods has increased much during the last three decades, and it is predicted to rise further because of climate change (Flexas et al., 2014; Matesanz and Valladares, 2014; Percival, 2017). Plants living in the Mediterranean basin frequently experience severe drought conditions, since rainfall scarcity occurs in concomitance with intense heat waves under excessive solar irradiance (Bussotti et al., 2014; Matesanz and Valladares, 2014). The negative impact of multiple stressors on plant performance will be exacerbated by climate change (Allen et al., 2010), thereby increasing the risk of regional-scale mortality in both forest and urban Mediterranean areas (Giorgi and Lionello, 2008). There is recent evidence, indeed, that in response to drought stress, Mediterranean plants display a more negative predawn leaf water potential (ψ_w) compared to not only temperate and tropical species, but also to desert plants (Martinez-Vilalta et al., 2014).

Plants inhabiting the Mediterranean areas adopt different strategies to cope with the severe scarcity of water available to the roots during the summer period (Lo Gullo and Salleo 1988; Quero et al., 2011), which originates from both rainfall scarcity and high evapo-transpiration demand. Some species display a near-anisohydric stomatal behavior in response to drought stress, which is manifested by low stomatal sensitivity to vapor pressure deficit (VPD) an active osmotic adjustment, and marked changes in cell wall elasticity and xylem traits (Kozlowski and Pallardy, 2002). This results in near-anisohydric plants actively decreasing their leaf ψ_w to similar or even greater extent than the drop in soil water potential, thereby maintaining soil-to-leaf water flux and a positive net carbon assimilation, even at very negative soil water potentials (Kozlowski and Pallardy, 2002; Roman et al., 2015). Instead, near-isohydric species display early depression in stomatal conductance to maintain ψ_w within a narrow range and avoid embolism, but at expenses of photosynthetic gas exchange (Quero et al., 2011; Tattini et al., 2015). This large reduction in the use radiant energy for carbon fixation enhances greatly the generation of reactive oxygen species (ROS), and imposes severe photo-oxidative stress to leaves (Hernández et al., 2012; Tattini et al., 2015).

Highly integrated biochemical adjustments operate in plants to effectively limit ROS formation and scavenge ROS once they are formed in response to drought (Noctor et al., 2014). These include the activation of a network of 'antioxidant' defenses, primarily constituted by photosynthetic pigments, antioxidant enzymes and low molecular weight antioxidants (Apel and Hirt, 2004; Pintó-Matijuan and

Munné-Bosch, 2014; Esteban et al., 2015a,b). This metabolic plasticity is of crucial significance for the survival of near-isohydric plants to the environmental challenges imposed by the Mediterranean climate (Tattini et al., 2015). There is still uncertainty about the effectiveness of such antioxidant system in preserving leaves from irreversible photooxidative damage during severe drought stress (Fini et al., 2012; Noctor et al., 2014; Tattini et al., 2015).

For instance, loss of chlorophyll may reduce centers of light absorption under excessive sunlight, but contemporarily limits the plant capacity to assimilate carbon and to promote new growth. There is controversial evidence that reduction in chlorophyll concentration is effective in reducing drought-induced photooxidative damage (Munné-Bosch et al., 2001; 2003), and chlorophyll loss has been associated to species or genotypes that display low resistance to drought (Colom and Vazzana 2003). In contrast, increases in carotenoid biosynthesis in response to several abiotic stresses have been documented in several species (possibly through ROS-mediated signaling, Fanciullino et al., 2014), with a very few exceptions (e.g. when leaves suffer from very severe dehydration, Colom and Vazzana 2003). This conforms to the notion that the ratio of carotenoids, particularly of violaxanthin cycle pigments (VAZ) to chlorophyll, increases when the radiation use efficiency is limited by a wide range of environmental constraints, including drought (Esteban et al., 2015a; Tattini et al., 2015). Carotenoids may indeed serve functions that go well beyond their mere ability to quench the excess energy in the chloroplast, when plants face severe drought (Davison et al., 2002; Demmig-Adams and Adams, 2006; Ramel et al*.,* 2012; Esteban et al., 2015a,b). Zeaxanthin (Zea) may enhance the rigidity of thylakoid membranes, thus limiting lipid peroxidation (Jahns and Holzwarth, 2012; Domonkos et al., 2013; Havaux and Garcia-Plazaola, 2014; Esteban et al., 2015a, Tattini et al., 2015). Similarly, β-carotene (β-car), due to its specific location in the core complexes of PSI and PSII, may effectively quench the highly reactive singlet oxygen ($^1O_2$), rather than deactivate the chlorophyll triplet state ($^3Chl^*$) (Ramel et al., 2012, 2013).

Similarly, the effectiveness of antioxidant enzymes to control cellular redox homeostasis has been questioned in some instances (Peltzer and Polle, 2001; Peltzer et al., 2002; Fini et al., 2011, 2012). There is evidence that the activities of catalase and ascorbate peroxidases substantially decrease in plants facing a severe light excess (Mubarakshina et al., 2010; Fini et al., 2011, Agati et al., 2012; Fini et al., 2012), as observed in isohydric plants suffering from multiple stressors associated to Mediterranean summer conditions (Tattini et al., 2015).

Celtis australis, native of thermophile mixed deciduous forests in the South Mediterranean is largely used in reforestation plans in semiarid riparian zone as well as in restoration programs of natural Mediterranean ecosystems (Schirone et al., 2011). In addition to its importance in natural Mediterranean ecosystems, *C. australis* is also widely planted in the green infrastructure in cities across the Southern Mediterranean (Oliveira et al., 2011; Gratani et al., 2016). Despite the large utilization of this species to extensively replace ornamental trees that are not native to the Mediterranean (Konijnendijk, 2008), the responses of *C. australis* to drought have been poorly investigated.

Therefore, in our study we investigated the physiological and biochemical strategies adopted by *C. australis* to cope with drought stress of increasing severity during Mediterranean summer. We analyzed (1) water relations, gas exchange and PSII performance; (2) relevant biochemical traits involved in photoprotection mechanisms, such as the concentration of photosynthetic pigments, the activity and the concentration of primary antioxidants and (3) markers of photo-oxidative damage, related to the oxidation of membrane lipids and proteins.

2. Material and Methods

2.1. Plant material and growth conditions

Two–year-old *Celtis australis* L. plants were grown in 8-L pots with a peat/pumice substrate (50:50, v:v), and grown outside in screen houses in Florence, Italy (43° 46' N, 11° 15' E). Screen houses were covered with a 100 µm ETFE fluoropolymer film (NOWOFLON® ET-6235, NOWOFLON® Kunststoffprodukte GmbH & Co. KG, Siegsdorf, Germany), as reported in Agati et al. (2011). Drought stress was imposed by withholding water (WS plants) whereas control plants (WW) were irrigated daily to pot capacity, over a two-week experimental period. Pots were weighed daily, and actual water content (AWC) of the substrate, a parameter depicting available moisture, was calculated as described in Fini et al. (2013). Solar irradiance and air temperature, over the experimental period, were recorded at the Institute of Biometeorology of the National Research Council of Italy (http://www.lamma.rete.toscana.it/en/weather-stations-data) located 200 m away from the experimental site. The experiment was performed in July, under minimum/maximum temperatures of 18.3 ± 1.5 / 33.1 ± 2.6 °C, and midday photosynthetic photon flux density (PPFD) of 1780 ± 140 µmol quanta m^{-2} s^{-1} (mean ± standard deviation, n = 15). Measurements were conducted one and two weeks after withholding water in fully developed leaves.

2.2. Analysis of water relations, gas exchanges and chlorophyll fluorescence

Measurements of predawn water (ψ_w) and osmotic (ψ_π) potentials, and relative water content (RWC) were conducted as in Tattini et al. (2002). Net assimilation rate (A_N), stomatal conductance (g_s), and intercellular CO_2 concentration (C_i) were measured using a LI-6400 portable photosynthesis system (Li-Cor, Lincoln, NE, USA). Measurements were conducted at 1200 μmol photons m^{-2} s^{-1} , a leaf temperature of 30°C, and external CO_2 concentration of 400 μmol mol^{-1}. Irradiance was provided by the Li-Cor integrated light source. To draw photosynthetic response curves to internal CO_2 concentration (A_N/C_i), measurements were first recorded at 400 μmol mol^{-1} external CO_2 concentration (C_a). Then, C_a was decreased stepwise to 50 μmol mol^{-1}, returned to 400 μmol mol^{-1}, finally increased to 1800 μmol mol^{-1} as reported in Tattini et al. (2015). The apparent maximum carboxylation rate allowed by Rubisco ($V_{c,max}$), and the apparent maximum electron transport rate contributing to ribulose-1,5-bisphosphate (RuBP) regeneration (J_{max}) were calculated from A_N/Ci curves, as described by Sharkey et al. (2007). A quantitative analysis of stomatal (SL), and non-stomatal (NSL) limitations to A_N was performed from A_N/Ci curves as described previously (Lawlor, 2002; Long and Bernacchi, 2003). Briefly, SL were assessed as: SL = (A''-A')/A'', where A' is net CO_2 assimilation at ambient CO_2 concentration and A'' is CO_2 assimilation assuming Ci = Ca (i.e. infinite gs). NSL were assessed as NSL = (A – B)/A where A and B denote CO_2 assimilation at ambient CO_2 concentration in unstressed and drought stressed plants, respectively.

Chlorophyll-fluorescence kinetics analysis was conducted using an Imaging-PAM chlorophyll fluorometer (Heinz Walz, Effeltrich, Germany), as detailed in Tattini et al. (2015). Minimum fluorescence (F_0) was determined after a 0.8 μmol m^{-2} s^{-1} measuring light in dark-adapted leaves (over a 30-min period), and maximum fluorescence in the dark-adapted state (F_m) using saturating pulses (0.5 s) of red light (8000 μmol m^{-2} s^{-1}). Maximum PSII photochemistry (F_v/F_m) was then calculated as $F_v/F_m = (F_m - F_0)/F_m$. Steady state fluorescence (F_s) was recorded under actinic light of 1000 μmol m^{-2} s^{-1}, then the maximum fluorescence under actinic light (F_m') was recorded following saturating light pulses. Nonphotochemical quenching (NPQ) was calculated as NPQ= $(F_m - F_m')/F_m'$) and actual quantum yield of PSII (Φ_{PSII}) as $\Phi_{PSII} = (F_m' - F_s)/F_m'$. The electron transport rate (ETR) was calculated as ETR = 0.5 × Φ_{PSII} × PPFD × leaf absorptance. The factor 0.5 assumes an equal distribution of photons between PSI and PSII. Leaf absorptance of 0.87 was determined using a Li-Cor 1800 spectroradiometer equipped with a Li-Cor 1800-125 integrating sphere, as previously reported (Tattini et al. 2005).

2.3. Analysis of photosynthetic pigments

Photosynthetic pigments were analyzed following the protocol of Tattini et al. (2015). Fresh leaf material (300 mg) was extracted with 2 × 5 mL acetone (with the addition of 0.5 g L^{-1} $CaCO_3$) and injected (15 µL) in a Perkin Elmer Flexar liquid chromatograph equipped with a quaternary 200Q/410 pump and a LC 200 photodiode array detector (PAD) (Perkin Elmer, Bradford, CT, USA). Photosynthetic pigments were separated in a 250 × 4.6 mm Agilent Zorbax SB-C_{18} (5 µm) column operating at 30°C, and eluted with a linear gradient solvent system, from 100% CH_3CN/MeOH (95/5 with 0.05% of triethylamine) to 100% MeOH/ethyl acetate (6.8/3.2), at a flow rate of 1 mL min^{-1} over a 18-min run. Violaxanthin-cycle pigments (violaxanthin, Vio; antheraxanthin, Ant; zeaxanthin, Zea), neoxanthin (Neo), lutein (Lut), β-carotene (β-car), chlorophylls *a* and *b*, were identified using spectral characteristics and retention times. Pigments were quantified using authentic standards from Extrasynthese (Lyon-Nord, Genay, France) and from Sigma Aldrich (Milan, Italy), respectively.

2.4. Analysis of antioxidant enzymes and ascorbic acid

The activities of antioxidant enzymes and the concentrations of reduced (ASA) and oxidized (DHA) ascorbic acid concentration were determined spectrophotometrically on 500 mg fresh leaf material, frozen in liquid nitrogen and extracted with 2 mL of 100 mM potassium phosphate buffer (pH 7.0) with the addition of ethylene diamine tetra-acetic acid (EDTA), as recently reported (Tattini et al., 2015). Protein content was determined using the Protein Assay Kit (Bio Rad®, Hercules, CA, USA). Catalase (*CAT*, EC 1.11.1.6) activity was measured at 270 nm by measuring the rate of conversion of H_2O_2 to O_2 and H_2O. The activity of ascorbate peroxidase (*APX*, EC 1.11.1.11) was determined by monitoring at 265 nm the H_2O_2-dependent ASA oxidation in a reaction mixture (in phosphate buffer, pH 6.4) consisting of 50 µM ASA, 90 µM H_2O_2, 50-100 µg protein. Non-enzymatic H_2O_2-dependent and H_2O_2-independent ASA oxidation were subtracted to correct *APX* activity. The analysis of superoxide dismutase (*SOD*; EC 1.15.1.1) activity was conducted by measurements at 560 nm of the inhibition of nitroblue tetrazolium (NBT) reduction by SOD. The amount of enzyme required to reduce the NBT reduction state by 50% was defined as one unit of SOD. The concentrations of ASA and DHA were determined as in Tattini et al. (2015).

2.5. Analysis of lipid peroxidation and protein oxidation

The lipid peroxidation was determined spectrophotometrically based on the formation of malondialdehyde (MDA) using the thiobarbituric acid (TBA) reaction, whereas protein-carbonyl content

was determined by the reaction with 2,4-dinitrophenylhydrazine, following the protocols reported in Tattini et al., 2015.

2.6. Experimental design and statistical analysis

The experiment was a completely randomized block design, with four blocks (screen houses), each consisting of eight plants per water treatment. Measurements were conducted when AWC of WS plants reached 25-30% (moderate stress, 7 days after withholding water), and when AWC < 10% (severe stress, 15 days after withholding water). Water relations, gas exchange and chlorophyll fluorescence kinetics were analyzed in four replicate plants per treatment between 11:30-14:00 h. Each replicate consisted of two leaves. Identification and quantification of metabolites and the activities of antioxidant enzyme were conducted on four replicate plants (two leaves per replicate) sampled at 11:30 and 14:00 h, and pooled together prior to analysis. Data were analyzed using repeated measures with ANOVA (SPSS v.20, IBM, NY, USA), with water treatment as the between-subjects factor and time as the within-subjects factor. Significant differences among means were determined with Tukey's test at the 5% level.

3. Results

3.1. Drought effects on water relations, gas exchange and PSII performance

C. australis did not suffer from severe leaf water unbalance during drought stress (Fig. 1). Predawn leaf water potential declined already after one week of drought stress, and decreased further, though to lesser extent, as drought stress progressed (Fig. 1a). Turgor potential decreased in WS plants (ψ_p varied from −1.45 in WW to −1.18 MPa in WS leaves at day 15, data not shown), since osmotic potential did not vary between WW and WS plants (Fig. 1b). Nonetheless, relative water content (RWC) was unaffected by the water treatment (Fig. 1c).

Net photosynthesis (A_N, −29%, Fig. 2a) and particularly stomatal conductance (g_s, −48%, Fig. 2b) decreased substantially already at moderate drought, and declined further as the stress became more severe, with slightly greater reductions in g_s than in A_N. Drought-induced depressions in A_N were mostly due to stomatal limitations (SL, Fig. 3), irrespective of the severity of drought. This is in line with the observation that the intercellular CO_2 concentration (C_i) was lower in WS leaves than in WW leaves throughout the experiment (Fig. 2c). Nonetheless, NSL to A_N (i.e. mesophyll resistance to CO_2 diffusion to chloroplasts plus biochemical constraints to CO_2 carboxylation, Fini et al., 2016) considerably increased from moderate to severe drought (inset in Fig. 3).

The apparent rate of carboxylation by Rubisco ($V_{c,max}$, Fig. 2d) did not vary, whereas the apparent maximum electron transport rate contributing to RuBP regeneration (J_{max}, Fig. 2e) declined significantly (-26%) at moderate drought in WS leaves. Both parameters, particularly J_{max} decreased steeply (-68%) from moderate to severe drought in WS leaves. The electron transport rate (ETR, Fig. 2f) did not vary at moderate drought, and the reduction in ETR at severe drought was less than the declines in other photosynthetic parameters.

Maximum efficiency of PSII photochemistry (F_v/F_m, Fig. 4a) decreased similarly at moderate stress and severe stress (-5% and -10%, compared to well-watered trees), because of increases in the fluorescence yield of open reaction centers (i.e., F_0/F_m increased by 25% in WS leaves at severe drought, Fig. 4b). The removal of excess energy estimated by NPQ significantly increased at moderate stress (+54%), but did not change further at severe drought (Fig. 4c).

3.2. Drought effects on photosynthetic pigments

The concentration of total chlorophyll (Chl_{tot}, Fig. 5a) did not differ between WS and WW leaves, whereas the concentration of carotenoids (Car_{tot}) was higher in severely WS leaves than in the WW ones (Fig. 5b). In WW leaves, all carotenoids, with the exception of Vio (Fig. 5g) and β-car (Fig. 5d), decreased in concentration throughout the experiment. While the concentrations of Lut (+20%, Fig. 5e), Neo (+34%, Fig. 5f) and Zea (+34%, Fig. 5h) increased in WS leaves from moderate to severe drought, the reverse was observed regarding the concentration of β-car. The concentration of VAZ pigments in WS leaves largely exceeded that in WW leaves, not only on Chl_{tot} basis (on average +51%, Fig. 5c), but also on leaf mass basis (+39%, data not shown). The de-epoxidation state of VAZ pigments (DES, Fig. 5i), which was on average 90% higher in WS than in WW leaves, increased already at mild drought, but much less from mild to severe drought.

3.3. Drought effects on enzymatic and non-enzymatic antioxidants, and markers of oxidative damage

The activities of antioxidant enzymes, with the exception of CAT, varied significantly early during drought stress imposition (on average by 45% between WW and WS leaves at moderate drought, Fig.6 a-c). In contrast, the activities of all antioxidant enzymes greatly increased at severe drought (on average + 130%). We did not observe changes in the concentration of ascorbic acid (ASA) between WW and WS

leaves (Fig. 6d), whereas the ratio of ASA to DHA (dehydroascorbic acid) was significantly higher in WW compared to WS leaves at severe drought (Fig. 6e).

Moderately droughted leaves experienced greater photooxidative damage, here estimated by the peroxidation of leaf membrane lipids (MDA, Fig. 7a) and by the formation of carbonyl groups due to protein oxidation (Fig. 7b), compared WW leaves. Instead, both MDA and carbonyl group concentration did not differ between WW and severely droughted WS leaves.

4. Discussion

4.1. Celtis australis displays near-isohydric behavior under drought and diffusional limitations mostly constrain photosynthesis

Our study indicates *C. australis* does not tolerate dehydration since leaf osmotic potential did not vary between WW and WS leaves (Kozlowski and Pallardy, 2002). Instead, markedly higher reductions in A_N and g_s than in both ψ_w and tissue hydration (RWC), irrespective of the severity of drought, are consistent with a near-isohydric behaviour regulating the response of *C. australis* to low water availability. Data of our study suggests that C. *australis* adopts a conservative use of water (Moreno-Gutierrez et al., 2012) to cope with water deficit. Drought stressed leaves display a higher intrinsic water use efficiency (iWUE = A_N/g_s, was on average 105.4 ± 8.3 mmol CO_2 mol^{-1} H_2O, mean ± SD, n = 8) compared to WW leaves (iWUE averaged 75.2 ± 5.6). Water and CO_2 exchange between the atmosphere and the sub-stomatal chamber were hindered by high stomatal limitations occurring in both WW (on average SL accounted for 36%) and WS leaves (SL reached 44%), regardless of soil water availability. Indeed, in our study, saturating photosynthesis and stomatal conductance did not exceed 7.85 μmol m^{-2} s^{-1} and 108.4 mmol m^{-2} s^{-1}, respectively, under well-watered conditions. Though we have not measured morphological traits of leaf surface, the dense indumentum of non-glandular trichomes coupled with a low frequency of paracytic and small-sized stomata previously reported in *Celtis* (Abrams et al., 1994) might have contributed to high stomatal limitations to photosynthesis observed in our study.

Albeit stomatal closure mostly limited A_N in drought stressed *C. australis* leaves, our study also evidences that both the apparent decreases in both maximum Rubisco carboxylation ($V_{c,max}$), and particularly the RuBP regeneration (J_{max}) contributed in limiting photosynthesis at severe drought (Medrano et al., 2002; Flexas et al., 2014). The markedly greater declines in $V_{c,max}$ and J_{max} than in ETR, coupled with small reductions in F_v/F_m, suggest down-regulation rather than permanent impairment of

the photosynthetic apparatus occurred in *C. Australis* even at severe drought (Murchie and Lawson, 2013). In our study, drought-induced declines in F_v/F_m resulted from large enhancements in F_0/F_m. Since Chl_{tot} did not vary between WW and WS plants, we suggest that drought stress mostly affected the photochemical efficiency of PSII reaction centers, possibly due to the physical separation of PSII reaction centers from both light harvesting complexes and from the donor side of PSII (Murchie and Lawson, 2013).

Regulatory mechanisms aimed dissipating the excess energy in the chloroplast are evident in our study. Drought-induced increase in ETR/A_N (on average ETR/A_N varied from 14.2 ± 2.4 in WW to 20.1 ± 2.1 in WS leaves, mean ± s.d., n = 8) was paralleled by an increased amount of energy dissipated as heat, as revealed by the increase in NPQ. However, we note that ETR/A_N significantly increased (P = 0.007) in WS plants from moderate (ETR/A_N = 17.7 ± 1.8, mean ± S.D. n = 4) to severe drought (ETR/A_N = 24.2 ± 1.9) without a parallel enhancement in NPQ. We hypothesize that alternative dissipation processes, such as photorespiration, (see section *4.3* for further details) may have additionally contributed in dissipating excess electrons.

4.2. Carotenoids only in part contributed to nonphotochemical quenching in severely droughted leaves

The concentration of carotenoids decreased in WW, but not in WS leaves during the experiment. The carotenoid reduction in WW leaves was possibly due to the concomitant effect of high both solar irradiance and air temperature to which leaves were exposed in our study, as also recently reported by (Tattini et al. (2015). Since fresh assimilated carbon available to the biosynthesis of Methylerythritol 4-phosphate (MEP)-derived products progressively decreased, we suggest that an increased flux of fresh assimilated carbon was devoted to the biosynthesis of photoprotective pigments in WS leaves, as drought stress became more severe. This may have relevant functional reasons, since WS leaves retained Chl_{tot} at the level of WW leaves, and hence were exposed to photo-oxidative stress of increasing severity as drought stress progressed.

Our hypothesis is further corroborated by the observation that the composition, not only the bulk of carotenoid pigments underwent large variations because of the severity of drought. Firstly, the significantly higher concentration of VAZ, coupled with the greater contribution of Zea to the VAZ pool, sustained the superior dissipation of excess energy through NPQ observed in WS than in WW leaves. It is worth nothing, however, that the concentration of VAZ pigments relative to Chl_{tot} was high enough

(even in WW leaves) to exceed largely their potential binding sites in antenna proteins (Esteban et al., 2015a). This suggests that Zea likely served antioxidant functions in the chloroplast (Havaux et al., 2007; Peguero-Pina et al., 2013; Esteban et al., 2015a,b), the significance of which increased along with the severity of drought stress. This is in line with the observation that while the VAZ pool and de-epoxidation state of VAZ significantly increased, NPQ did not vary in from moderately to severely droughted, WS leaves. Secondly, the increase in Zea concentration was not paralleled by a decrease in Vio concentration, but by a decrease in β-car concentration, as drought stress progressed. We hypothesize that Zea may have been in part synthesized from β-car (through the action of β-car hydroxylase, Davison et al., 2002) in severely droughted plants. Indeed, the ratio of Zea to β-car increased from 0.72 in moderately to 1.38 in severely droughted leaves. This likely enhanced the rigidity of thylakoid membranes and limited membrane lipid peroxidation (Gruszecki and Strzałka 2005; Domonkos et al., 2013), thereby contributing to drought resistance. We cannot exclude that the decrease in β-car concentration, as the drought become more severe, may have resulted from its direct oxidation by $^1O_2$, with formation of oxidation products (which is known to occur also in low-light conditions, Ramel et al., 2012) that have not been examined in our study. However, β-car oxidation unlikely fully accounted for the large decrease in β-car concentration (0.09 μmol on leaf mass basis) from moderate to severe drought in WS leaves. Finally, the increases in the concentration of other major components of the xanthophyll pool, such as Lut and Neo, during drought stress progression may have also relevant functional reasons. Lut has the greatest ability to quench ^{3}Chl* compared to other xanthophylls, because of its specific ability to bind at the L1 site of the major LHCII complex (Mozzo et al., 2008), whereas the location of Neo at the periphery of the PSII super complexes may be an effective scavenger of superoxide anion (O_2^-, Dall'Osto et al., 2007).

4.3 Antioxidant enzymes effectively preserve drought stressed leaves from oxidative damage

Data of our study suggest a central role of *SOD*, *CAT*, and *APX* in protecting *C. australis* against intense drought stress, since their activities increased considerably and markers of oxidative damage did not vary as drought stress progressed. Data of our study are in contrast with the marked declines in the activities of APX and CAT observed in species with wider geographical distribution when exposed to long periods of drought stress and high air temperature (Peltzer and Polle, 2011; Peltzer et al., 2002; Fini et al., 2012; Tattini et al., 2015). Our data offer additional experimental support to previous suggestions that the depression in the activities of antioxidant enzymes occurs when leaves are challenged by a severe excess of excitation energy (Mullineaux and Karpinski, 2002; Mubarakshina et al., 2010; Fini et al., 2012; Tattini et al., 2015). Indeed, in our experiment, A_N was still appreciable and ETR/A_N did not

increase steeply even at severe drought. Since drought stress did not affect the ASA concentration, whereas the ASA/DHA ratio decreased relatively little (-18%) even in severely droughted WS leaves, it is suggested that the chloroplasts were equipped with an efficient system for the removal of H_2O_2 (Jubani-Mari et al., 2010). The observation of the steep enhancement of CAT activity from moderate to severe drought stress is interesting, and may further corroborate our hypothesis that photorespiration might have represented an effective dissipation process for excess reducing power in severely droughted leaves (Noctor et al., 2014). In fact, because CAT is mainly located in peroxisomes, enhanced activity of this enzyme may be particularly important to metabolize photorespiratory H2O2, which, as noted above, can be produced at higher rates when water becomes limiting (Noctor et al., 2014).

5. Conclusions

C. australis displays a very effective -near-isohydric- strategy to cope with the scarcity of soil water, and diffusional limitations mostly constrain photosynthesis even at severe drought. Our study reveals an effective down-regulation rather than permanent impairment of PSII photochemistry in response to drought stress of increasing severity. It is therefore conceivable that prompt recovery of photosynthetic performance will occur upon the removal of stomatal limitations, when water is newly available to the roots. We have shown that *C. australis* did not suffer from severe photo-oxidative damage, here estimated based on the products of both lipid and protein oxidation, even when challenged by severe drought. We offer evidence of a major role of de-epoxided xanthophylls in sustaining the thermal dissipation of excess radiant energy through NPQ at moderate drought, whereas at severe drought both Zea (which likely was partially synthesized thorough hydroxylation of β-car) and Neo likely served prominent antioxidant functions. Our study also reveals pivotal roles of antioxidant enzymes and ascorbic acid in ROS detoxification, the significance of which increased along with the severity of drought.

Our study supports the view that *C. australis*, an isohydric species with a conservative use of use, is effectively equipped to match the oxidative load generated by the reductions in CO_2 availability for photosynthesis during drought. This makes *C. australis* an excellent candidate for forest and urban plantings in sites experiencing extended periods of drought stress.

Funding

This study was funded by the Project TreeCity, PRIN (2010-2011), Rome, Italy.

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Figure captions

Figure 1. Predawn leaf water (Ψ_w, a) and osmotic (Ψ_π, b) potentials, and relative water content (RWC, c) in well-watered (WW, open bars) or drought stressed (WS, grey bars) leaves of *Celtis australis* sampled at moderate (7 d) and severe (15 d) drought stress. Data (means ± SD, $n = 4$) were analyzed with repeated measures with ANOVA, and bars with different letters differ significantly at $P < 0.05$, using Tukey's test.

Figure 2. Photosynthesis (A_N, a), stomatal conductance (g_s, b), intercellular CO_2 concentration (C_i, c), apparent maximum both carboxylation rate allowed by Rubisco ($V_{c,max}$, d), electron transport rate contributing to ribulose-1,5-bisphosphate (RuBP) regeneration (J_{max}, e), and the actual electron transport rate (ETR, f) in well-watered (WW, open bars) and drought stressed (WS, grey bars) leaves of *Celtis australis*, measured at moderate (7 d) and severe (15 d) drought stress. Data (means ± SD, $n = 4$) were analyzed with repeated measures with ANOVA, and bars with different letters differ significantly at $P < 0.05$, using Tukey's test.

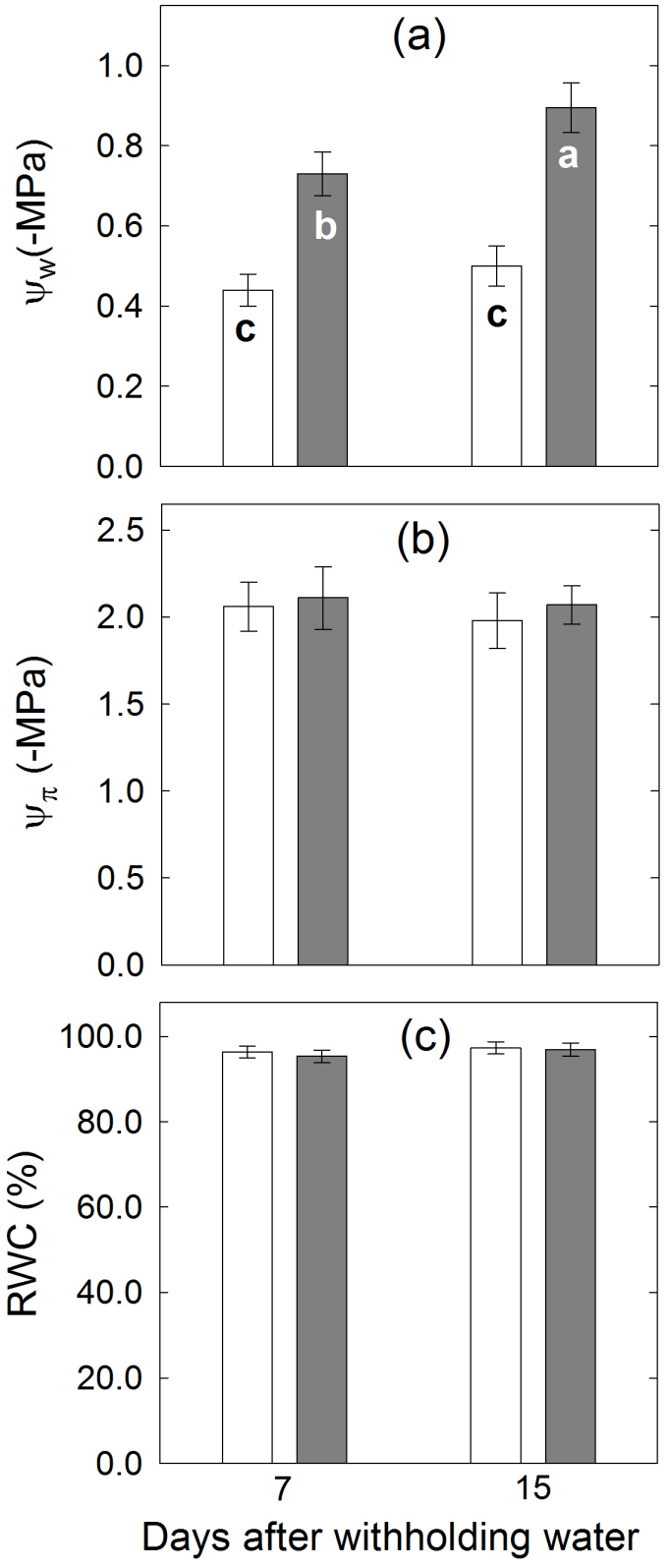
Figure 3. Stomatal limitations (SL, in percent) to photosynthesis (A_N) in well-watered (WW, open bars) or drought stressed (WS, grey bars) leaves of *Celtis australis* sampled at moderate (7 d) and severe (15 d) drought stress. Graph in the inset shows the contribution of non-stomatal limitations (NSL, in percent) to A_N in droughted leaves. Data (means ± SD, $n = 4$) were analyzed with repeated measures with ANOVA, and bars with different letters differ significantly at $P < 0.05$, using Tukey's test.

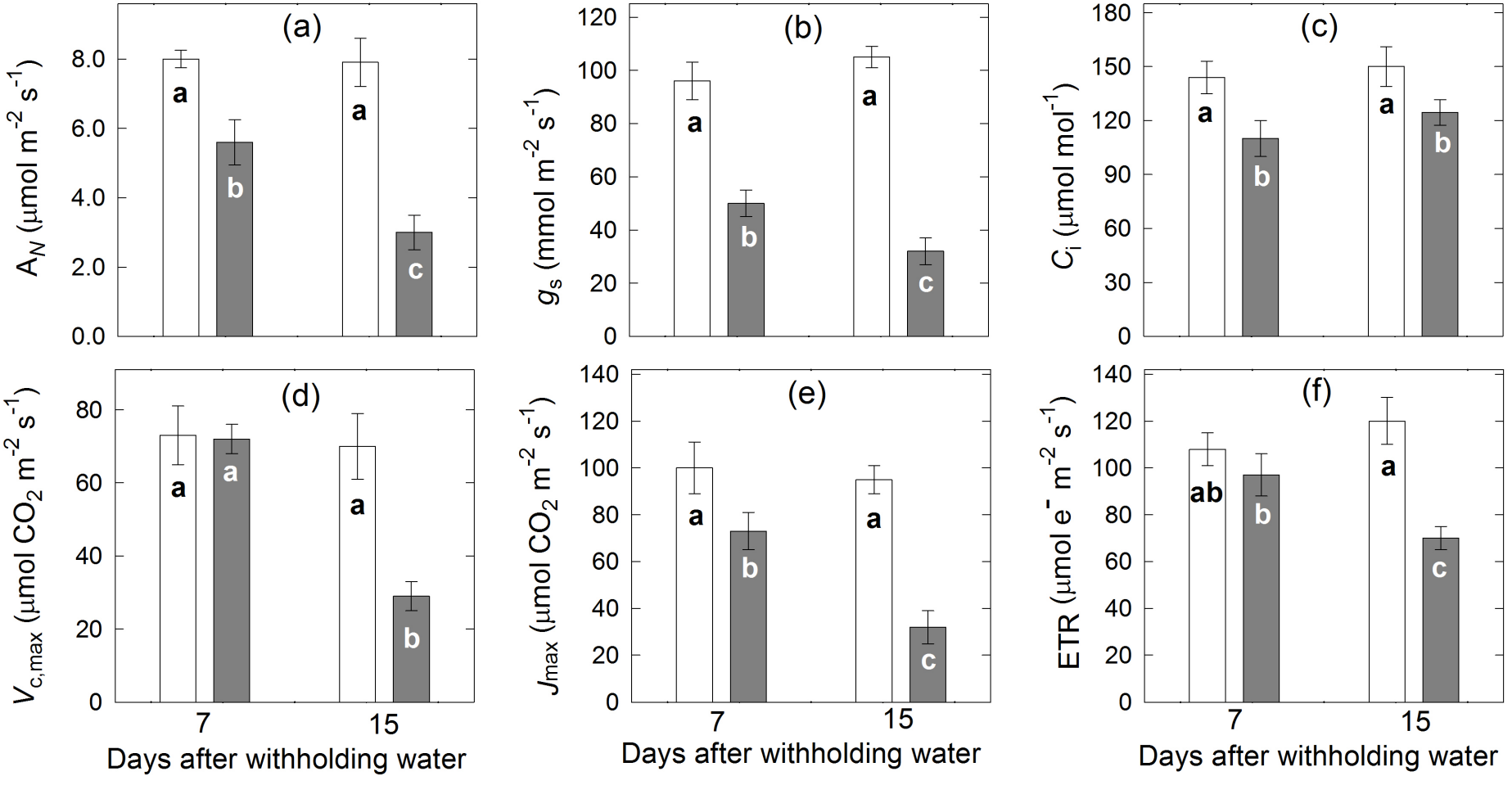
Figure 4. The maximum efficiency of PSII photochemistry (F_v/F_m, a), the ratio of minimum (F_0) to maximum (F_m) fluorescence (F_0/F_m, b), and the nonphotochemical quenching (NPQ, c) in well-watered (WW, open bars) or drought stressed (WS, grey bars) leaves of *Celtis australis* sampled at moderate (7 d) and severe (15 d) drought stress. Data (means ± SD, $n = 4$) were analyzed with repeated measures with ANOVA, and bars with different letters differ significantly at $P < 0.05$, using Tukey's test.

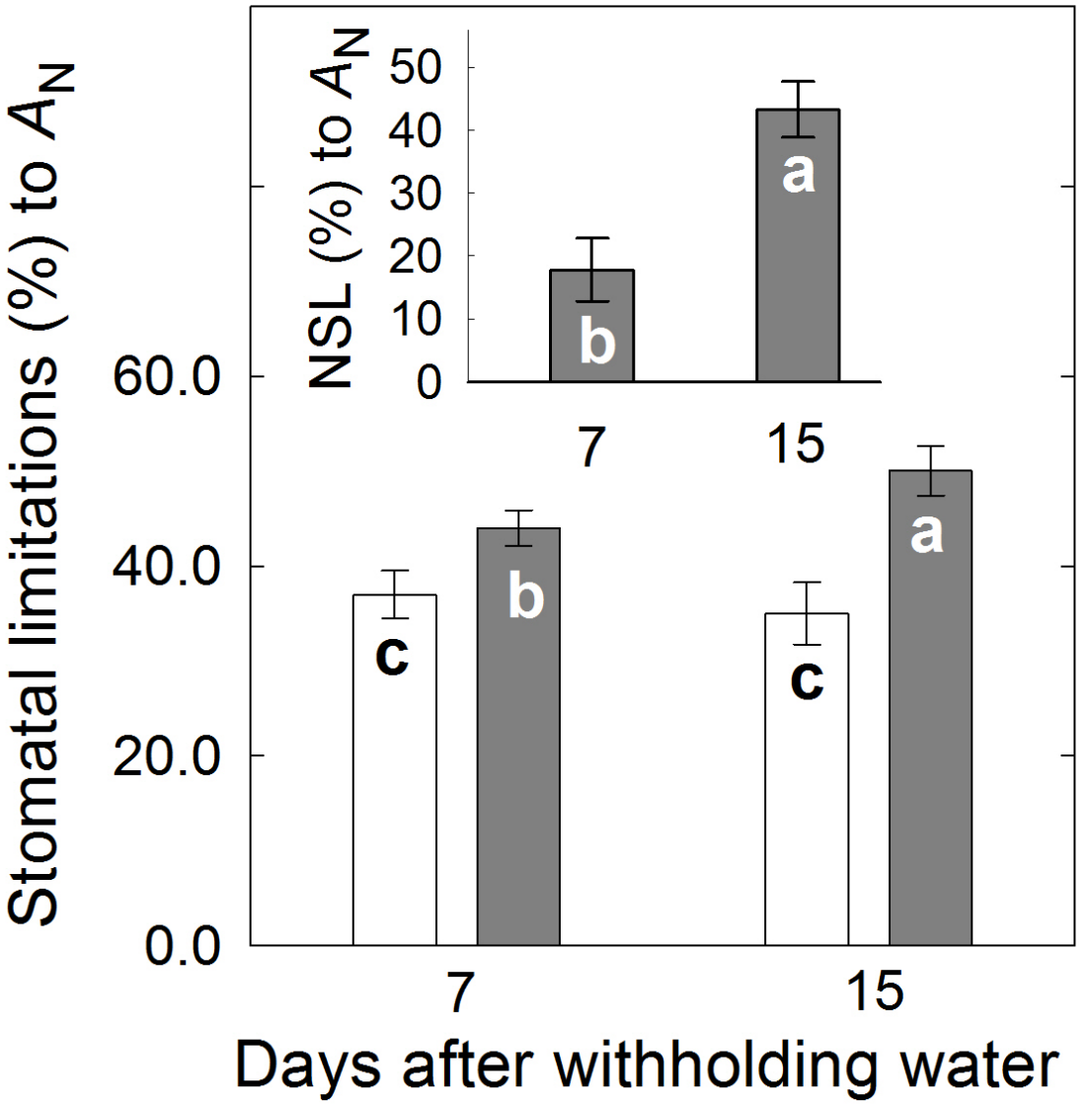
Figure 5. The concentrations of total chlorophyll (Chl_{tot}, a) and total carotenoids (Car_{tot}, b), the concentration of violaxanthin-cycle pigments (VAZ) relative to Chl_{tot} (c), the concentrations of lutein (d), β-carotene (e), neoxanthin (f), violaxanthin (g), zeaxanthin (h), and the de-epoxidation state (DES) of VAZ ($(DES = (0.5A + Z)(V + A + Z)^{-1}$, i) in well-watered (WW, open bars) or drought stressed (WS, grey bars) leaves of *Celtis australis* sampled at moderate (7 d) and severe (15 d) drought stress. Data (means ± SD, $n = 4$) were analyzed with repeated measures with ANOVA, and bars with different letters differ significantly at $P < 0.05$, using Tukey's test.

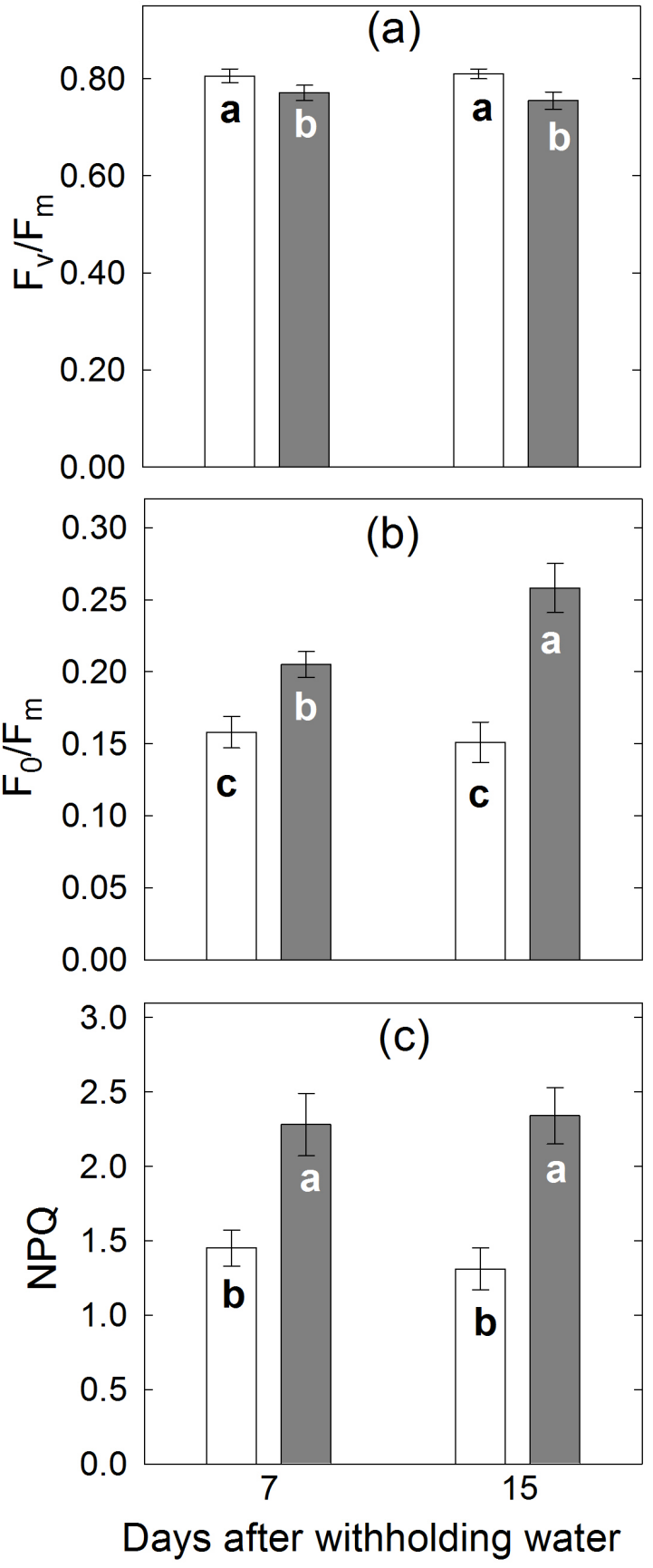
Figure 6. The activities (on protein, p., basis) of (a) superoxide dismutase (SOD,), (b) ascorbate peroxidase (APX,) and (c) catalase (CAT), (d) the concentration (on DW basis) of reduced ascorbate (ASA) and (e) the ratio of ASA to oxidized ascorbate (DHA) (ASA/DHA) in well-watered (WW, open bars) or drought stressed (WS, grey bars) leaves of *Celtis australis* sampled at moderate (7 d) and severe (15 d) drought stress. Data (means ± SD, $n = 4$) were analyzed with repeated measures with ANOVA, and bars with different letters differ significantly at $P < 0.05$, using Tukey's test.

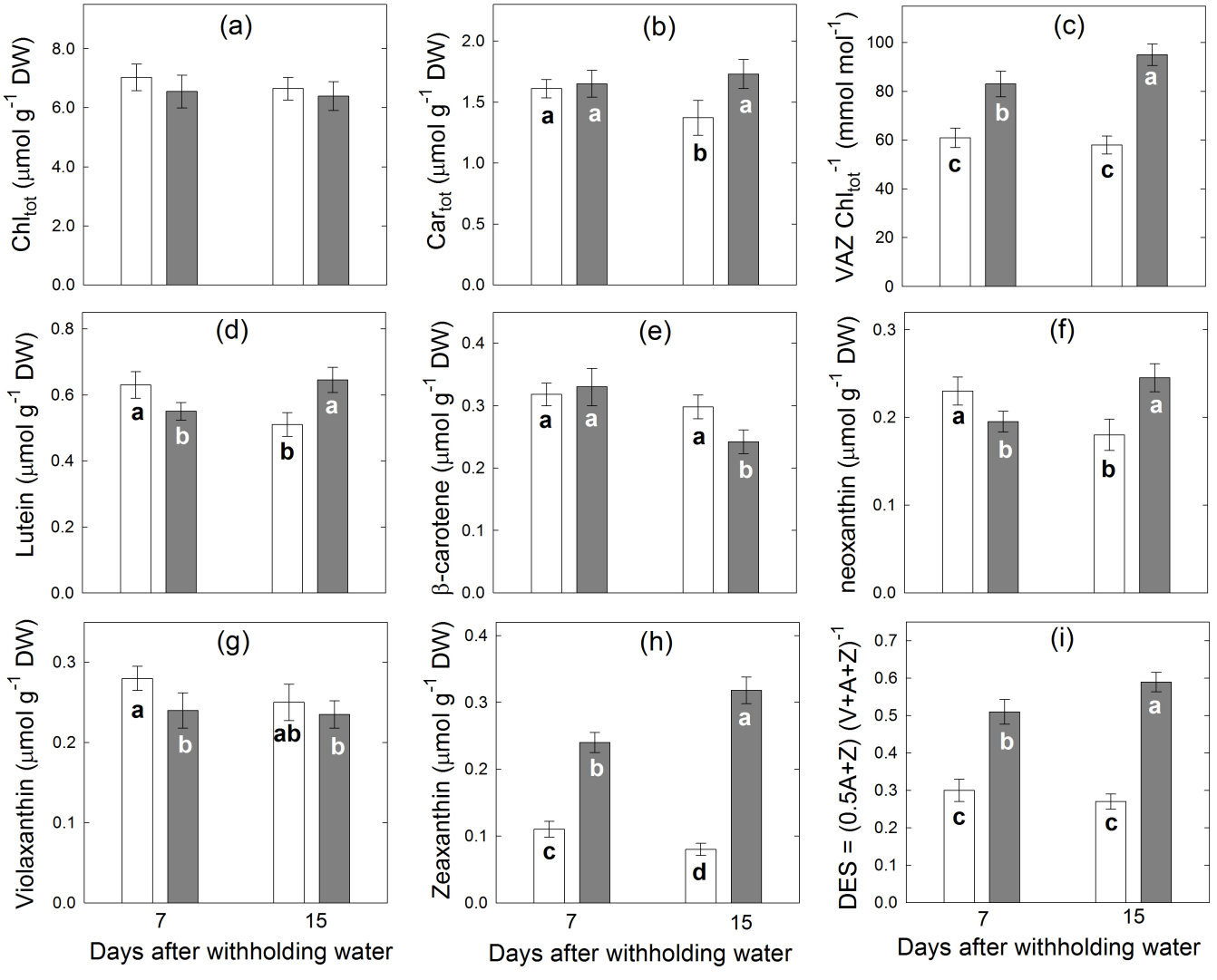
Figure 7. The concentrations of malondialdehyde (MDA, a) and of carbonyl groups (b) in well-watered (WW, open bars) or drought stressed (WS, grey bars) leaves of *Celtis australis* sampled at moderate (7 d) and severe (15 d) drought stress. Data (means ± SD, $n = 4$) were analyzed with repeated measures with ANOVA, and bars with different letters differ significantly at $P < 0.05$, using Tukey's test.

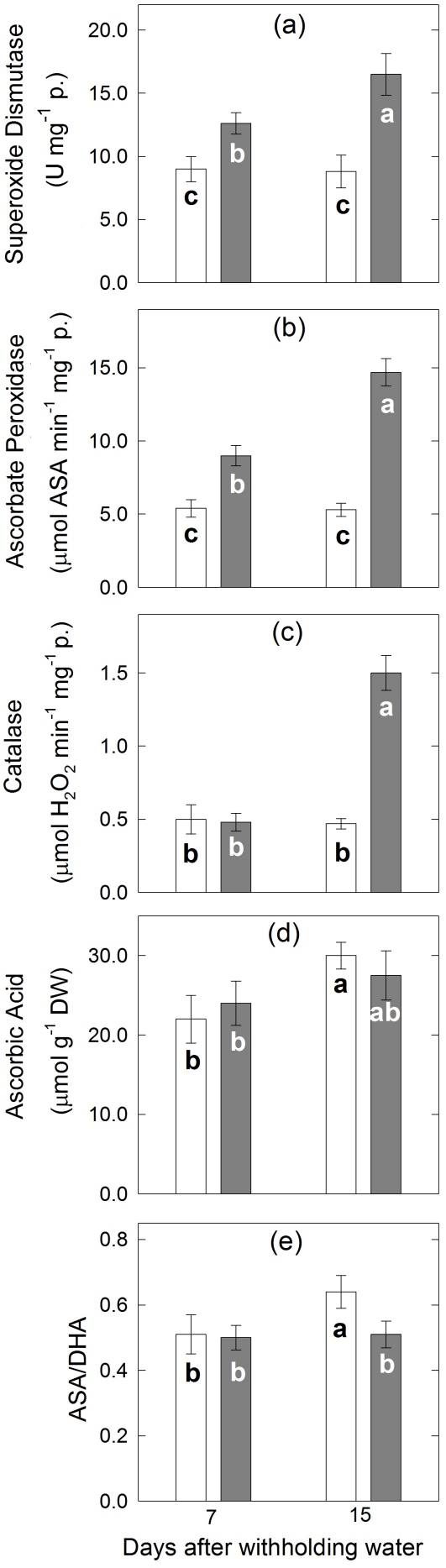

(a)
1.0
0.8
0.6
0.4
0.2
0.0
ψ_w(-MPa)
c
b
c
a
(b)
2.5
2.0
1.5
1.0
0.5
0.0
ψ_π (-MPa)
(c)
100.0
80.0
60.0
40.0
20.0
0.0
RWC (%)
7
15
Days after withholding water

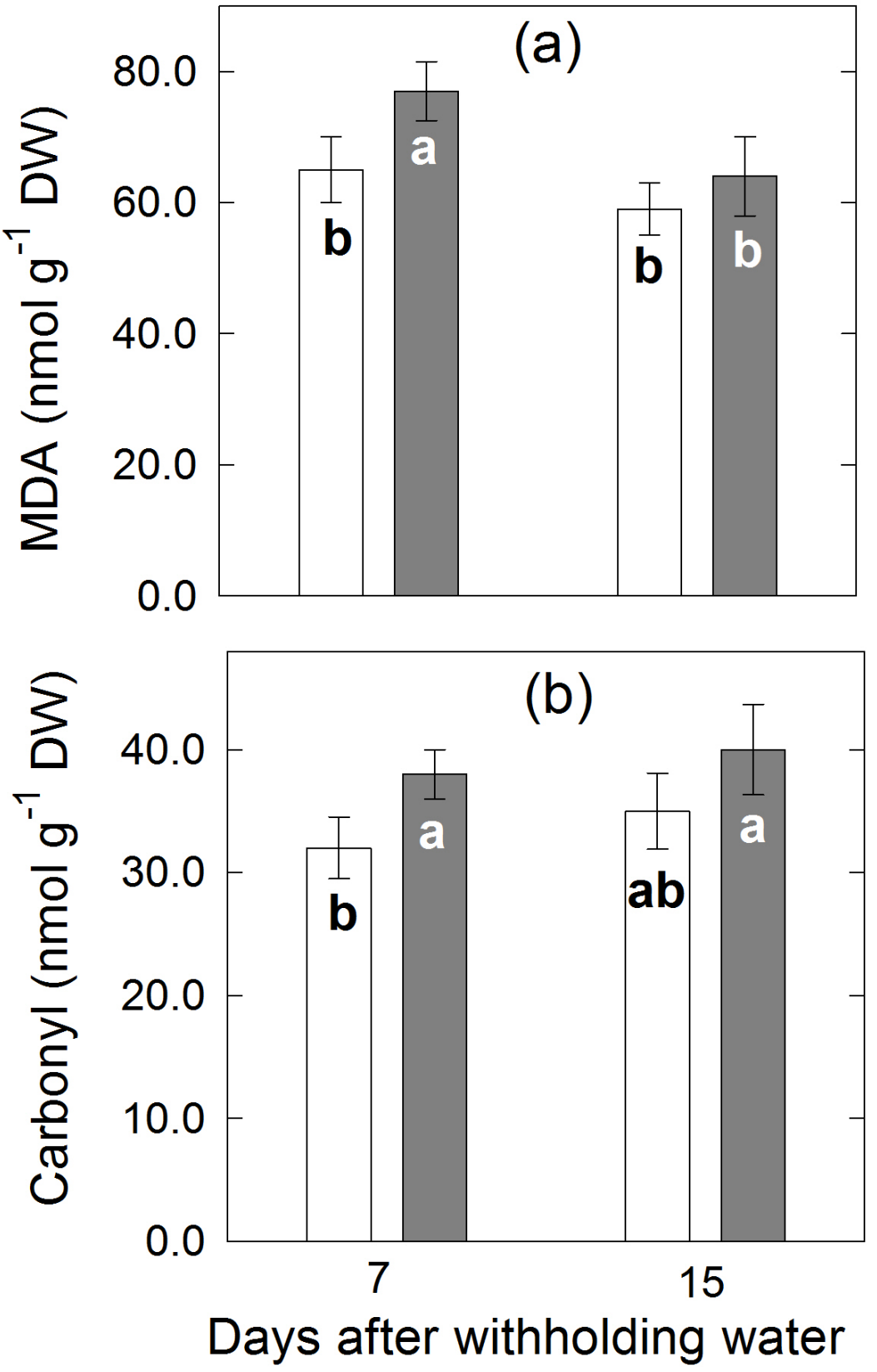

(a)
A_N (μmol m^{-2} s^{-1})
8.0
6.0
4.0
2.0
0.0
a
b
a
c
(b)
g_s (mmol m^{-2} s^{-1})
120
100
80
60
40
20
0
a
b
a
c
(c)
C_i (μmol mol^{-1})
180
150
120
90
60
30
0
a
b
a
b
(d)
$V_{c,max}$ (μmol CO$_2$ m^{-2} s^{-1})
80
60
40
20
0
a
a
a
b
7
15
Days after withholding water
(e)
J_{max} (μmol CO$_2$ m^{-2} s^{-1})
140
120
100
80
60
40
20
0
a
b
a
c
7
15
Days after withholding water
(f)
ETR (μmol e$^-$ m^{-2} s^{-1})
140
120
100
80
60
40
20
0
ab
b
a
c
7
15
Days after withholding water


Stomatal limitations (%) to A_N
60.0
40.0
20.0
0.0
7
15
Days after withholding water
c
b
c
a
NSL (%) to A_N
50
40
30
20
10
0
7
15
b
a


(a)
0.80
0.60
0.40
0.20
0.00
F_v/F_m
a
b
a
b
(b)
0.30
0.25
0.20
0.15
0.10
0.05
0.00
F_0/F_m
c
b
c
a
(c)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
NPQ
b
a
b
a
7
15
Days after withholding water


(a)
Chl$_{tot}$ (µmol g^{-1} DW)
(b)
Car$_{tot}$ (µmol g^{-1} DW)
(c)
VAZ Chl$_{tot}^{-1}$ (mmol mol^{-1})
(d)
Lutein (µmol g^{-1} DW)
(e)
β-carotene (µmol g^{-1} DW)
(f)
neoxanthin (µmol g^{-1} DW)
(g)
Violaxanthin (µmol g^{-1} DW)
(h)
Zeaxanthin (µmol g^{-1} DW)
(i)
DES = (0.5A+Z) (V+A+Z)$^{-1}$
7
15
Days after withholding water


(a)
Superoxide Dismutase
(U mg-1 p.)
20.0
15.0
10.0
5.0
0.0
c
b
c
a
(b)
Ascorbate Peroxidase
(µmol ASA min-1 mg-1 p.)
15.0
10.0
5.0
0.0
c
b
c
a
(c)
Catalase
(µmol H2O2 min-1 mg-1 p.)
1.5
1.0
0.5
0.0
b
b
b
a
(d)
Ascorbic Acid
(µmol g-1 DW)
30.0
20.0
10.0
0.0
b
b
a
ab
(e)
ASA/DHA
0.8
0.6
0.4
0.2
0.0
b
b
a
b
7
15
Days after withholding water


(a)
MDA (nmol g-1 DW)
80.0
60.0
40.0
20.0
0.0
b
a
b
b
(b)
Carbonyl (nmol g-1 DW)
40.0
30.0
20.0
10.0
0.0
b
a
ab
a
7
15
Days after withholding water