Environmental and Experimental Botany 125 (2016) 20–30

Contents lists available at ScienceDirect

Environmental and Experimental Botany

journal homepage: www.elsevier.com/locate/envexpbot

Acclimation to heat and drought—Lessons to learn from the date palm (*Phoenix dactylifera*)

Leila Arab[a,*], Jürgen Kreuzwieser[a], Jörg Kruse[a], Ina Zimmer[b], Peter Ache[c], Saleh Alfarraj[d], Khaled A.S. Al-Rasheid[d], Jörg-Peter Schnitzler[b], Rainer Hedrich[c,d], Heinz Rennenberg[a,d]

[a] *Institute of Forest Sciences, University of Freiburg, Georges-Köhler-Allee 53/54, 79110 Freiburg, Germany*
[b] *Helmholtz Zentrum München, Research Unit Environmental Simulation, Institute of Biochemical Plant Pathology, Ingolstädter Landstrasse 1, 85764 Neuherberg, Germany*
[c] *Institute for Molecular Plant Physiology and Biophysics, University of Würzburg, Julius-von-Sachs Platz 2, 97082 Würzburg, Germany*
[d] *College of Sciences, King Saud University, P.O. Box 2455, Riyadh 11451, Saudi Arabia*

ARTICLE INFO

Article history:
Received 28 September 2015
Received in revised form 6 January 2016
Accepted 15 January 2016
Available online 21 January 2016

Keywords:
Ascorbate
Dehydroascorbate reductase
Fatty acids
Gas exchange
Glutathione
Glutathione reductase
Isoprene
Photosynthesis
Thiols

ABSTRACT

In the present study, we investigated the responses of date palm (*Phoenix dactylifera*) to drought and heat as single stressors and in combination. We tested the hypotheses (i) that heat and drought enhance the capacity of the antioxidative system, and (ii) that due to the high stress tolerance of date palm, the plants' redox state will be widely unaffected, and (iii) that heat but not drought changes the plants' fatty acid composition and biosynthesis of isoprene, both contributing to the stabilization of membrane integrity. Photosynthesis was only weakly affected by both stresses, whereas the levels of the antioxidants ascorbate and glutathione in leaves dropped. This drop was, however, over-compensated by increased activities of glutathione reductase, an important enzyme of the antioxidative system. The plants' redox state was unaffected by stress as indicated by unchanged H_2O_2 levels. Because we do not know the concentration of isoprene at its site of action, isoprene emission might provide indirect hints on its possible functions. Isoprene emission strongly increased due to heat indicating its possible role as an antioxidant and for stabilization of thylakoid membranes. Fatty acids only reacted in response to drought. We conclude that the high heat and drought tolerance of date palm is the consequence of a concerted action of the antioxidative system, mainly based on enzyme activities and the assumed antioxidative effects of isoprene as well as adjustments in the fatty acid composition.

1. Introduction

Climate change will lead to an increase in global temperatures of at least 2 °C and strongly reduced summer precipitation compared to 1986–2005 levels (IPCC, 2014) in the near future. There is substantial evidence that this climate change also leads to an increase in frequency and intensity of extreme events such as heat waves and summer drought (Perkins et al., 2012). Coumou and Rahmstorf (2012) showed that in the last fifteen years five extreme heat wave events occurred world-wide, four of which were observed in Europe. In order to identify physiological traits that can reduce the sensitivity of trees to climate change by preventing or scavenging injurious effects of these environmental constrains, analysis of woody plants exposed to extreme climate conditions in their natural environment appears to be a promising approach. In this context, date palms (*Phoenix dactylifera*) are of particular significance, since they can experience both, temperature extremes and prolonged periods of drought under the arid and semi-arid conditions of their natural environment (Shabani et al., 2012). However, the physiological and molecular mechanisms that enable date palms to grow and develop under these conditions have so far not been elucidated.

Both heat and drought can result in enhanced production of reactive oxygen species (ROS) (Rennenberg et al., 2006). However, ROS production is not restricted to stress reactions. Plants have to cope permanently with the formation of ROS, inevitably evolving during metabolism under aerobic conditions in both, autotrophic

Abbreviations: ROS, reactive oxygen species; DW, dry weight; FW, fresh weight.
* Corresponding author.
E-mail address: leila.arab@ctp.uni-freiburg.de (L. Arab).

http://dx.doi.org/10.1016/j.envexpbot.2016.01.003
0098-8472/

and heterotrophic cells. Therefore, plant cells are well equipped with antioxidative systems capable to scavenge ROS. Generation of ROS can increase at potentially harmful rates, if redox disequilibria build up between redox carriers of electron transport chains in thylakoid and mitochondrial membranes (Dietz, 2015). Such imbalances frequently occur as a consequence of fluctuating leaf temperature (Scheibe and Dietz, 2012) and in response to heat and drought stress, in particular when rates of photophosphorylation and $NADP^+$-reduction exceed rates of ATP and NADPH-consumption in the Calvin-cycle. This is generally observed at low intracellular CO_2 availability due to stomatal closure (Rennenberg et al., 2006). Reducing the production of ROS as well as highly efficient ROS scavenging may therefore constitute strategies to cope with these environmental constrains (Rennenberg et al., 2006).

Scavenging of ROS is achieved in plant cells mainly by chemical reactions with antioxidants such as ascorbate and glutathione, but also by the enzymatic reactions of the Foyer–Halliwell–Asada pathway using the same metabolites as co-substrates (Munne'-Bosch et al., 2014; Noctor et al., 2012). Hence, antioxidative capacity of cells depends on the activity of antioxidative enzymes in different cellular compartments as well as on the pool sizes of antioxidants in these compartments (Tausz et al., 2001). In this context, levels and redox state of ascorbate and glutathione as well as glutathione reductase (GR) and dehydroascorbate reductase (DHAR) activity are thought to be of pivotal importance (Rennenberg et al., 2006).

Plants have developed several additional mechanisms to scavenge ROS, including the accumulation of secondary metabolites (Bartwall et al., 2013). For example, enhanced biosynthesis of isoprene in response to heat can protect the photosynthetic machinery from functional damage (1) by quenching and/or regulating ROS formation (Velikova et al., 2012), and (2) by stabilizing thylakoid membrane structures (Velikova et al., 2011). Even though the exact mechanisms, by which isoprene emission influences ROS and antioxidant levels are still unknown, its emission during heat stress can be substantial for many plant species (Kivimäenpää et al., 2013). Maintaining membrane integrity of plant cells and organelles is thought to be a particular challenge under heat and drought. This may be achieved by modifying the structure of existing membrane components, for example, by integration of isoprene (Velikova et al., 2011, 2012), and/or by changing the level of unsaturated fatty acids in order to adjust membrane fluidity (Zhong et al., 2011). Currently, it is unknown if these or other mechanisms to achieve ROS homeostasis and to maintain membrane integrity have been developed in date palms.

The present study was aimed at elucidating physiological mechanisms that allow date palms to cope with elevated temperatures and water deprivation. For this purpose, we exposed young date palm plants to heat and mild drought, single and in combination. We analyzed central parameters of the antioxidative system including levels and redox states of glutathione and ascorbate as well as the apparent activities of GR and DHAR in leaves and roots. As parameters affecting membrane integrity and fluidity, we further studied the fatty acid composition and isoprene emission in response to heat and drought.

With these approaches, we tested the hypotheses that (i) heat and drought stress in date palm is ameliorated by enhanced capacity of the Foyer–Halliwell–Asada pathway, particularly by increased activities of the responsible enzymes, (ii) the redox state of the palm trees is therefore widely unaffected by heat and drought, and (iii) membrane integrity during heat but not drought stress is maintained by increased isoprene biosynthesis and adjustments in fatty acid composition.

2. Materials and methods

2.1. Plant material and growth conditions

Two-year old date palm (*Phoenix dactilyfera*) seedlings were purchased from a commercial supplier ('Der Palmenmann', Bottrop, Germany). Two months before the start of experiments, plants were repotted (2 L pots; peat–sand–perlite mixture, 20:30:50 (vol%)), and grown under greenhouse conditions (15–25 °C, 60–70% rH). Because of the low nutrient content of peat, 10 g of NPK fertilizer were mixed to the substrate. Plants were irrigated every second day towards the end of the light period (*c.* 200 mL per pot). After two months, plants were transferred to two climate-controlled chambers (Heraeus, Vötsch, Germany). One chamber was set at 20 °C during the light and 15 °C during the dark period (16 h/8 h; 70 ± 3% rH at day and night), while in the second chamber plants were exposed to enhanced growth temperature during the light period (35 °C at day/15 °C at night; 60 ± 8% rH at day and 70 ± 3% at night). It was taken care that incident light was the same between chambers, reaching a photosynthetic active radiation (PAR) of 200–300 μmol photons $m^{-2}\,s^{-1}$ at leaf level. We choose this light intensity to simulate shading conditions in order to exclude light stress in addition to heat and drought stress.

In a first set of experiments, plants continued to be irrigated every second day, including the night before gas exchange measurements ('well-watered' conditions). Plants were given two weeks' time to adjust to different temperature regimes between chambers. Experimental setup ensured that plants were exposed for the same time to different growth temperatures. After two weeks, temperature and light responses of net photosynthesis and isoprene emission were determined. Plants were placed back into the climate-controlled chambers and harvested the following day—always exactly 6 h after onset of light. Plant material was frozen in liquid N_2 and stored at −80 °C until further analysis.

The above experiment was replicated with one modification: after two weeks of acclimation at the two different growth temperatures, the irrigation of the 35 °C-grown plants was stopped for 4–5 days, and that of the 20 °C-grown plants was stopped for 7–8 days prior to gas exchange measurements (mild 'drought' conditions). Because of lower relative humidity in the 35 °C-chamber, the duration of water deprivation was 3 days longer for 20 °C-grown plants. We thereby tested if temperature effects were modified by plant water availability.

2.2. Leaf and roots hydration measurements

Leaf and root hydration (H; g $H_2O\,g^{-1}$ DW) was determined as (FW − DW)/DW, where FW is the fresh mass and DW is the dry mass after drying the samples in an oven at 60 °C for 48 h (Contin et al., 2014).

2.3. Determination of total N and total C

Total N and total C contents were determined in leaves and roots according to Dannenmann et al. (2009). Briefly, oven dried (48 h, 60 °C) and ground plant material (aliquots of 0.5–1.0 mg) was transferred into tin capsules (IVA Analysentechnik, Meerbusch, Germany). Samples were analyzed using an elemental analyzer (Vario EL, Elementar Analysensysteme GmbH, Hanau, Germany).

2.4. Gas exchange measurements

A portable gas exchange measuring system (GFS 3000, Walz GmbH, Effeltrich, Germany) was used for the determination of leaf gas exchange (net photosynthesis, stomatal conductance) and collection of concomitantly emitted isoprene. Date palm leaves

were placed into the 8 cm^2 cuvette of the system which was flushed with synthetic air (80% N_2, 20% O_2, Linde Gas, Stuttgart, Germany) at a defined flow rate of 700 $\mu mol\ s^{-1}$. Temperature response of light-saturated photosynthesis (PAR: 1200 $\mu mol\ m^{-2}\ s^{-1}$) was determined in 5 °C steps at ambient CO_2 (400 ppm), ranging from 20 °C to 45 °C. A total of 13.000 ppm H_2O was added to the dry, incoming air (GFS 3000), independent of incubation temperature. Relative humidity in the cuvette thus decreased from 55% at 20 °C to 15% at 45 °C incubation temperature. The temperature response of net photosynthesis (A_T) was fitted to a parabolic function (Gunderson et al., 2010).

After steady state rates of photosynthesis were reached and recorded, sampling of isoprene was started. For this purpose, outlet air of the cuvette was drawn over glass tubes packed with 20 mg Tenax (60/80 mesh), 30 mg Carbotrap (20/40 mesh) and 40 mg Carboxen 569 (20/45 mesh) (Supelco, Bellafonte, PA). Trapping of isoprene was performed for 30 min at a flow rate of 150 mL min^{-1}. Immediately after sampling, the glass tubes were sealed and stored at 4 °C until analysis.

Sensitivity of photosynthesis and isoprene emission to incident light was assessed via light response curves, ranging from 0 to 1500 $\mu mol\ m^{-2}\ s^{-1}$ PAR. Light responses were either determined at 20 °C, or at 35 °C incubation temperature. Both incubation temperatures were applied to well-watered plants that were acclimated to 20 °C or 35 °C growth temperature. The light response of photosynthesis (A_Q) can be fitted to a non-rectangular hyberbola (Thornley and Johnson, 1990):

$$A_{(Q)} = \frac{\alpha \times Q + A_{sat} - \sqrt{(\alpha \times Q + A_{sat})^2 - 4\alpha \times Q \times A_{sat} \times \Theta}}{2 \times \Theta} - R_l \quad (A.1)$$

where Q is the intensity of incident light (μmol quanta $m^{-2}\ s^{-1}$), α is the quantum use efficiency ($\mu mol\ CO_2\ \mu mol^{-1}$ quanta), A_{sat} is the light-saturated rate of photosynthesis ($\mu mol\ m^{-2}\ s^{-1}$), R_l is the mitochondrial respiration occurring in the light ($\mu mol\ m^{-2}\ s^{-1}$), and Θ is a curvature factor (dimensionless).

2.5. Analysis of isoprene

Isoprene samples were analyzed on a gas chromatograph (model 7890A, Agilent Technologies Böblingen, Germany) equipped with a mass-selective detector (5975C, Agilent Technologies Böblingen, Germany) and a thermodesorption/cold injection system (TDU-CIS) (Gerstel, Germany). Isoprene was thermodesorbed from the sampling tubes at 240 °C, cryofocused at – 100 °C and – after heating the CIS to 240 °C – injected onto the separation column (DB-624, Agilent Technologies, Böblingen, Germany). For isoprene identification and quantification, external isoprene standards (Linde Gas, Stuttgart, Germany) were used. Emission rates were calculated based on the isoprene concentration in the cuvette, the flow rate through the cuvette and the projected leaf area.

2.6. Determination of in vitro activities of glutathione reductase (GR) and dehydroascorbate reductase (DHAR)

For determination of the apparent GR and DHAR *in vitro* enzyme activities, proteins from frozen plant material (100 mg of finely ground leaf and root powder) were extracted with 1.5 mL of ice-cold extraction buffer, containing 100 mM potassium phosphate (pH 7.8), 80 mg polyvinylpolypyrrolidone (PVPP) and 1% Triton X-100 (v/v). Extracts were passed through a Sephadex G-25 column (NAP-5 column, GE Healthcare Life Science) to collect the protein fraction. The activity of GR was determined by monitoring glutathione dependent oxidation of 1.25 mM NADPH at 340 nm as described by Polle et al. (1990).

DHAR activity was assayed directly by following the increase in absorbance at 265 nm, resulting from GSH-dependent production of ascorbate (Polle et al., 1990). The assay mixture consisted of 8 mM DHA, 10 mM GSH and 100 mM potassium phosphate buffer (pH 6.1). Enzyme measurements were conducted at 20 °C and 35 °C, for both 20 °C- and 35 °C-grown plants. The substrate concentrations in the enzyme assays were shown to be sufficient to achieve maximum activity irrespective of measurement temperature.

Q10 is the factor by which the reaction rate increases when the temperature is raised by ten degrees. The Q10 value of the substrate-saturated *in vitro* activities (GR and DHAR) were calculated from measurements at 20 °C and 35 °C, according to Atkin and Tjoelker (2003).

$$Q10_{enzyme} = \left(\frac{A_{35}}{A_{20}}\right)^{\left[\frac{10}{35-20}\right]} \quad (A.2)$$

where A_{35} and A_{20} are the *in vitro* activities measured at 35 °C and 20 °C, respectively.

2.7. Extraction and quantification of thiols

Thiol extraction and quantification were performed as described by Strohm et al. (1995). Aliquots of 50 mg finely ground frozen plant material were transferred to pre-cooled tubes (4 °C), containing 1.5 mL 0.1 N HCl and 100 mg of pre-washed PVPP (Sigma–Aldrich Inc., Steinheim, Germany). Samples were vigorously shaken and subsequently centrifuged at 14,000 rpm. For the analyses of reduced plus oxidized (=total) thiols, aliquots of 180 μL of the supernatant were added to 240 μL of 200 mM 2-(*N*-cyclohexylamino) ethanesulphonic acid (CHES) buffer (pH 9.3) and 40 μL of 10 mM dithiothreitol (DTT) for the reduction of oxidized thiols. Oxidized thiols were determined in 180 μL aliquots treated with 240 μL of 200 mM CHES buffer and 40 μL of 5 mM *N*-ethylmaleimide (NEM) for 10 min prior to the reduction with DTT in order to block reduced thiols. After incubation at room temperature for 60 min, the samples were derivatized in darkness for 15 min by adding 30 μL of 30 mM monobromobimane (Thiolyte, Calbiochem, Bad Soden, Germany). Subsequently, monobromobimane derivatives were stabilized by adding 260 μL 10% (v/v) acetic acid. Thiol derivatives were separated and quantified by HPLC (Beckman Gold System, Beckman, Fullerton, CA, USA) using a C-18 column (ODS-Hypersil 250 × 4.6 mm id, 5 μm particle size; Bischoff Chromatography, Leonsberg, Germany) and fluorescence detection with a Shimadzu RF-535-Fluorescence monitor (Shimadzu Europe GmbH, Duisburg, Germany). Peaks were identified and quantified with a standard solution containing 0.2 mM cysteine, 0.1 mM γ-glutamylcysteine (γ-EC) and 1 mM glutathione.

2.8. Extraction and quantification of ascorbic acid

For the determination of ascorbic acid, aliquots of 50 mg frozen homogenized leaf and root material were extracted in 500 μL 5% meta-H_3PO_4 solution vortexed and centrifuged for 30 min at 4 °C and 12,000 rpm. Aliquots of 100 μL of the supernatant were mixed with 20 μL 1.5 M triethanolamine and 100 μL sodium phosphate buffer (150 mM, pH 7.4) in two separate safe seal microtubes (2 mL, Sarstedt AG & CO., Nümbrecht, Germany) for each sample, one to determine the amounts of reduced ascorbate and the other to quantify total ascorbate contents. Total ascorbate contents were measured after reduction by adding 50 μL DTT (10 mM) and incubation at room temperature for 15 min. The excess DTT was

removed by adding 50 μL NEM (0.5%). Samples for the determination of both reduced and total ascorbate contents were further prepared by adding 200 μL trichloroacetic acid (10%), 200 μL orthophosphoric acid (44%), 200 μL 2,2′-dipyridil (4% in ethanol) and 100 μL $FeCl_3$ (3%). All sample solutions were mixed and incubated for 60 min at 37 °C. The absorption of the solutions was determined with a UV-DU650 spectrophotometer (Beckman Coulter Inc., Fullerton, CA, USA) at 525 nm. L-Ascorbic acid (Sigma–Aldrich, Steinheim, Germany, 1.5 mg mL^{-1}) was used as a standard.

2.9. Hydrogen peroxide determination

H_2O_2 content of leaf and root material was measured by applying the methodology described by Cheeseman (2006). For this purpose, tissue samples were homogenized in 0.1 M K-phosphate (pH 6.4) supplemented with 5 mM KCN as extraction medium. The assay mixture contained 250 μM ferrous ammonium sulphate, 100 μM sorbitol, 100 μM xylenol orange, and 1% ethanol in 25 mM H_2SO_4. H_2O_2 contents were determined by the difference in absorbance between 550 nm and 800 nm at least 40 min after mixing the solutions and quantified using a standard curve in the range of 1.25–10 μmol H_2O_2.

2.10. Fatty acid analysis

Extraction and derivatisation of plant samples were performed essentially as described by Lytovchenko et al. (2009). Leaf and root material (about 50 mg fresh weight) was homogenized and extracted in 700 μL 100% methanol at 70 °C for 15 min and centrifuged at 14,000 rpm for 5 min. The supernatants were transferred to new tubes and 1 mL double distilled water/chloroform were added, tubes were vigorously shaken and centrifuged at 14,000 rpm for 5 min. Aliquots of 200 μL of the chloroform phase were dried in a speed-vac (RVC 2-25, Christ, Osterode, Germany) and derivatised. For derivatisation 50 μL methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA; Sigma, Munich, Germany) with 20 μL pyridine were added and samples were incubated at 37 °C for 30 min. Subsequently, sample reaction solutions were transferred to glass vials suitable for the Gerstel MultiPurpose Sampler (MPS2-XL, Gerstel, Mülheim, Germany). 1 μL aliquots were injected into the system and run on a capillary column (HP-5MS, length: 30 m, diameter: 0.25 mm, film thickness: 0.25 μm; Agilent Technologies, Palo Alto, CA, USA) at a helium flow of 1 mL min^{-1}. Fatty acid abundance was determined by GC-EIMSD (Agilent 7890A GC coupled to an Agilent 5975C MS; Agilent Technologies, Frankfurt, Germany) with the GC/MS settings previously described by Jaeger et al. (2009). For fatty acid identification and quantification, the Golm metabolome database (Hummel et al., 2010; Kopka et al., 2005) and available authentic external standards of known concentration were used. Peak identification and deconvolution of chromatograms was performed using AMDIS 2.71 ("Automated Mass Spectral Deconvolution and Identification System" freely available from www.amdis.net) and the web-based platform "SpectConnect" (www.spectconnect.mit.edu) (Styczynski, 2007). Quantification of fatty acids was obtained by analysis of dilution series of standard compounds (Table S3) dissolved in chloroform (usually 5–10 mg mL^{-1} stock). From the abundance of unsaturated fatty acids the double bond index (DBI) was calculated according to People et al. (1978).

2.11. Statistical analysis

The experiment was performed with 14 plants per temperature and irrigation treatment, summing up to 56 plants in total. Plants were divided into leaves and roots for determination of hydration, total N, and contents of thiols, ascorbate, and hydrogen peroxide. *In vitro* enzyme activities were measured at 20 °C and 35 °C incubation temperature, in order to derive Q10-values and assess treatment effects on Q10.

Instantaneous temperature responses of gas exchange and isoprene emission were replicated with 8 plants per growth temperature and irrigation treatment. Light responses of photosynthesis and isoprene emission were replicated with 8 plants. Light responses were performed with well-watered plants only, at 20 °C and 35 °C incubation temperature (Fig. 1). Data were positively tested for homoscedasticity, and subsequently subjected to 2-way ANOVA to assess the significance of treatment effects (Sigmaplot 11.0; Systat Software GmbH, Erkrath, Germany). In the case of significant interactive effects between growth temperature and irrigation treatment (*i.e.*, $T \times D < 0.05$), Tukey HSD post-hoc test helped to reveal groups that were significantly different from one another (at $P < 0.05$).

Fatty acid analysis was performed with 7 plants per treatment. The patterns of fatty acid composition of leaves and roots of date palms were assessed by principal component analysis (PCA) and partial least square discriminant analyses (PLSDA) to describe the different compositions of fatty acids from plants differently treated (well-watered, drought, growth at 20 °C and at 35 °C) in an objective and unsupervised (PCA) or supervised (PLSDA) manner. Analysis was carried out with MetaboAnalyst 2.0 (www.metaboanalyst.ca; Xia et al., 2012). For the analyses, data sets of fatty acid contents from each individual biological replicate were used as X. Before analysis, data were pre-processed by log transformation [$X = \log(X+1)$]. PCA and PLSDA models were then calculated and score plots were used to visualize classification results. 95% confidence areas were illustrated by ellipses in score plots.

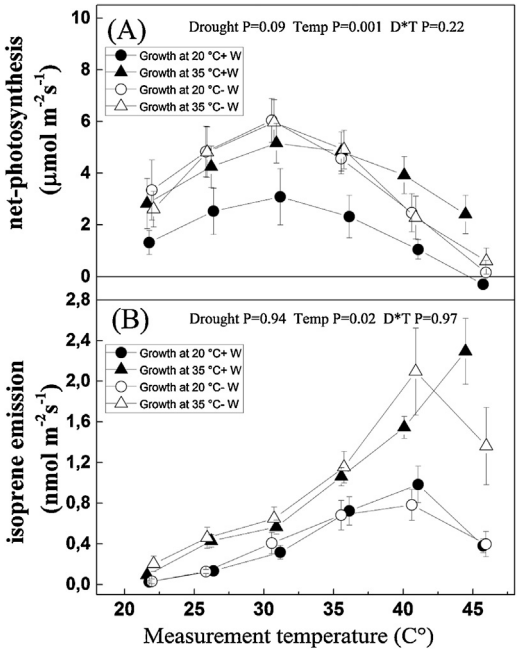

Fig. 1. Temperature sensitivity of net-photosynthesis (A) and isoprene emission (B). Circle and triangles represent growth at 20 °C and 35 °C, respectively. Open symbols: water-deprived plants (−W); closed symbols: well-watered plants (+W). Data shown are averages ± S.D ($n = 8$). Significant treatment effects on net-photosynthesis and isoprene emission were calculated by ANOVA.

Table 1
Biometric and physiological characteristics of date palm tissue, grown under heat and drought. Data shown are averages ± S.D (n = 14). Significant treatment effects on plant characteristics were calculated by ANOVA. SLA: specific leaf area; G_{growth}: stomatal conductance measured at respective growth temperature; c_i: substomatal [CO_2] at respective growth temperature.

	Well-watered		Drought	
	20 °C	35 °C	20 °C	35 °C
Leaf hydration (g H_2O g^{-1} DW)	1.53 ± 0.12a	1.67 ± 0.17ab	1.95 ± 0.17c	1.73 ± 0.20b
SLA (cm^2 g^{-1} DW)	35.4 ± 0.8cb	37.3 ± 1.0b	41.2 ± 0.9a	38.1 ± 1.4ab
Root hydration (g H_2O g^{-1} DW)	1.57 ± 0.20a	1.27 ± 0.47a	1.49 ± 0.20a	1.37 ± 0.32a
Leaf-N (mg g^{-1} DW)	2.21 ± 0.30bc	2.0 ± 0.20c	2.6 ± 0.14a	2.23 ± 0.12b
Root-N (mg g^{-1} DW)	3.02 ± 0.3a	2.14 ± 0.27b	2.8 ± 0.28a	3.15 ± 0.4a
Leaf—C/N ratio	20.7 ± 2.6b	23.1 ± 2.5a	18.1 ± 0.9c	20.0 ± 1.6bc
Root—C/N ratio	13.7 ± 1.9bc	19.3 ± 2.4a	14.4 ± 1.2b	12.2 ± 1.9c
G_{growth} (mmol m^{-2} s^{-1})	17.8 ± 3.7a	47.8 ± 8.7a	43.1 ± 15.3a	43.0 ± 11.1a
c_i at T_{growth} (ppm)	270 ± 30a	235 ± 10c	250 ± 15b	180 ± 15d

3. Results

3.1. Influence of heat and drought on total N contents, C/N ratio and hydration

Total N contents in leaves decreased due to heat and increased upon water-deprivation. In roots, temperature-related decline of total N was only observed for well-watered plants (significant $T \times D$ interaction; Tables 1 and S4). These effects of drought and heat on N contents of leaves and roots resulted in changed C/N ratios. In the leaves, the C/N ratios increased with elevated growth temperature irrespective of water availability and decreased with drought stress irrespective of growth temperature. In the roots an increase of the C/N ratio with growth temperature was only observed for well-watered plants; upon drought, the C/N ratio declined with increasing growth temperature (Tables 1 and S4). Leaf hydration was significantly increased by drought, but the strength of this effect depended on growth temperature. However, a significant effect of both stressors on roots hydration was not observed (Tables 1 and S4).

3.2. Growth temperature affects gas exchange and isoprene emission

Acclimation of photosynthesis to the enhanced growth temperature was assessed by a dual approach, firstly via measurement of photosynthetic temperature responses (A/T-responses, Fig. 1A). The temperature–response of net photosynthesis can be described by a bell-shaped optimum curve, where the peak rate of photosynthesis (A_{opt}) is achieved at a distinct optimum temperature (T_{opt}). Acclimation to contrasting growth temperature had little effect on T_{opt}. This optimum was already high in 20 °C-grown plants (c. 29.5 °C), and increased by only 2 °C in 35 °C-grown plants (p = 0.08; Table S1). A_{growth} significantly increased in plants exposed to heat and in water-deprived plants (Table S1). At the same time, stomatal conductance at growth temperature was largely unaffected by temperature and irrigation treatments. Apparently, variation in (instantaneous) c_i was mainly driven by temperature- and drought-dependent effects on biochemical demand for CO_2, while CO_2-supply via stomata remained low irrespective of treatment (Table 1). On average, c_i was reduced from 260 ppm CO_2 at 20 °C to 210 ppm at 35 °C growth temperature, and it was reduced from 255 ppm CO_2 in well-watered to 215 ppm in water-deprived plants. These trends were similar to those observed for A_{growth} (Table S1).

In a second approach, we determined the light response of photosynthesis of well-watered plants as affected by growth and incubation temperatures (A/Q-responses, Fig. 2A). Photosynthesis at saturating light (A_{sat}) intensities was hardly affected by incubation temperature (*i.e.*, 20 °C versus 35 °C cuvette temperature), but was significantly enhanced in 35 °C-grown plants as compared to 20 °C-grown plants (Fig. 2A and Table S1)—similar to observations made for A/T-responses.

Rates of dark respiration increased with measurement temperature, averaging 0.3 μmol m^{-2} s^{-1} at 20 °C and 0.9 μmol m^{-2} s^{-1} at 35 °C. This effect of incubation temperature, however, was modified by acclimation to contrasting growth temperature. Plants grown at 35 °C exhibited lower respiration rates than 20 °C-grown plants, in particular, when measured at 35 °C (Table S1).

Isoprene was emitted from date palm leaves in a temperature-dependent manner (Fig. 1B). Maximum emission rates of trees grown at moderate temperatures of 20 °C amounted to ca. 0.8–1.0 nmol m^{-2} s^{-1} irrespective of water supply. Growth at elevated air temperature of 35 °C increased isoprene emission two-fold as compared to 20 °C-grown plants. In well-watered plants grown at

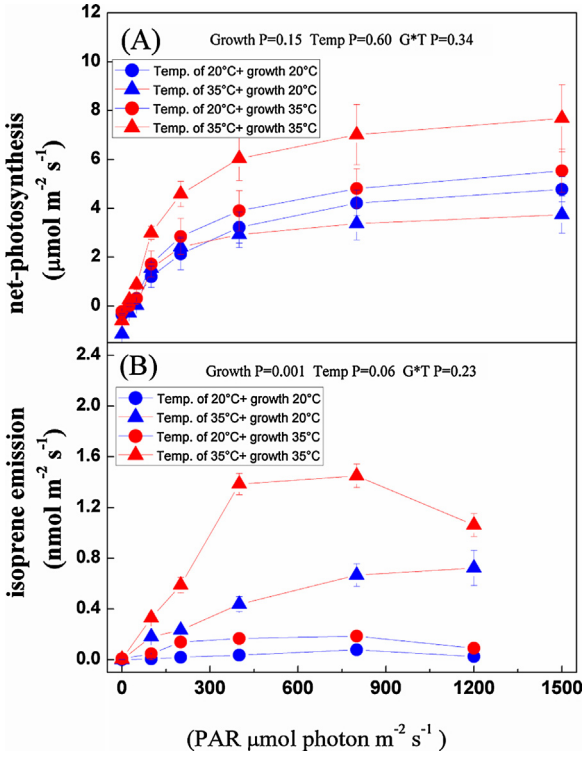

Fig. 2. Light response of net-photosynthesis (A) and isoprene emission (B). Circles and triangles in A and B represent measurement temperatures of 20 °C and 35 °C, respectively. Blue and red colors represent growth at 20 °C and 35 °C, respectively. Data shown are averages ± S.D (n = 8). Significant treatment effects on net-photosynthesis and isoprene emission were calculated by ANOVA. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

elevated temperatures also the pattern of the temperature response differed considerably. Under well-watered conditions the emission of isoprene increased with leaf temperature up to 45 °C, whereas the emission dropped at temperatures >40 °C in leaves from the other treatments. Drought therefore reduced isoprene emission at leaf temperatures above 40 °C in plants grown at elevated temperature. As expected, the temperature optima of isoprene emission were significantly higher than those of net photosynthesis.

Light saturation of isoprene emission was observed at PAR of ca. 300–500 μmol photons m^{-2} s^{-1} (Fig. 2B). Clearly, plants grown at elevated air temperature displayed a higher capacity to produce and emit isoprene than trees grown at 20 °C air temperature. This became evident irrespective of leaf temperature during the measurements.

3.3. Anti-oxidant levels and anti-oxidative enzymes in leaves and roots are affected by heat and drought treatment

The heat and drought treatments apparently did not affected the ROS homeostasis in leaves and roots, since in both tissue the H_2O_2 levels remained unchanged (Fig. 3). This result is surprising, because elevated air temperature caused a significant decrease in cysteine, γ-EC and GSH levels in the leaves of both, well-watered and drought-treated plants (Fig. 4A, C and E). In contrast, the combination of drought stress and elevated temperature resulted in a significant increase in cysteine and γ-EC contents in roots (Fig. 4B and D). GSH contents in roots, significantly increased upon water-deprivation, at both 20 °C and 35 °C growth temperature (Fig. 4F). Despite these changes in the thiol levels in response to the heat and drought treatment, the redox state of glutathione remained unchanged with GSSG contributing 12.47 ± 6.02% to total glutathione in leaves and 5.12 ± 4.8% to total glutathione in roots (data not shown).

In a similar way, total and reduced ascorbic acid contents in leaves of date palms significantly decreased if plants were grown at elevated air temperature irrespective of the water availability (Fig. 5A and C). The redox state of ascorbate, however, was not affected by this treatment (Fig. 5E). In roots neither temperature- nor irrigation treatment affected pool sizes of ascorbate (Fig. 5B and D). Their redox state also remained stable (Fig. 5F).

From these results, it may be inferred that acclimation to heat enhanced turnover of antioxidants, in spite of reduced pool sizes. To test this hypothesis, we analyzed *in vitro* activities of key-enzymes of the antioxidative system, namely glutathione reductase (GR) and dehydroascorbate reductase (DHAR) at standard (20 °C) and elevated (35 °C) incubation temperature (Table 2). Unsurprisingly, *in vitro* GR activities were higher at 35 °C as compared to 20 °C incubation temperature. In leaves, elevated growth temperature reduced GR activity, when measured at 20 °C incubation temperature (Tables 2(A) and S5). Conversely, acclimation to heat increased GR-activity, when measured at 35 °C (Tables 2(A) and S5). Consequently, acclimation to heat strongly increased the Q10 of GR-activity, whereas water-deprivation had little effect on this Q10. A different picture emerged from measurements of GR-activities from root extracts. Acclimation to heat did not affect the Q10 of root GR-activity, but water-deprivation significantly enhanced this Q10 (Tables 2(A) and S5).

DHAR *in vitro* activities in leaves and roots were hardly affected by temperature and irrigation treatments (Tables 2(B) and S5). Only for DHAR activity from leaf extracts, we observed a slight, but significant, increase of Q10-values in response to drought (Tables 2(B) and S5).

3.4. Fatty acid composition of leaves and roots changes in response to drought but not to heat

Determination of fatty acid composition revealed significant abundance of 17 different compounds mainly including saturated, but also a few unsaturated fatty acids. Multivariate analysis demonstrated relatively high similarities of the fatty acid composition – and therefore common clustering – of the well-watered plants, *i.e.*, no significant differences between plants grown at 20 °C and 35 °C were observed (Figs. 6A and S1). In contrast, drought-stressed plants separated from the well-watered plants, forming own clusters; such clusters were also independent of growth temperature. The fatty acid composition pattern in root extracts showed similar effects. Drought stress in combination with elevated temperature resulted in fatty acid patterns significantly different from that of well-watered plants. The fatty acid pattern of drought stressed date palms, which were grown at 20 °C, however, did not differ from any other treatment (Fig. 6B). The fatty acids determining separation into the different clusters are shown in loading plots (Figs. 6C and D and S1). In leaves, hexadecanoic acid (“8”), eicosanoic acid (“12”), octadecanoic acid (“11”) and octacosanoic acid (“17”) were of greatest importance, whereas in roots octanoic acid (“3”), hexadecanoic acid (“8”), octadecanoic acid (“11”), pentacosanoic acid (“15”) and hexacosanoic acid (“16”) were responsible for forming clusters (Table S2). The double bond indices (DBIs) of leaves decreased; this effect was accompanied by a decrease of the level of 9,12-octadecanoic acid and 9,12,15-octadecanoic acid due to drought. However in the roots, DBIs were significantly increased in response to drought mainly because of an increase of the amount of 9,12-octadecanoic acid. In contrast, heat exerted no effects on DBIs (Table S2).

4. Discussion

Heat stress is usually accompanied by drought, such that individual effects on plant physiological performance are hardly

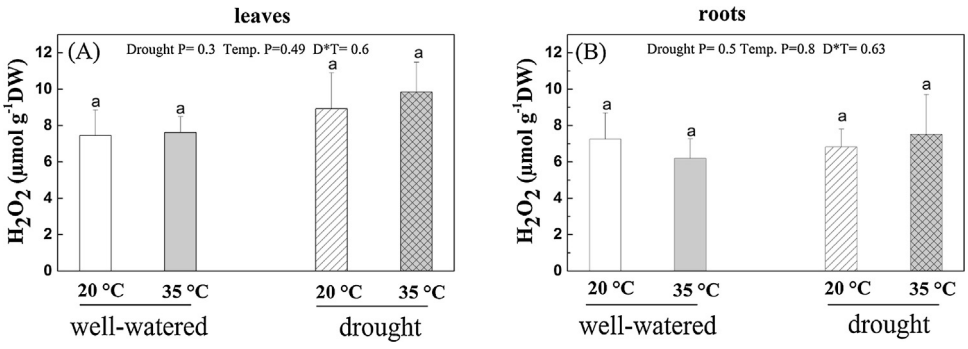

Fig. 3. H_2O_2 contents in leaves and roots of date palm plants treated by heat and drought. Data shown are means ± S.D (n = 14). Significant treatment effects on H_2O_2 contents were calculated by ANOVA.

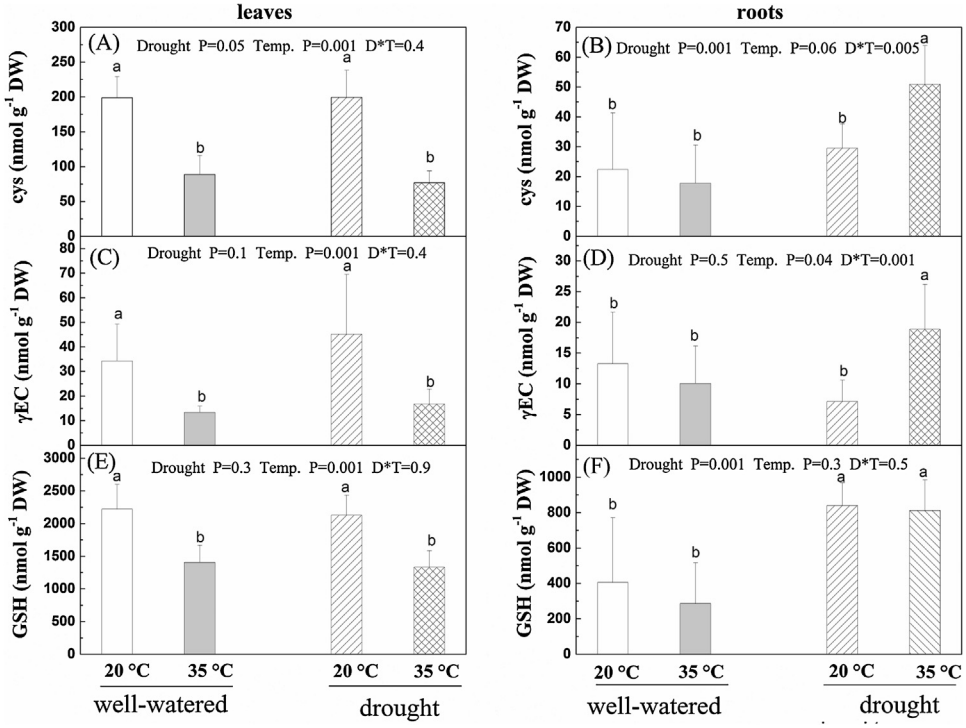

Fig. 4. Effects of heat and drought on thiol concentrations in leaves and roots of date palm trees. (A) Cysteine contents of leaves. (B) Cysteine contents of roots. (C) γ-EC contents of leaves. (D) γ-EC contents of roots. (E) GSH contents of leaves (GSH, γ-glutamyl-cysteinylglycin). (F) GSH contents of roots. Data shown are means ± S.D. (n = 14). Significant treatment effects on thiol contents were calculated by ANOVA.

distinguishable under field conditions. Heat generally promotes transpiration, thereby accelerating soil desiccation, which, in turn, down-regulates stomatal conductance. In most temperate woody species, this physiological response causes a drop in c_i, and enhanced formation of ROS. The combination of heat and drought seems to be more detrimental to plants than each stress individually (Dreesen et al., 2012). The present study attempts to separate the effects of heat and water-deprivation on physiological performance of date palm, paying particular attention to interactive effects between both stressors. Why does date

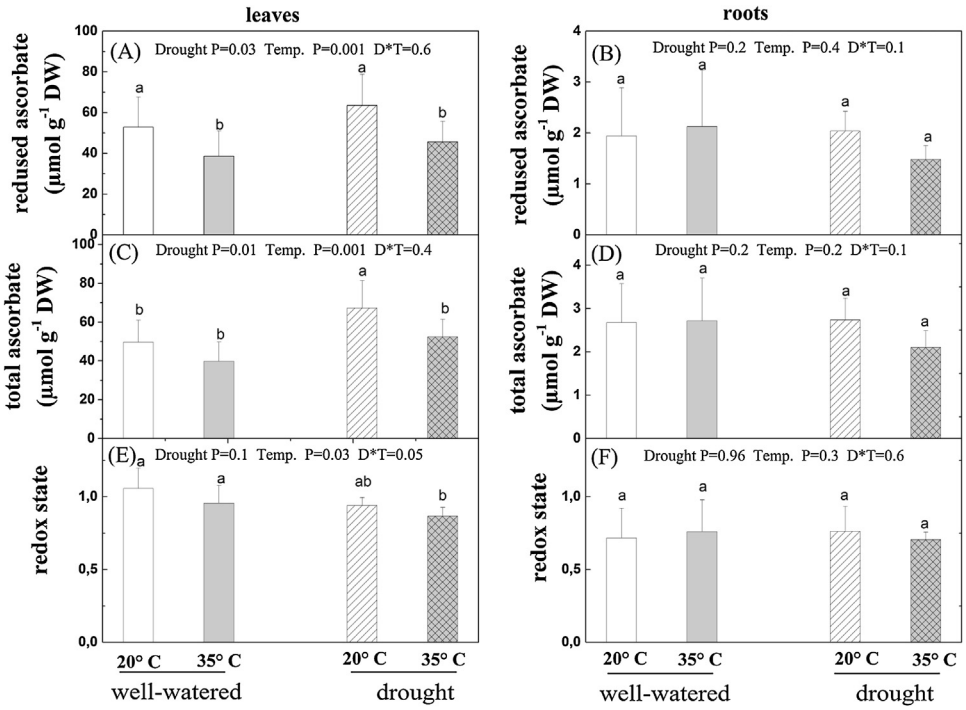

Fig. 5. Effects of heat and drought on ascorbate concentrations in leaves and roots of date palm trees. (A and B) Contents of reduced ascorbate in leaves and roots of date palm trees. (C and D) Contents of total ascorbate in leaves and roots of date palm trees. (E and F) Quotient of reduced: total ascorbate ('redox state') in leaves and roots of date palm trees. Data shown are means ± S.D. (n = 14). Significant treatment effects on ascorbate contents and the redox state were calculated by ANOVA.

Table 2
In vitro activities of antioxidative enzymes measured under substrate saturation. (A) *In vitro* activity of (iso-) enzymes involved in glutathione reduction, measured at two contrasting measurement temperatures ($T_{measure}$: 20 and 35 °C). (B) *In vitro* activity of (iso-) enzymes involved in dehydroascorbate reduction, measured at two contrasting measurement temperatures ($T_{measure}$). Data shown are means ± S.D. (n = 14). Significant treatment effects on enzyme activity were calculated by ANOVA.

(A) Glutathione reductase activity (μmol min^{-1} g^{-1} DW)

	Well-watered		Drought	
	20 °C	35 °C	20 °C	35 °C
Leaves				
$T_{measure}$ = 20 °C	3.97 ± 0.77ab	2.74 ± 0.3c	4.06 ± 0.53a	3.29 ± 0.5bc
$T_{measure}$ = 35 °C	4.95 ± 1.05b	5.84 ± 0.5ab	5.41 ± 0.9ab	6.15 ± 0.98a
Q_{10}	1.16 ± 0.12b	1.67 ± 0.12a	1.21 ± 0.14b	1.52 ± 0.12a
Roots				
$T_{measure}$ = 20 °C	1.09 ± 0.34a	0.61 ± 0.2b	0.79 ± 0.2b	0.6 ± 0.12b
$T_{measure}$ = 35 °C	2.41 ± 0.86a	1.43 ± 0.41c	2.22 ± 0.4ab	1.65 ± 0.35bc
Q_{10}	1.68 ± 0.14b	1.79 ± 0.3ab	2.01 ± 0.2a	1.99 ± 0.22a

(B) Dehydroascorbate reductase activity (μmol min^{-1} g^{-1} DW)

	Well-watered		Drought	
	20 °C	35 °C	20 °C	35 °C
Leaves				
$T_{measure}$ = 20 °C	0.68 ± 0.13a	0.68 ± 0.1a	0.76 ± 0.09a	0.64 ± 0.02a
$T_{measure}$ = 35 °C	1.34 ± 0.17b	1.39 ± 0.22b	1.68 ± 0.13a	1.51 ± 0.14a
Q_{10}	1.58 ± 0.17a	1.61 ± 0.18a	1.69 ± 0.12a	1.77 ± 0.14a
Roots				
$T_{measure}$ = 20 °C	0.27 ± 0.03a	0.27 ± 0.01a	0.29 ± 0.03a	0.25 ± 0.01a
$T_{measure}$ = 35 °C	0.6 ± 0.03ab	0.57 ± 0.03b	0.62 ± 0.04a	0.53 ± 0.03c
Q_{10}	1.68 ± 0.19a	1.64 ± 0.07a	1.67 ± 0.22a	1.61 ± 0.04a

palm succeed in an environment, where most other species would certainly fail?

4.1. Heat and drought had little effect on stomatal conductance, but affected photosynthesis

Two weeks of exposure to different growth temperature had little effect on T_{opt} of photosynthesis, which was already high in plants grown at 20 °C (i.e., 29.5 °C). T_{opt} of other species respond more quickly and stronger to shifts in growth temperature (Yamori et al., 2010). Rates of photosynthesis measured at growth temperature (A_{growth}), however, were generally higher in plants exposed to heat, in particular under well-watered conditions. Considering the evolutionary background of date palm with adaptation to dry and hot desert conditions, such behavior of photosynthesis is not surprising. Another result that may appear counter-intuitive was the effect of water-deprivation on A_{growth}. A_{growth} did not decline under drought, as one might have expected, but even slightly increased compared to well-watered plants.

Neither heat nor water-shortage had any significant effect on stomatal conductance of date palm, measured at growth temperature (G_{growth}). Stomatal conductance of date palm was already low under well-watered conditions compared to temperate woody species. Date palm is a slow-growing species with conservative water-usage, which appears to be a major feature of this species adapted to xeric environments. The duration of water-withdrawal (5–8 days), was apparently not stressful for date palms. The drop of c_i observed during water-shortage was caused by enhanced A_{growth}, and not by reduced G_{growth}. This response is contrary to temperate plant species. The leaf-N contents showed a similar pattern to net photosynthesis; *i.e.*, increased values at reduced water availability. Such effect was also observed in *Pinus sylvestris* where total N content significantly increased under drought (Turtola et al., 2003).

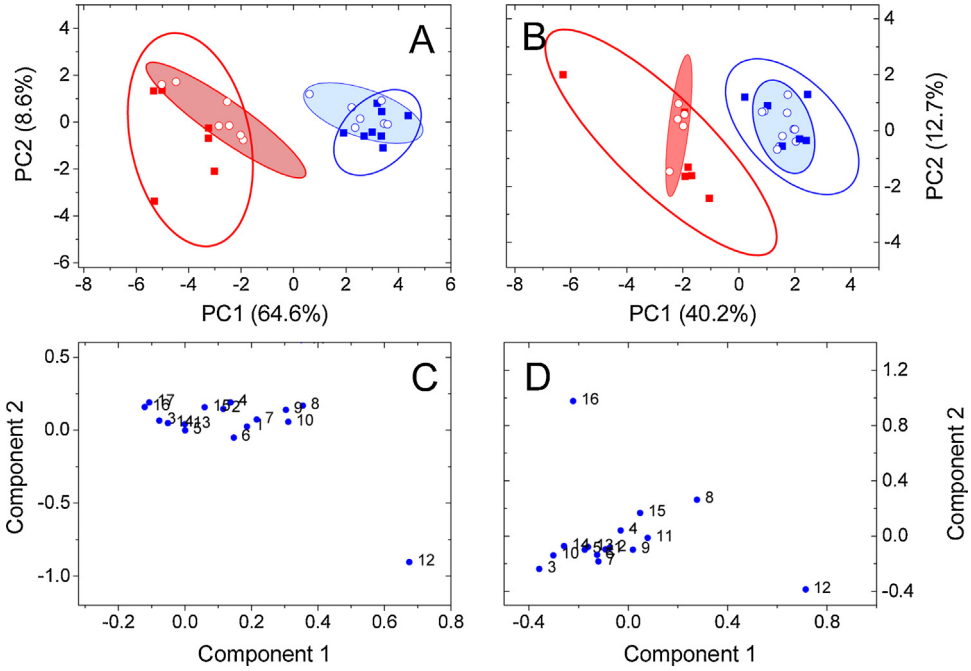

Fig. 6. Two-dimensional score (A and B) and scaled-loading plots (C and D) of partial least square discriminant analysis (PLSDA) calculated for fatty acid contents of leaves (A and C) and roots (B and D) of well-watered and drought stressed date palm grown at 20 °C or 35 °C. Plants (n = 7 per treatment) were divided into four treatment groups (drought, growth at 20 °C: red squares; drought, growth at 35 °C: red circles; well-watered, growth at 20 °C: blue squares, well-watered, growth at 35 °C: blue circles). Ellipses in (A) indicate the 95% confidence level; ellipses of clusters indicating treatment at elevated temperature are coloured. (C and D) indicates the relative importance of individual compounds for the principal components. Numbers given in the loading plot (C and D) indicate the compounds provided in Table S2. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

The increased levels of photosynthesis might therefore be a consequence of enhanced Rubisco abundance (Salvucci and Crafts-Brandner, 2004). In the present study, leaf C/N ratios increased by elevated temperature under well-watered and drought treatments, apparently due to decreased N contents. Similar results have been shown in other species (Albert et al., 2011). Surprisingly, leaf hydration increased in the present study by drought. However, this result is consistent with observations by Silva et al. (2011) which determined relative leaf water contents from 73% to 79% for sensitive sugarcane genotypes and from 85 to 87% for tolerant genotypes under water deficit conditions in the field. Apparently, tolerant plants possess a higher capacity to save water during drought, although the mechanisms responsible are not understood.

4.2. ROS abundance and the anti-oxidative system

ROS formation occurs in cells under normal as well as a wide range of stressful conditions (Munne'-Bosch et al., 2014). However, in consistence with our hypothesis (ii) neither heat nor drought and the combination of both affected H_2O_2 contents in date palm. ROS levels in plant tissues are a product of their rates of production and scavenging (Goh et al., 2012). Thus, unchanged H_2O_2 levels can be due either to an unaffected production rate or to an increased scavenging efficiency of the antioxidative system. This system consists of non-enzymatic (*i.e.*, antioxidants) and enzymatic components to efficiently quench ROS (Gill and Tuteja, 2010). Surprisingly, in leaves of date palms, the concentrations of GSH and its precursors cysteine and γ-EC as well as reduced and total ascorbate concentrations significantly decreased due to heat independent of water supply. In the roots, in contrast, drought caused increased levels of GSH independent of growth temperature. Cysteine concentrations in roots increased in response to the combination of drought and heat. These changes can be considered a mechanism to deal with enhanced ROS production during stress in the roots (Jozefczak et al., 2012). In contrast, the reduced levels of ascorbate and glutathione in leaves of stressed date palm might suggest an overload of the antioxidative capacity of the plants. In the present experiment, the apparent *in vitro* GR activity increased under elevated growth temperature and drought at 35 °C, therefore supporting our hypothesis (i). This is in consistence with previous research indicating that heat can stimulate GR activity (Sánchez-Rodríguez et al., 2010). Moreover, also drought caused higher GR activity in several species (Hossain et al., 2013; Ratnayaka et al., 2003). In the present experiments, heat and drought did not affect DHAR activity as well as the respective Q10 values, suggesting that another antioxidant defense mechanism enables date palms to face the adverse challenges of oxidative stress. The higher activities of GR indicate that although concentrations of glutathione decreased due to stress, the turnover through the antioxidative system was enhanced, thereby ensuring effective scavenging of ROS produced during the stress period (Jozefczak et al., 2012), hence, supporting our hypothesis (ii). We assume that the higher Q10 values for GR under drought are a consequence of an altered pattern of isozymes compared to well-watered plants. This assumption has to be tested in future studies by transcriptomic and proteomic approaches.

4.3. Heat but not drought causes increased isoprene emission

In consistence with our hypothesis (iii), highest isoprene emission rates were detected at saturating light intensity and at almost 45 °C (*i.e.*, 44.46 °C) leaf temperature in well-watered trees. This was different in drought-stressed trees where isoprene emission dropped at temperatures slightly above 45 °C. Such temperatures were clearly above the optimum temperature of photosynthesis. Several studies have demonstrated that isoprene can ameliorate heat stress effects on photosynthesis (Sharkey and Singsaas, 1995) most probably by either quenching ROS thereby reducing leaf internal levels of damaging ROS (Loreto and Velikova, 2001) or by interaction with thylakoid membranes thereby increasing the cohesiveness of the phospholipid bilayer (Velikova et al., 2011). This could indirectly be seen by altered chlorophyll fluorescence (Weis and Berry, 1987). As in our study, isoprene emissions were determined and not isoprene concentrations at the thylakoid membranes, the significance of our conclusions remains limited.

In the present study heat but not drought stimulated isoprene emission, which fits well with other research. In several species, drought reduced stomatal conductance and, subsequently, photosynthesis but did not show effects on isoprene emission (Brüggemann and Schnitzler, 2002; Fortunati et al., 2008). Only severe drought reduced isoprene emission most probably because precursors for isoprene biosynthesis could no longer be synthesized due to strongly reduced photosynthesis (Fortunati et al., 2008). The drought treatment in the present study was obviously too mild to cause effects on isoprene biosynthesis.

Longer term changes in growth temperature are often alleviated by altered fatty acid composition of membrane phospholipids (Harwood et al., 1994). However, in the present study, not heat but drought caused changes in fatty acid contents in date palm leaves—and to a less extent also in the roots, partially supporting our hypothesis (iii). The content of numerous fatty acids decreased in response to drought stress; and, as a consequence, the degree of unsaturation expressed as DBI (double bond index), was higher in leaves of well-watered plants and less in roots as compared to drought stressed palm trees (Table S2) indicating adjustment of the membrane composition to the stress. Current literature suggests that specific adjustments in the fatty acid composition and unsaturation level of lipids under drought stress could help plants maintaining membrane integrity (Zhong et al., 2011). Consistent with our results on leaf fatty acids, drought altered total fatty acid contents in leaves of other plants (Gigon et al., 2004). Earlier studies also revealed altered portions of saturated and unsaturated fatty acids in response to drought (Zhong et al., 2011), which was, however, not observed in our work. Surprisingly, in our study, fatty acid contents in leaves and roots did not respond to elevated growth temperature, and also the ratio of unsaturated to saturated C16 and C18-fatty acids remained constant (data not shown). Usually, growth at higher temperatures favors the incorporation of saturated C16 and C18 fatty acids into phospholipids, thus enhancing membrane stability (Falcone et al., 2004). We assume that in date palms the exposure to drought and to high temperatures was too short to induce strong modifications at the level of fatty acid composition and negative effects of heat were fully ameliorated by isoprene production.

5. Conclusions

Date palm revealed high tolerance against heat, short-term drought and the combination of these stresses. Such stress tolerance does not seem to be mediated by one physiological feature alone, but by a well-orchestrated network including several important tolerance mechanisms. Certainly, date palms possess an efficient antioxidative system that is able to quench ROS formed during periods of oxidative stress. On the other hand, the temperature-dependent increase in isoprene biosynthesis might contribute to the stabilization of thylakoid membranes under conditions of short-term heat pulses, whereas drought caused significant changes in fatty acid contents. Further studies should elucidate if other defense systems also contribute to the strong tolerance of date palm. To obtain a comprehensive overview of the

features involved in stress tolerance mechanisms of date palms, the temporal pattern of the stress response should be elucidated in more detail; in addition genome wide analyses are urgently required.

Acknowledgment

The authors extend their sincere appreciations to the Deanship of Scientific Research at King Saud University for its funding this Prolific Research group (PRG-1436-24).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.envexpbot.2016.01.003.

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