RESEARCH PAPER

Climate and development modulate the metabolome and antioxidative system of date palm leaves

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Abstract

Date palms are remarkably tolerant to environmental stresses, but the mechanisms involved remain poorly characterized. Leaf metabolome profiling was therefore performed on mature (ML) and young (YL) leaves of 2-year-old date palm seedlings that had been grown in climate chambers that simulate summer and winter conditions in eastern Saudi Arabia. Cultivation under high temperature (summer climate) resulted in higher YL H_2O_2 leaf levels despite increases in dehydroascorbate reductase (DHAR) activities. The levels of raffinose and galactinol, tricarboxylic acid cycle intermediates, and total amino acids were higher under these conditions, particularly in YL. The accumulation of unsaturated fatty acids, 9,12-octadecadienoic acid and 9,12,15-octadecatrienoic acid, was lower in ML. In contrast, the amounts of saturated tetradecanoic acid and heptadecanoic acid were increased in YL under summer climate conditions. The accumulation of phenolic compounds was favored under summer conditions, while flavonoids accumulated under lower temperature (winter climate) conditions. YL displayed stronger hydration, lower H_2O_2 levels, and more negative $\delta^{13}C$ values, indicating effective reactive oxygen species scavenging. These findings, which demonstrate the substantial metabolic adjustments that facilitate tolerance to the high temperatures in YL and ML, suggest that YL may be more responsive to climate change.

Keywords: Amino acids, antioxidants, fatty acids, leaf age, membrane fluidity, metabolomics, phenolics, reactive oxygen species, temperature responses.

Introduction

Long-term, recurrent patterns of incident radiation, growth temperature, and precipitation define climate, and largely determine geographical distribution and productivity of plant species (Walther *et al.*, 2002; Chen *et al.*, 2011; Shabani *et al.*, 2012). Over shorter time periods, plants also have to cope with diurnal, weekly, and seasonal variation of meteorological conditions, in particular that of growth temperature (Rennenberg *et al.*, 2006; Chen *et al.*, 2011; Kruse *et al.*, 2011). Even slight variation in growth temperature can trigger a wide range of metabolic responses, for example changes in the accumulation of osmolytes, compatible solutes, antioxidants, and signaling molecules (Kaplan *et al.*, 2004; Guy *et al.*, 2008; Nishizawa *et al.*, 2008). Many of these changes correlate with increased tolerance to stressful abiotic conditions (Rennenberg *et al.*, 2006), known as acquired thermo- and chilling tolerance (Guy *et al.*, 2008). As poikilothermic organisms, plants must be able to adjust physiologically to changing temperature, in order to coordinate metabolism with growth and development, and to avoid metabolic imbalances that could cause 'stress' (Rennenberg *et al.*, 2006; Kruse *et al.*, 2011, 2017).

The capability to withstand temperature-induced stress depends on climate of plant origin (Aranda *et al.*, 2005; Chaves *et al.*, 2011; Du *et al.*, 2014). Generally, plants from tropical and subtropical climates with significant changes in temperature and solar radiation are more prone to chilling stress (Kyparissis *et al.*, 2000; Aranda *et al.*, 2005; Cunningham and Read, 2006). Instead, boreal and temperate species are more prone to heat stress (Cunningham and Read, 2006; Hozain *et al.*, 2010). Moreover, numerous studies showed that mature and developing leaves differ in their resistance and resilience to climate variations. In general, young developing leaves with greater metabolic activity are more vulnerable to climatic stress, as observed for Douglas fir (*Pseudotsuga menziesii*) (Du *et al.*, 2014; Jansen *et al.*, 2014), European beech (*Fagus sylvatica*) (Polle *et al.*, 2001), soybean (*Glycine max*) (Jiang *et al.*, 2005), and tobacco (*Nicotiana tabacum*) leaves (Gullner and Tyihák, 1991). At the same time, however, developing leaves are also more plastic than mature leaves, in terms of both structural and physiological features—which might allow for more effective acclimation to changing environmental conditions (Urban *et al.*, 2008; Sperdouli and Moustakas, 2012).

Date palms (*Phoenix dactylifera* L.) are remarkably tolerant to environmental stresses, and significantly contribute to economic and natural wealth of predominantly arid and semiarid regions. Its plantations are still expanding (Shabani *et al.*, 2012; Al-Redhaiman, 2014), even though more frequent drought periods and temperature extremes are projected to restrict productivity in a changing climate (Allbed *et al.*, 2017). Thermal acclimation of date palms is well documented for leaf gas exchange (Kruse *et al.*, 2016, 2017, 2019). The capacity of leaf dark respiration, for example, is generally down-regulated upon exposure to warmer temperature, in order to compensate for accelerated rates of biochemical reactions (Kruse *et al.*, 2011). As a result, respiratory CO_2 flux remains more or less homeostatic irrespective of changing growth temperature. Compensatory processes are partly reflected in altered transcript and protein abundances of mitochondrial enzymes (Arab *et al.*, 2016; Kruse *et al.*, 2017; Safronov *et al.*, 2017). As another example, photosynthetic acclimation is often associated with shifts in optimum temperature of CO_2 assimilation, tracking changes in growth temperature (Kruse *et al.*, 2019). Mechanisms conferring this kind of acclimation response remain poorly understood, but might be related to altered lipid composition of thylakoid membranes for adjustment of membrane fluidity to prevailing growth temperature—and could be revealed by changing abundances of specific fatty acids (Penfield, 2008; Arab *et al.*, 2016; Safronov *et al.*, 2017).

In the present study, sufficiently watered date palm seedlings were grown for 3 weeks under meteorological conditions that are typically experienced in Saudi Arabian winter or summer. We monitored temperature-induced changes in foliar metabolite profiles (including fatty acids), with the aim to elucidate thermal acclimation strategies in both young expanding leaves and mature fully developed leaves. Specifically, we tested the following hypotheses: (i) foliar reactive oxygen species (ROS) levels are largely conserved in different climates due to enhanced ROS-scavenging capacity, particularly in summer-acclimated plants with increased enzyme activity and antioxidant abundances of the antioxidant system; and (ii) acclimation to summer temperatures will change abundances of fatty acids. We expected to find greater concentrations of saturated fatty acids, conferring greater viscosity to membranes. We further hypothesized that (iii) temperature-induced changes in antioxidant concentrations and fatty acid profiles will be more pronounced for young compared with mature leaves.

Materials and methods

Plant material and experimental set-up

Two-year old seedlings of date palm were purchased from a commercial supplier ('Der Palmenmann', Bottrop, Germany) and potted into 3.3 liter pots filled with a peat–soil–sand mixture (3:1:7 v/v/v). Each pot was supplied with ~10 g of Osmocote fertilizer (16% N, 9% P_2O_5, 12% K_2O). Plants were grown under greenhouse conditions [day length 12 h, 25/15 °C, 20/30% relative humidity (day/night)] and irrigated once per week (~150–200 ml per pot). After 2 months, on 10 January 2014, seedlings were transferred to four fully automatized, climate-controlled walk-in growth chambers. Conditions in the four exposure chambers were slowly adjusted to match typical Saudi Arabian summer and winter conditions. Final climatic conditions were attained 9 d after the start of the experiment. At this time, average noon temperatures peaked at ~40 °C in summer and ~25 °C in winter according to the temperature averages during 10 years (2003–2012; relative humidity data were available for 2013 only) in Alahsa, Saudi Arabia. Maximum irradiance was similar in summer and winter climate conditions (i.e. photon flux density: 600 μM m^{-2} s^{-1}; for more detailed information, refer to Kruse *et al.*, 2019). Each day, plants were watered with 40 ml of de-ionized water (at 06.00 h). To achieve similar soil water availability (~20% of soil volumetric water content), plants in summer climate were supplied with an additional 40 ml of de-ionized water (every evening at 18.00 h). Fully developed mature leaves (ML) and still developing young leaves (YL) from six individual plants (per season) were harvested at midday, after 3 weeks of acclimation to differing growth temperature (i.e. on 31 January 2014; for more detailed information, refer to Kruse *et al.*, 2019). Plant material was immediately frozen and homogenized in liquid nitrogen, and kept at −80 °C until further analyses.

Leaf hydration measurements

Leaf hydration (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 (Du *et al*., 2018).

Total carbon (C) and nitrogen (N) contents, and δ^{13}C, δ^{15}N signatures

Between 1.5 mg and 2.0 mg of dried, pulverized leaf material was weighed into tin capsules (IVA Analysentechnik, Meerbusch, Germany), and combusted in an elemental analyzer (NA 2500; CE Instruments, Milan, Italy) coupled to an isotope ratio mass spectrometer (Delta Plus/ Delta Plus XL; Finnigan MAT, Bremen, Germany) by a Conflo II/III interface (Thermo–Finnigan, Bremen, Germany) for leaf C, N, and isotope signature analysis as described previously in Peuke *et al*. (2006).

Thiols and ascorbate measurement

Glutathione (GSH), cysteine, and γ–glutamylcysteine contents were extracted from ~40 mg of frozen leaf powder with 1 ml of 0.1 M HCl containing 100 mg of pre-washed polyvinylpolypyrrolidone (PVP 6755; Sigma-Aldrich Chemie, Steinheim, Germany) as described by Schupp and Rennenberg (1988). Determination of oxidized glutathione (GSSG) was based on the irreversible alkylation of the free thiol groups of the GSH present with *N*-ethylmaleimide (NEM), and the subsequent reduction of GSSG with DTT (Strohm *et al*., 1995). Reduced thiols were derivatized with monobromobimane as previously described (Schupp and Rennenberg, 1988). Thiol derivatives were separated on an ACQUITY UPLC® HSS (Waters, Eschborn, Germany) with a C-18 column (2.1×50 mm; 1.18 µm mesh size) applying a solution of potassium acetate (100 mM) in methanol (100%) for elution. Concentrations of thiols were quantified according to a mixed standard solution consisting of GSH, cysteine, and γ–glutamylcysteine subjected to the same reduction and derivatization procedure (Samuilov *et al*., 2016).

Total and reduced ascorbate were determined with a UV-DU650 spectrophotometer (Beckman Coulter, Fullerton, CA, USA) using a colorimetric method previously described (Arab *et al*., 2016). Concentrations of total and reduced ascorbate were calculated according to a standard curve using dilutions of 1.5 mg ml^{-1} L-ascorbic acid (Sigma-Aldrich).

Determination of hydrogen peroxide (H_2O_2) contents and in vitro *activities of glutathione reductase (GR) and dehydroascorbate reductase (DHAR)*

Foliar H_2O_2 contents were measured according to Velikova *et al*. (2000) as described in Du *et al*. (2018). The absorbance of H_2O_2 was measured at 390 nm (UV-DU650 spectrophotometer, Beckman Coulter Inc.) and the concentration was quantified using a standard curve ranging from 0 to 100 µM H_2O_2.

In vitro GR (EC 1.8.1.7) and DHAR (EC 1.8.5.1) activities of leaves were determined as described previously (Arab *et al*., 2016). GR activity was quantified by monitoring glutathione-dependent oxidation of 1.25 mM NADPH at 340 nm; DHAR activity was analyzed directly by following the increase in absorbance at 265 nm, resulting from GSH-dependent production of ascorbate (Polle *et al*., 1990). Enzyme activities were measured at both 25 °C and 40 °C in accordance with midday peak temperatures experienced under winter and summer climate simulated in the chambers.

Total amino acids, soluble protein, pigment contents, and structural N calculation

Total soluble amino acids (TAAs) were extracted and determined as described in Simon *et al*. (2010) by a modification of the method of Winter *et al*. (1992). Glutamine was used as a standard for the calculation of TAA contents.

Total soluble protein contents were determined as previously described (Du *et al*., 2014) by the absorbance of the mixture of 5 µl of extract with 200 µl of Bradford reagent (Amresco, Solon, OH, USA) measured at 595 nm on a 96-well microplate reader (Sunrise Basic, Tecan, Grödig, Austria).

Foliar Chl *a*, Chl *b*, and carotene contents were extracted and calculated according to the method of Lichtenthaler and Wellburn (1983). For this purpose, 25 mg of frozen leaf powder was extracted with 8 ml of 80% acetone, and the extraction was incubated at 4 °C overnight with constant shaking. The supernatant containing the pigments was separated from the pellet by centrifugation and measured at 470, 646, and 663 nm using a UV-DU650 spectrophotometer (Beckman Coulter).

The structural N content in leaves was calculated by subtracting the N fractions of soluble proteins, amino acids, and pigments from total N in leaf bulk material as described previously by Du *et al*. (2014).

Determination of anions

The anions nitrate (NO_3^-), phosphate (PO_4^{3-}), and sulfate (SO_4^{2-}) were determined in aqueous extracts from homogenized frozen material by automated anion chromatography as described previously by Peuke *et al*. (2006). Separation of anions was achieved on an ion exchange column (AS12A, 4 mm, Dionex, Idstein, Germany) with 2.7 mM Na_2CO_3 and 0.3 mM $NaHCO_3$ as mobile phase. Detection and quantification were performed with a pulsed amperometric detector (Electrochemical detector ED 40 Dionex). Sodium salts of nitrate, phosphate, and sulfate were used as standards.

Metabolites analyzed by GC-MS

Extraction and derivatization of water-soluble metabolites and fatty acids from leaf samples were performed by a modified method of Lisec *et al*. (2006). Aliquots of 50 mg of homogenized frozen leaf powder were weighed into pre-frozen 2 ml Eppendorf tubes and 600 µl of cold 100% methanol was added as extraction medium. Then, 60 µl of ribitol (0.2 mg ml^{-1} in ddH_2O) was added as internal standard. Tubes were rapidly heated to 70 °C and shaken at 1200 *g* for 10 min. After centrifugation, 500 µl aliquots of the supernatant were transferred to new microfuge tubes and combined with the same volume of ddH_2O and chloroform. The combined solutions were intensively shaken and centrifuged at 14 000 *g* at 4 °C for 5 min. Aliquots of 100 µl of the supernatant methanol phase were transferred into 1.5 ml Eppendorf tubes and dried in a freezer drier (Alpha 2–4, Martin Christ Gefriertrocknungsanlagen, Osterode am Harz, Germany) for analysis of polar metabolites. Aliquots of 300 µl of supernatant of the chloroform phase were mixed with 30 µl of nonadecanoic acid methyl ester (0.2 mg ml^{-1} in chloroform) as internal standard and dried in a speed-vacuum device (RVC 2-25, Martin Christ Gefriertrocknungsanlagen) for fatty acid analysis.

Dried extracts from the methanol phase were methoximated by adding 20 µl of a solution containing 20 mg ml^{-1} methoxyamine hydrochloride in anhydrous pyridine (Sigma-Aldrich); the mixtures were incubated at 30 °C for 90 min and shaken at 1400 *g*. For trimethylsilylation, 35 µl of *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA; Sigma-Aldrich) was added to each tube. The mixtures were incubated at 37 °C for 30 min and shaken at 1400 *g*. A 5 µl aliquot of *n*-alkane retention index calibration standard (*n*-alkane mix, C10–C40, 50 µg ml^{-1} in *n*-hexane; Sigma-Aldrich) was added to each sample. After brief vortexing, reaction mixtures were centrifuged (14 000 *g*, 20 °C) for 2 min, and 50 µl of supernatant were transferred to GC-MS vials with low volume inserts and screw-top seals (Agilent Technologies, Palo Alto, CA, USA) for GC-MS analysis. Derivatization of dried extracts from the chloroform phase was achieved by adding 70 µl of MSTFA reagent. A 5 µl aliquot of FAMEs (fatty acid methyl ester mixture, 1 mg ml^{-1} from Sigma-Aldrich) was added as internal standard. The reaction solutions were incubated with shaking at 37 °C and 1200 *g* for 30 min. Finally, 50 µl aliquots were transferred into GC-MS glass vials with inserts, and sealed for GC-MS analysis.

The abundance of polar and non-polar metabolites was determined on a GC-MS system as previously described (Du *et al*., 2016). Analysis conditions as well as MS settings were as described by Kreuzwieser *et al*. (2009). Peak identification and deconvolution of chromatograms were performed using the Quantitative Analysis Module of the MassHunter software (Agilent Technologies). For metabolite identification, the Golm metabolome database (Hummel *et al*., 2010) was used. Peak areas were

normalized using the peak area of the internal standards, namely ribitol and nonadecanoic acid methyl ester (Sigma–Aldrich) for polar metabolites and fatty acids, respectively, and the dry weight of the samples. Abundance of metabolites was indicated by normalized peak areas. Artifact peaks and common contaminants were identified by analysis of 'blank' samples prepared in the same manner as biological samples. Signals corresponding to these artifacts were omitted from interpretation.

Statistical analysis

Two–way ANOVA followed by the Holm–Sidak method was employed to examine effects of climate, leaf age, and their interaction on physiological leaf traits (SigmaPlot 12.0; Systat Software, Erkrath, Germany). Data obtained via GC–MS were $\log_{10}$ transformed to meet the criterion of normal distribution.

Sparse partial least square discriminant analysis (sPLS-DA) was conducted using a public web tool (MetaboAnalyst 4.0, http://www.metaboanalyst.ca/) (Lê Cao *et al.*, 2011; Chong *et al.*, 2018) after $\log_{10}$ transformation and mean-centering. Missing values were replaced by half the minimum abundance of the respective compounds, assuming that their concentrations were below the detection limit. Data shown in figures and tables represent means ±SE (*n*=4–6) on a dry weight basis.

Results

Exploratory analysis indicates effects of climate and development on the foliar metabolome and antioxidative system

The sPLS-DA clearly indicated a clustering of date palm leaves according to climate conditions (15% explained by PC2) and

leaf development (25% explained by PC1) (Fig. 1). Overall, climate and development had different effects on metabolites; that is, climate mainly affected some sugar, polyol, and amino acid abundances, wherea, ML and YL mostly differed in levels of H_2O_2, enzyme activities, and antioxidant abundances (Supplementary Table S1 at *JXB* online).

Climate affects the foliar metabolome and antioxidative system

Significant effects between summer and winter climate conditions ($P<0.05$) were mostly documented in YL, which had 28% higher H_2O_2 levels, 31% higher TAA contents, but 23% less total ascorbate under summer compared with winter climate (Tables 1, 2). Moreover, DHAR activities were significantly increased under summer climate in YL at both 25 °C and 40 °C assay temperature (Fig. 2). Only very minor effects of climate on leaf hydration, other antioxidant levels, and basic C and N fractions were observed. Pigment contents were not significantly affected by climate, except for the Chl *a*/*b* ratio in YL that was significantly decreased in summer climate (Table 2). Leaf contents of nitrate, sulfate, and phosphate were slightly increased under summer climate (Table 2).

Compared with winter climate conditions, the summer climate caused a higher abundance of raffinose, galactinol, 6-kestose, and gulono-1,4-lactone, but a lower abundance of glucose-6-phosphate, xylose, sorbose, galactose, lactose, sorbitol,
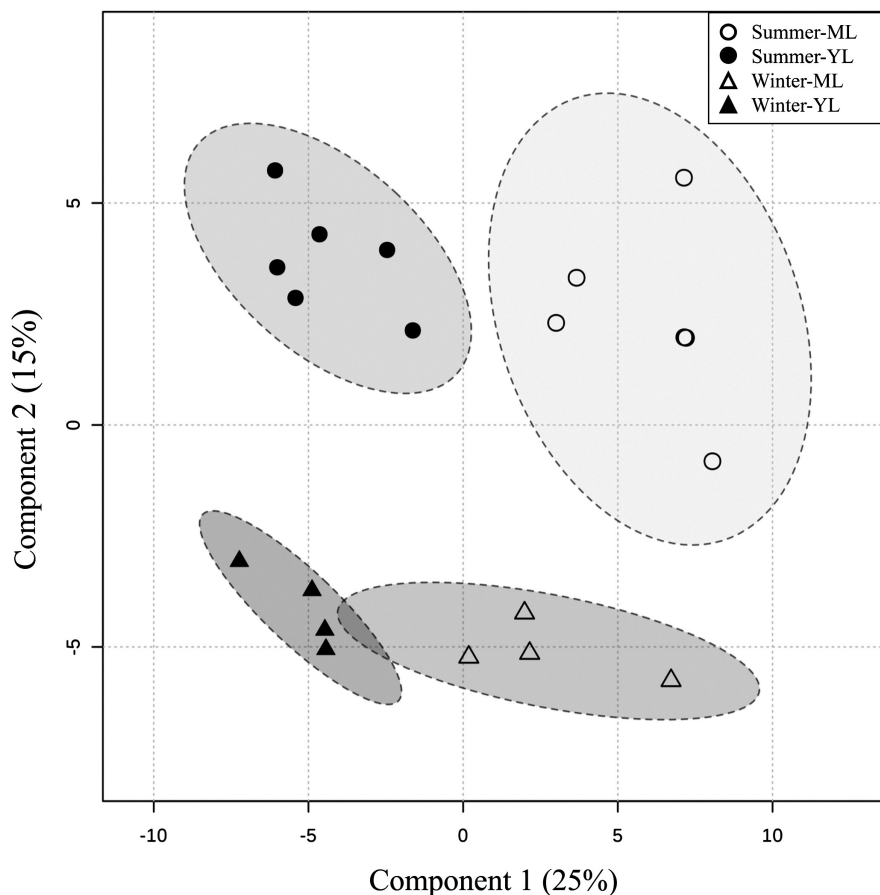

Fig. 1. Clustering of date palm seedlings based on metabolites in mature leaves (ML, open symbols) and young leaves (YL, filled symbols) of plants grown under summer (circles) and winter (triangles) climate, respectively. Semi-transparent shadings indicate 95% confidence regions.

Table 1. *Leaf hydration, hydrogen peroxide, ascorbate, and thiol contents in mature and young leaves of date palm seedlings under summer and winter climate*

Parameters	Leaf age	Climate	
		Winter	Summer
Leaf hydration (g H_2O g^{-1})	Mature	**1.27±0.03**	**1.31±0.08**
	Young	**1.42±0.03**	**1.54±0.05**
Hydrogen peroxide (µM g^{-1})	Mature	**7.46±1.57**	**10.21±0.77**
	Young	**3.37±0.22** a	**4.33±0.28** b
Total ascorbate (µM g^{-1})	Mature	22.45±0.51	21.38±1.12
	Young	24.39±1.59 a	18.72±1.87 b
Reduced ascorbate (µM g^{-1})	Mature	22.95±0.35	22.19±1.22
	Young	24.78±1.53	19.15±1.93
Total glutathione (µM g^{-1})	Mature	804.31±46.19	817.41±65.42
	Young	747.73±48.52	845.10±73.15
Oxidized glutathione (µM g^{-1})	Mature	183.36±28.65	135.27±26.13
	Young	171.62±18.63	158.25±23.78
GSSG/GSH (%)	Mature	22.54±2.42	15.85±2.25
	Young	23.17±2.62	19.09±2.79
γ-Glutamylcysteine (µM g^{-1})	Mature	2.39±0.78	**1.65±0.21**
	Young	3.40±0.50	**4.00±1.39**
Cysteine (µM g^{-1})	Mature	37.18±3.01	36.41±3.08
	Young	38.61±3.60	36.04±2.57

Data shown are the mean ±SE, *n*=4–6. Numbers in bold indicate significant differences (*P*<0.05) between mature and young leaves within the same climate. Different lower case letters indicate significant differences between winter and summer climates of young leaves. No significant interaction between leaf age and climate was found

Table 2. *Basic C and N metabolite composition and anions of date palm leaves*

Parameters	Leaf age	Climate	
		Winter	Summer
Total C (mg g^{-1})	Mature	485.12±7.66	477.81±6.56
	Young	482.72±15.77	477.69±2.54
Total N (mg g^{-1})	Mature	16.06±1.95	15.65±0.85
	Young	17.33±1.31	19.07±1.16
C/N ratio	Mature	32.10±5.31	30.97±1.65
	Young	28.37±2.42	25.57±1.75
Soluble protein N (mg g^{-1})	Mature	4.97±0.63	5.18±0.46
	Young	5.93±0.66	5.58±0.57
Total amino acid N (mg g^{-1})	Mature	0.87±0.03	**1.12±0.09**
	Young	1.15±0.22 a	**1.54±0.08** b
Chl *a* (mg g^{-1})	Mature	7.89±0.43	6.85±0.64
	Young	7.52±1.11	7.94±0.89
Chl *b* (mg g^{-1})	Mature	1.93±0.13	1.84±0.14
	Young	1.84±0.30	2.12±0.21
Chl *a/b*	Mature	4.10±0.08	3.71±0.09
	Young	4.12±0.10 a	3.73±0.06 b
Carotene (mg g^{-1})	Mature	2.08±0.08	1.84±0.13
	Young	1.80±0.25	1.88±0.20
Structural N (mg g^{-1})	Mature	10.22±1.33	9.35±0.50
	Young	10.25±0.79	11.95±0.89
δ ^{13}C (‰)	Mature	**-27.78±0.19**	**-27.59±0.28**
	Young	**-29.27±0.41**	**-28.70±0.16**
δ ^{15}N (‰)	Mature	1.29±0.68	0.95±0.35
	Young	1.06±0.49	1.75±0.26
Nitrate (µM g^{-1})	Mature	1.08±0.03	3.17±1.73
	Young	1.77±0.96	3.40±1.35
Phosphate (µM g^{-1})	Mature	25.63±6.05	30.74±6.27
	Young	31.75±8.91	46.13±8.62
Sulfate (µM g^{-1})	Mature	38.31±5.92	51.46±14.38
	Young	31.29±8.71	45.44±9.61

Data shown are the mean ±SE, *n*=4–6. Numbers in bold indicate significant differences (*P*<0.05) between mature and young leaves within the same climate. Different lower case letters indicate significant differences between winter and summer climates of young leaves. No significant interaction between leaf age and climate was found

galactonic acid, glyceric acid, dehydroascorbate (DHA) dimer, and isoascorbic acid in both leaf age classes (Fig. 3).

Primary metabolites involved in the tricarboxylic acid (TCA) cycle, namely citrate, succinate, fumarate, and malate, were generally up-regulated under the summer climate, with the exception of succinate in YL. Likewise, the abundances of the amino acids glycine, phenylalanine, alanine, threonine, γ-aminobutyric acid (GABA), glutamate, and glutamine were increased under summer climate, whereas lysine and *N*-acetyl-ornithine were more abundant in leaves under winter climate (Fig. 3). Summer climate also increased the abundances of *N*-acetylneuraminic acid (Neu5Ac), nicotinic acid, and phosphoric acid compared with winter climate. The abundance of glycerol-3-phosphate declined under summer climate in both leaf types (Fig. 3).

Shikimate as well as its metabolic flavonoid products taxifolin, catechin, and epicatechin were decreased in leaves under summer climate, whereas the contents of the phenylpropanoid *trans*-sinapic acid and the benzoic salicylic acid were generally enhanced. Four fatty acids, namely hexanoic acid, dodecanoic acid, tetradecanoic acid, and 5,8,11,14–eicosatetraenoic acid, as well as stearyl alcohol, α-tocopherol, and β-sitosterol were increased under summer climate in both ML and YL (Fig. 3). Compared with the winter climate conditions, ML had lower levels of the fatty acids hexadecanoic acid, 9,12-octadecadienoic acid, 9,12,15-octadecatrienoic acid, and heptadecanoic acid, and the amino acid proline under summer climate, whereas YL showed generally increased abundances of these metabolites

under summer climate. YL had significantly lower abundances of myo-inositol and monomethyl phosphate in summer than winter climate, whereas the level of these compounds showed an opposite effect in ML (Fig. 3).

Leaf development affects the foliar metabolome and antioxidative system

Compared with ML, YL were more hydrated and had lower H_2O_2 contents (Tables 1, 2), as well as more negative δ ^{13}C values (*P*<0.05) irrespective of climate (Table 2). *In vitro* enzyme activities of GR and DHAR were significantly higher (*P*<0.05) in YL than in ML (Fig. 2A). No significant differences of ascorbate and thiol levels between ML and YL were observed, except for γ-glutamylcysteine, which was higher in YL under summer climate (Table 2).

Higher TAA contents in YL than in ML, particularly under the summer conditions (*P*<0.05) (Table 2), could be attributed
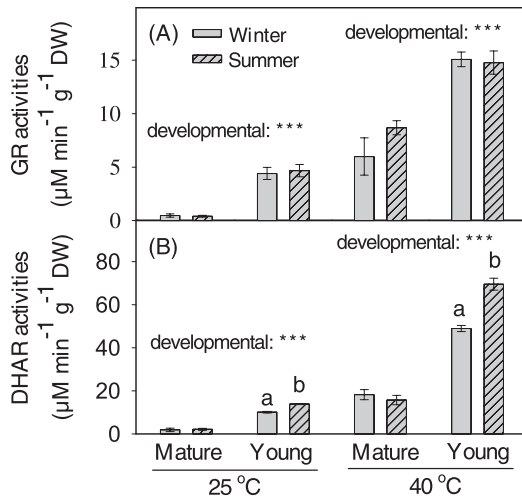
Fig. 2. *In vitro* activities of glutathione reductase (GR) and dehydroascorbate reductase (DHAR) in mature and young leaves of date palm seedlings grown under winter (gray bar) and summer (hatched bar) climate measured at 25 °C (left panel) and 40 °C (right panel), respectively. Different lower case letters indicate significant differences ($P<0.05$) between winter and summer climate. The enzyme activities in young leaves are significantly higher than those in mature leaves irrespective of climate conditions at both assay temperatures (developmental: ***$P<0.001$). Significant interactions between development and climate were observed in DHAR activities measured at 25 °C ($P=0.003$) and 40 °C ($P<0.001$), respectively. No significant interaction between development and climate was found for GR activities. The enzyme activities measured at 40 °C were all significantly higher ($P<0.01$) than those measured at 25 °C.

to a number of specific amino acids: (i) serine and glycine derived from 3-phosphoglycerate; (ii) proline, arginine, *N*-acetyl-glutamate, pyroglutamate, *N*-acetyl-ornithine, and GABA derived from 2-oxoglutarate; (iii) alanine and β-alanine derived from pyruvate; (iv) aspartate and threonine derived from oxaloacetate; and (v) phenylalanine derived from shikimate. Proline, alanine, threonine and *N*-acetyl-glutamate ($P<0.05$) in particular were responsible for the difference between YL and ML in summer climate (Fig. 3). Generally, also in winter climate higher contents of these amino acids were observed, except for proline, serine, and pyroglutamate. In addition, tryptophan and glutamine also showed higher contents in YL compared with ML under the cooler conditions (Fig. 3). No significant effects of leaf development were apparent on total C, total N, soluble protein, pigment, structural N and anion contents, the C/N and Chl *a/b* ratios, and the $\delta^{15}N$ signature, either in summer or under winter climate (Table 2).

Concentrations of most soluble sugars were higher in ML than in YL in both climates, except for xylose and 6-kestose, as well as glucose and erythrose under summer climate (Fig. 3). Galactinol, myo-inositol, sorbitol, and the sugar acids galactonic acid and glyceric acid were less abundant in ML, particularly in winter climate. Lower levels of shikimate were recorded in YL compared with ML irrespective of climate. In ML, the fatty acids hexanoic acid, dodecanoic acid, and tetradecanoic acid were more abundant in summer and winter climate, whereas lower abundances of heptadecanoic acid, hexadecanoic acid, 9,12-octadecadienoic acid, and 9,12,15-octadecatrienoic acid were observed in particular in summer climate. In contrast to lysine, Neu5Ac, glycerol-3-phosphate, gulono-1,4-lactone,

4-hydroxy-benzoic acid, *trans*-caffeic acid, salicylic acid, nictonic acid, α-tocopherol, and β-sitosterol, which were more abundant in ML, urea, maleamate, 2*R*,5*R*-dihydroxymethyl-3*R*,4*R*-dihydroxy-pyrrolidine (DMDP), pyrophosphate, lumichrome, and an unknown compound of A143018 had higher abundances in YL irrespective of climate (Fig. 3). No significant interaction between leaf age and climate was found for these parameters.

Discussion

Seasonal climate traits of date palm leaves

In the present study, two contrasting climatic conditions (summer versus winter climate) typical for Saudi Arabia and mainly determined by differences in temperature were shown to significantly affect both foliar ROS levels and the antioxidative system of date palm leaves, which corroborated the increased expression of genes encoding ROS scavenging-related proteins in response to heat (Safronov *et al.*, 2017). H_2O_2 plays a dual role in plants, either acting as a signal molecule that mediates responses to abiotic and biotic stresses, or orchestrating programmed cell death when accumulated to high levels (Quan *et al.*, 2008). Although H_2O_2 is continually generated from various sources during normal plant metabolism, mitochondrial respiration and photoproduction of O_2^- at chloroplast thylakoids are considered to be the major intracellular sources of accidental ROS production (Asada, 2006; Giorgio *et al.*, 2007). Safronov *et al.* (2017) identified genes of date palms encoding ROS-scavenging-related proteins (e.g. ascorbate peroxidase, superoxide dismutase, dehydroascorbate reductase, and thioredoxins) targeted to the chloroplast and cytosol. These genes had increased expression, whereas the expression of genes encoding proteins (e.g. glutaredoxin and thioredoxins) targeted to mitochondria was decreased under either elevated temperature alone or in combination with drought. Compared with winter climate, summer temperatures caused 37% and 28% higher H_2O_2 contents in ML and YL, respectively ($P<0.05$ for YL), which is partly against our first hypothesis. Although ROS production was not directly measured in the present study, the increased ROS levels, particularly in YL, were most likely to be due to an enhanced production efficiency (Goh *et al.*, 2012), presumably used for signaling purposes (Foyer and Shigeoka, 2011). This is assumed from apparently stimulated photorespiration under summer climate, as indicated from slightly elevated glycine and serine abundances concurrent with the decreased abundance of its precursor glyceric acid (Yu *et al.*, 2012). Metabolite flux analyses in future studies will test this assumption. Stimulated photorespiration caused by the high temperature seems feasible in the present study, because samples were harvested at a peak temperature of 40 °C at midday, which is above the 36 °C optimum temperature (A_{opt}) of date palm photosynthesis recently reported (Kruse *et al.*, 2019). The present study also highlights that while A_{opt} was increased in summer (Kruse *et al.*, 2019), there was also a greater risk of metabolic perturbation.

The glutathione–ascorbate cycle is the most important metabolic pathway for the detoxification of H_2O_2 (Noctor and
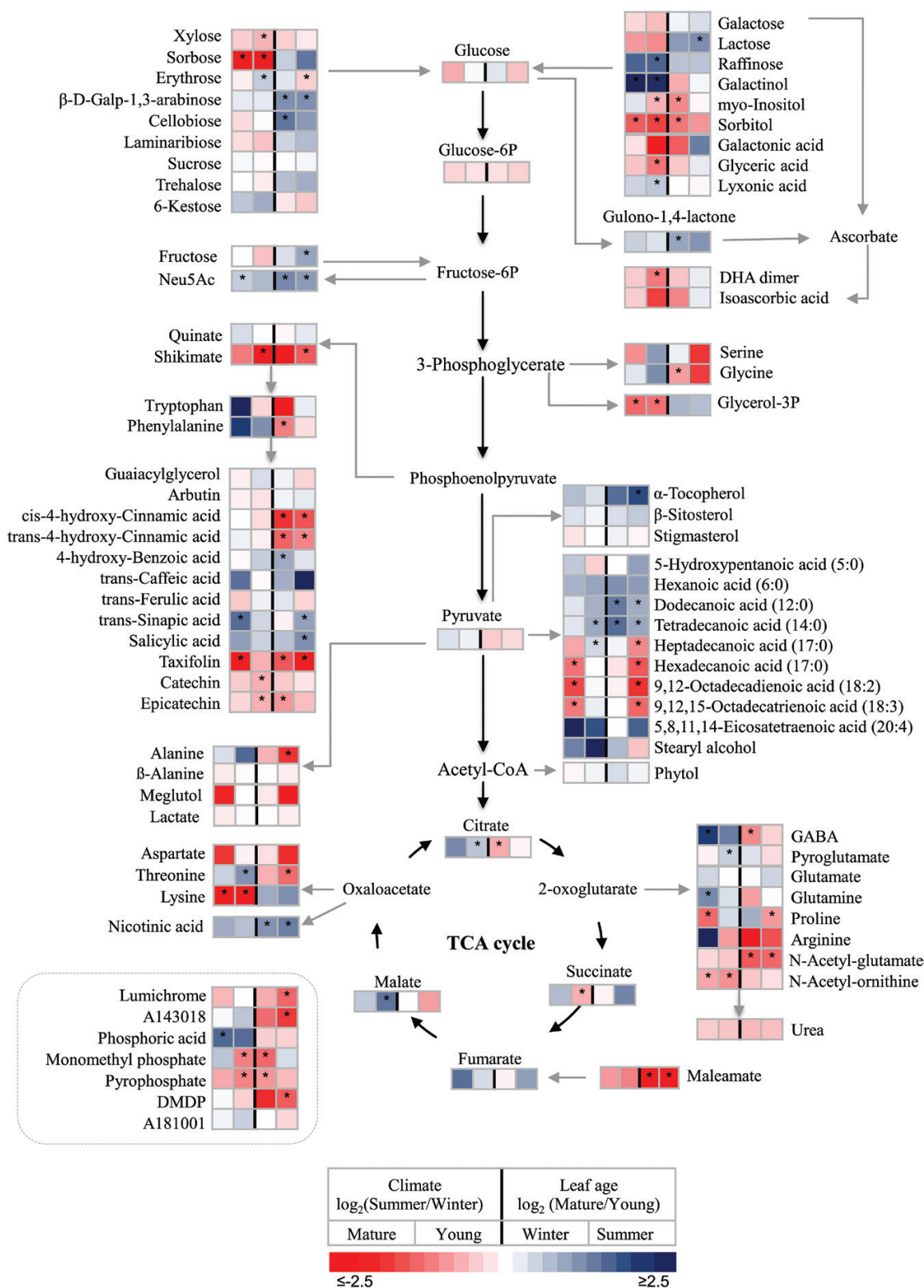
Fig. 3. Log$_2$-transformed relative abundances of low molecular weight metabolites between summer and winter climate in mature and young leaves, respectively (left panel), and between mature and young leaves in winter and summer climate, respectively (right panel). Asterisks indicate significant differences ($P<0.05$) between the two groups, i.e. between summer and winter in mature and young leaves, respectively (left panel), and between mature and young leaves under winter and summer, respectively (right panel). A143018, *N*-methyl *trans*-4-hydroxy-L-proline (2*S*,4*R*)-4-hydroxy-1-methyl pyrrolidine-2-carboxylic acid; DMDP, 2,5-dihydroxymethyl-3,4-dihydroxypyrrolidine; A181001, code of an unknown metabolite in the Golm library; GABA, γ-aminobutyric acid; P, phosphate. No significant interaction between leaf age and climate was found.

Foyer, 1998); their redox states and/or contents can be taken as indicators of oxidative stress inside the cell (Noctor *et al.*, 2016). Its constituent substrate ascorbate is the most abundant water-soluble redox compound in plants, particularly in leaves, where it can represent >10% of soluble carbohydrate (Noctor and Foyer, 1998; Ishikawa and Shigeoka, 2008). The ascorbate concentration in plant tissues is a consequence of balancing synthesis and catabolism. Recent evidence shows that although ascorbate can be synthesized in several different ways, the galactose pathway is responsible for the majority of ascorbate synthesized in plants (Bulley and Laing, 2016). This pathway is regulated by temperature and seasonal adaptations (Polle and Rennenberg, 1992; Bulley and Laing, 2016; Du and Rennenberg, 2018; Du *et al.*, 2018). In the current study, despite increased H_2O_2 levels in summer climate, total ascorbate contents were 30% less ($P<0.05$) in YL in summer than in winter climate, but increased DHAR activities in summer climate (Fig. 2) may have compensated for this decline. Also in this context, ROS and antioxidant flux analyses are required in future studies to test this assumption.

It is well known that oxidative stress can trigger increases in the total extractable activities of antioxidative enzymes, and their maximal extractable activities have long been recognized as indicators of cellular redox status (Noctor *et al.*, 2016). Apparently it seemed that the enhanced DHAR activity was insufficient to maintain H_2O_2 levels in summer climate at reduced total ascorbate contents. Therefore, our first hypothesis has to be rejected. Instead, reduced ascorbate synthesis (Bulley and Laing, 2016) may be responsible for this effect, since the level of its main metabolic precursor, galactose, was also diminished in summer climate. In part, these results are consistent with a previous study (Arab *et al.*, 2016) that reported decreased total and reduced ascorbate contents, but also decreased foliar cysteine, γ-glutamylcysteine, and GSH levels in date palm seedlings at elevated air temperature. In the present study, no clear differences in GSH contents and those of its precursors cysteine and γ-glutamylcysteine were found between plants that experienced summer and winter climate. Altogether, the present study indicates that date palm leaves might possess other physiological adaptations to enhanced temperature apart from the ascorbate–glutathione cycle (Noctor and Foyer, 1998; Rennenberg *et al.*, 2006; Arab *et al.*, 2016).

As observed by Arab *et al.* (2016) and Du *et al.* (2014) for date palm leaves and Douglas fir needles, respectively, no significant differences in foliar total N and soluble protein contents were documented between summer and winter climate in the present study. However, in summer climate, higher TAA contents were found in ML and particularly in YL in winter climate (Table 2). The enhanced TAA content in YL was mainly attributed to pyroglutamate and threonine, as well as the additive effect of slight accumulation of 3-phosphoglycerate-derived serine and glycine, pyruvate-derived alanine, glutamate, glutamine, proline, and GABA, from the glutamate family, and the aromatic amino acid phenylalanine (Fig. 3). Pyroglutamate, also named 5-oxoproline, is a precursor to glutamate synthesis and a major contributor to glutamate steady-state levels (Ohkama-Ohtsu *et al.*, 2008). Accumulation of pyroglutamate is thought to strengthen the antioxidant system (Marrs, 1996; Yu *et al.*, 2012). As also reported by other authors (Caldovic

and Tuchman, 2003; Fondi *et al.*, 2007; Xu *et al.*, 2007), date palm leaves in summer climate had lower foliar abundances of lysine, *N*-acetyl-glutamate, and *N*-acetyl–ornithine, the precursor of ornithine, than in winter climate, accompanied by a dramatic accumulation of arginine in ML in the present study (Fig. 3). In contrast to these results, reduced contents of TAA and organic acids involved in the TCA cycle were observed in current year Douglas fir needles at elevated air temperature (Du *et al.*, 2014; Jansen *et al.*, 2014), indicating species-specific responses of amino acid synthesis to increased temperature. The enhanced amino acid contents in YL under summer climate conditions observed may at least partially be a consequence of an improved N uptake at increased soil temperature (Dong *et al.*, 2001) and an up-regulation of the TCA cycle as revealed by enhanced citrate and malate contents (Yu *et al.*, 2012).

Compatible solutes are important for sustaining cell turgor by osmotic adjustment, and stabilization of enzymes under stress conditions (Kaplan *et al.*, 2004). Sugars, as key components of primary metabolism, have an eminent signaling role for metabolic regulation in date palm leaves in response to heat at the level of gene expression (Safronov *et al.*, 2017). In the current study, we did not see any significant climatic effect on sucrose or glucose and fructose contents in both leaf age classes. Instead, date palms accumulated more sorbose and xylose, as well as sorbitol, the precursor of sorbose, in leaves in winter than in summer climate. Galactinol and raffinose are thought to protect plant cells from oxidative damage caused by heat, drought, salinity, or chilling (Nishizawa *et al.*, 2008). Consistent with this assumption, we observed increased levels of galactinol and raffinose, concurrent with decreased levels of myo-inositol, the precursor of galactinol, in YL in summer climate (Fig. 3). In contrast to our observation, Douglas fir needles (Jansen *et al.*, 2014) and Arabidopsis leaves (Cook *et al.*, 2004; Maruyama *et al.*, 2009) showed reduced galactinol and raffinose levels at elevated temperature. Our findings support the view that a multiplicity of primary metabolites could act collectively as compatible solutes (Kaplan *et al.*, 2004).

In contrast, the report of Arab *et al.* (2016) that fatty acid composition was not impacted by heat, strong impacts of heat on fatty acid and flavonoid biosynthesis were revealed by gene expression analysis of date palm leaves (Safronov *et al.*, 2017). The latter result is to be expected, because the lipid composition determines the sensitivity of chloroplast membranes to thermal denaturation (Nahar *et al.*, 2015). In addition, changes in response to environmental conditions such as heat, cold, or drought, as well as during growth and development, are required to adjust the physical characteristics of the membrane structures to cope with stress conditions (Quinn, 1988). In agreement with the results of Safronov *et al.* (2017), climate markedly impacted the abundances of fatty acids in the present study, with decreased levels of the unsaturated 9,12-octadecadienoic acid (C18:2) and 9,12,15-octadecatrienoic acid (C18:3), and the saturated hexadecanoic acid in ML, and increased levels of saturated tetradecanoic acid and heptadecanoic acid in YL in summer climate (Fig. 3). Therefore, our second hypothesis is fully supported. Similarly, decreases in the levels of 9,12-octadecadienoic acid and 9,12,15-octadecatrienoic acid were observed in drought-treated date palm leaves (Arab *et al.*, 2016). Also in pea (*Pisum sativum*) leaves, more unsaturated

fatty acids were present in winter than in summer (Chapman *et al.*, 1983). Such changes can maintain membrane fluidity under more oxidative cellular environments (Kenchanmane Raju *et al.*, 2018) and improve the ability of plants to grow over a wide temperature range.

Phenolic compounds are essential for the synthesis of secondary cell wall constituents which have been reported to be significantly affected by temperature (Chaves *et al.*, 2011; Janmohammadi, 2012; Safronov *et al.*, 2017). The phenolic compounds sensitive to temperature include aromatic amino acids from the phenylpropanoid pathway that also act as precursors of phytohormones, electron carriers, enzyme cofactors, and antioxidants (Ribeiro *et al.*, 2014). In the present study, the accumulation of phenolic compounds was favored under summer conditions, as revealed by the stimulated abundances of *trans*-sinapic acid and *trans*-caffeic acid, while flavonoids accumulated under lower temperature (winter climate) conditions. Taxifolin, catechin, and epicatechin, as well as the precursor shikimate, were accumulated in winter compared with summer climate in both leaf age classes (Fig. 3). Similarly, low temperatures also induced the accumulation of catechin and enhanced the transcript levels of phenylalanine ammonialyase (PAL) and chalcone synthase (CHS) in leaves of the evergreen *Quercus suber* (Chaves *et al.*, 2011) and Arabidopsis (Leyva *et al.*, 1995).

Leaf developmental traits of date palms

In date palms, new leaves are developed in both summer and winter climate (Tripler *et al.*, 2011). In order to identify leaf developmental differences, young developing and fully developed ML were analyzed in the present study under both climate conditions. In line with our third hypothesis, strong developmental effects on leaf hydration, $\delta^{13}C$ signatures, ROS levels, and metabolite profiles were observed in summer and winter climate. Compared with ML, YL had stronger leaf hydration, more negative $\delta^{13}C$ values, but >40% lower H_2O_2 irrespective of the climate conditions, indicating effective ROS scavenging in YL (Peuke *et al.*, 2006). This view is supported by the extremely higher YL enzyme activities of DHAR and GR irrespective of climate (Fig. 2), as well as the significantly stimulated YL proline abundance under summer climate conditions (Fig. 3), the latter is considered a general response to abiotic stress in the date palm (Yaish, 2015). Similarly, lower H_2O_2 contents together with higher catalase activities were found in young tobacco (*Nicotiana tabacum*) leaves (Gullner and Tyihák, 1991). Ascorbate contents and GR activity were also usually greater in 1-year-old than in current year needles of spruce trees (Polle and Rennenberg, 1992).

Stored reserves originating from excess photosynthate are important for respiration and maintenance metabolism during stress defense (Rennenberg *et al.*, 2010; Hartmann and Trumbore, 2016). Generally, ML of evergreen perennial plants store more C and N as carbohydrates, lipids, amino compounds, and protein than younger foliage (Wendler *et al.*, 1995; Cerasoli *et al.*, 2004; Rennenberg *et al.*, 2010; Du *et al.*, 2014). In the present study with date palm seedlings, YL tended to have higher TAA contents than ML, particularly in summer climate, mainly due to the elevated abundances of amino acids from glutamate

and aspartate families, 3-phosphoglycerate-derived serine and glycine, and alanine (>2-fold; data not shown). Similarly, accumulations of these amino acids during summer were also observed in needles of red pine (*Pinus resinosa*) and white spruce (*P. glauca*) seedlings (Kim and Glerum, 1995). In date palm leaves, the enhanced amino acid content under the hot summer climate may be a combined effect of enhanced photosynthesis (Kruse *et al.*, 2019) and a stimulated TCA cycle (Fig. 3).

Phenolic compounds are involved in many interactions of plants with their biotic and abiotic environment. In the present study, hydroxycinnamic acid derivatives as well as their precursors shikimate and phenylalanine were constantly less abundant in ML than in YL, probably indicating enhanced *de novo* synthesis of lignin in the less hardened YL (Fig. 3) (Schnitzler *et al.*, 1996; Hutzler *et al.*, 1998). In addition, the flavonoid taxifolin also accumulated in YL (Fig. 3). This phenolic compound has a high antioxidative and radical-scavenging capacity, constitutes a good inhibitor of lipid peroxidation *in vitro* (Willför *et al.*, 2003), and was increased in date palm leaves upon exposure to ozone (Du *et al.*, 2018).

ML generally showed higher contents of the saturated fatty acids dodecanoic acid and tetradecanoic acid, as well as α-tocopherol and nicotinic acid, than YL. Also, the abundances of heptadecanoic acid, hexadecanoic acid, 9,12-octadecadienoic acid, and 9,12,15-octadecatrienoic acid were significantly higher in YL in summer climate, but the accumulation of these compounds in YL disappeared in winter climate (Fig. 3). Therefore, our third hypothesis has to be amended since fatty acid profiles in ML and YL were both markedly impacted by different climate. Leaf age-dependent changes of phospholipid contents were also reported in tobacco plants (Koiwai *et al.*, 1981). The authors speculated that these changes might be related to the degradation of organelles other than chloroplasts during leaf development. From the current study, we speculate that selective degradation of these lipids at lower temperature during winter is required to maintain membrane fluidity.

In conclusion, summer climate with high temperature constitutes stress conditions for date palm seedlings, and consequently results in elevated ROS levels. Enhanced DHAR activity accompanied by reduced ascorbate synthesis was not sufficient to maintain H_2O_2 levels in summer climate. In contrast to Douglas fir needles and Arabidopsis leaves, the enhanced level of compatible N-containing solutes, as well as galactinol and raffinose contributed to prevent oxidative damage to date palm leaves, indicating species–specific physiological responses to climate change. Besides the levels of ROS, antioxidants and compatible solutes, membrane fatty acids, and phenolic compounds, acting as cell wall constituents and ROS scavengers, play significant roles in date palm leaves in response to changing climate. Although we did not observe statistically significant interactions between climate and leaf age, the impact of changes in climate with distinct temperatures on substantial metabolic adjustments that facilitate tolerance to high temperatures are highly dependent on leaf development. We argue that YL of date palm are more responsive and probably more vulnerable than ML to changes in climate conditions. Given faster growth and development in summer, date palms are likely to face a more serious challenge in the context of climate change.

Supplementary data

Supplementary data are available at *JXB* online.

Table S1. Loadings of component 1 and component 2 of sparse partial least squares–discriminant analysis (sPLS-DA).

Acknowledgements

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