

Original Article

Heat stress in grapevine: the pros and cons of acclimation

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ABSTRACT

Heat stress is a major limiting factor of grapevine production and quality. Acclimation and recovery are essential to ensure plant survival, and the recovery mechanisms can be independent of the heat response mechanisms. An experimental set up with and without acclimation to heat followed by recovery [stepwise acclimation and recovery (SAR) and stepwise recovery (SR), respectively] was applied to two grapevine varieties, Touriga Nacional (TN), and Trincadeira (TR), with different tolerance to abiotic stress. Major differences were found between leaves of SAR and SR, especially after recovery; in SAR, almost all parameters returned to basal levels while in SR they remained altered. Acclimation led to a swifter and short-term antioxidative response, affecting the plant to a lesser extent than SR. Significant differences were found among varieties: upon stress, TN significantly increased ascorbate and glutathione reduction levels, boosting the cell's redox-buffering capacity, while TR needed to synthesize both metabolites, its response being insufficient to keep the redox state at working levels. TR was affected by stress for a longer period and the up-regulation pattern of antioxidative stress genes was more obvious. In TN, heat shock proteins were significantly induced, but the canonical heat-stress gene signature was not evident probably because no shutdown of the housekeeping metabolism was needed.

Key-words: Acclimation and recovery; AsA; chlorophyll fluorescence; GSH; photosynthesis; redox state; transcript expression.

INTRODUCTION

As sessile organisms, plants are unable to circumvent changes taking place in their surrounding environment. Therefore, environmental fluctuations of temperature, water availability and light intensity will have immediate repercussions on a plant's life cycle. Plants can endure moderately high temperatures for a period of time without injuries or death. Usually, the definition of heat stress (HS) is an increase in temperature above a threshold level that persists long enough to cause irreversible damage to plant growth and development (Wahid *et al.* 2007). Even a temporary increase of 10–15 °C above the ambient temperature can cause HS. However, we

must bear in mind that HS is a composite function of intensity (degrees of temperature), length and rate of increase in temperature (Wahid *et al.* 2007). HS is responsible for cell damages such as protein denaturation and aggregation, and increased fluidity of membrane lipids with loss of membrane integrity. Indirectly related to heat is the inactivation of enzymes in the chloroplast and mitochondria, a general inhibition of protein synthesis, accompanied by an increase of protein degradation (Wahid *et al.* 2007). These injuries will ultimately lead to inhibition of growth, reduced ion flux and production of reactive oxygen species (ROS; Wahid *et al.* 2007). However, plants are able to maintain growth and reproductive success by sensing changes in the surrounding environment and reacting through mechanisms at the cellular, physiological and developmental levels. These response mechanisms enable plants to react rapidly, within hours or days, to extremes in the environment that could otherwise be injuring or lethal (Jenks & Hasegawa 2014).

Thermo-tolerance, the ability to acclimate to otherwise lethal high temperatures, was described for the first time more than 40 years ago (Larkindale *et al.* 2005). Heat tolerance results from previous exposure to a moderate stress treatment, which can be a short and sub-lethal high temperature (Larkindale & Vierling 2008). A key and common element of acquired thermo-tolerance in all organisms is the induction and synthesis of molecular chaperones or heat shock proteins (HSPs; Vierling 1991). In fact, a number of HSPs coded by different gene families, as well as heat shock transcription factors (HSFs), are associated to heat acclimation and resistance to HS in *Arabidopsis* (Hong & Vierling 2000; Lee *et al.* 2005) and tomatoes (Frank *et al.* 2009). Small heat shock proteins (sHSPs) are a family of low-molecular mass HSPs (12–40 kDa). In plants, this family is more prevalent and more diverse in sequence similarity, cellular location and functions than the other HSP families (Waters *et al.* 1996). sHSPs can be found in the nuclear–cytoplasmic compartment and plastids, mitochondria, endoplasmic reticulum and peroxisomes (Kotak *et al.* 2007). In grapevine 13, sHSPs have been identified (Genoscope 12X, <http://www.genoscope.cns.fr/externe/GenomeBrowser/Vitis/>). Their role in the response to stress is distinct from that of the other HSPs. Functionally, they confer thermo-tolerance in an ATP-independent way by selectively binding and stabilizing proteins, preventing their aggregation at elevated temperatures, and protecting enzymes against heat-induced inactivation (Ganea 2001). The high diversification of plant sHSPs

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probably reflects molecular adaptations to stress conditions that are unique to plants. In fact, they respond to an extensive scope of environmental stresses such as heat, cold, drought, salinity and oxidative stress, and their accumulation is strongly correlated with tolerance to stress (Waters *et al.* 1996; Sun *et al.* 2002; Wang *et al.* 2003).

The response of the Arabidopsis transcriptome to HS has been thoroughly scrutinized (Rizhsky *et al.* 2004; Busch *et al.* 2005; Schramm *et al.* 2008) and more recently, that of grapevine also (Liu *et al.* 2012). Plant hormones, such as abscisic acid (ABA), salicylic acid and ethylene, and other metabolites, namely hydrogen peroxide and calcium signalling, have been identified as key regulators of the acquisition of thermotolerance in plants (Larkindale & Knight 2002; Larkindale *et al.* 2005).

The processes of acclimation and recovery from HS are essential to ensure plant survival and the recovery mechanisms, thus set in motion can be independent of the direct heat response mechanisms (Vinocur & Altman 2005). When gene expression is analysed in different plant species subjected to high temperatures under various conditions, including acclimation, recovery, duration of stress treatments, different tissues, developmental stages and growth conditions, common patterns of transcript expression can be identified, affecting *circa* 2% of the genome (Rizhsky *et al.* 2004). In grapevine leaves, the number of HS-regulated genes was almost twice that of recovery-regulated genes, with genes coding sHSPs and APX identified as playing important roles in thermotolerance to acute HS, while genes for HSF7 and HSF1 may act in the recovery phase (Liu *et al.* 2012). It is common that transcripts decreasing in response to stress conditions are overlooked, although at present, there are strong indications that down-regulation of specific transcripts is paramount in effective HS acclimation (Larkindale & Vierling 2008; Ghosh & Pugh 2011). Examples are transcripts associated with growth and general metabolism, which are down-regulated upon stress, indicating that acclimated plants can limit nonessential cellular processes until the return of temperatures favourable for growth (Larkindale & Vierling 2008).

Temperature is widely considered a foremost influencing factor of grapevine productivity, worldwide wine production and quality are severely limited by temperature stresses (Cramer 2010) and climate change scenarios predict this limitation to worsen. Photosynthesis is affected by temperatures above 35 °C (Salvucci & Crafts-Brandner 2004), and in wine-producing regions, midday air temperature can surpass 40 °C in the summer. Extreme temperatures are prone to reduce berry quality and decrease economic revenue (Howell 2001) because of deleterious effects on berry ripening (Liu *et al.* 2012). Natural field conditions are difficult to reproduce and to compare with controlled conditions; however, results obtained under extreme controlled conditions are also difficult to extrapolate to field ones. In order to make a bridge between natural conditions and the artificial treatments described in the literature, we set up two HS treatments, one with acclimation and recovery, respectively, before and after an acute stress period and another one only with recovery. Two grapevine varieties, Touriga Nacional (TN) and

Trincadeira (TR) were chosen because of their contrasting responses to stress. TN has higher capacity for heat dissipation via evaporative cooling than TR and thus can be considered as better adapted to warm climate conditions, without water restraint, both in the field (Costa *et al.* 2012) and in controlled conditions (Carvalho *et al.* submitted).

In leaves of the grapevine variety Aragonez, (syn Tempranillo) after acute HS using a high-throughput transcriptomic grapevine array, we verified that the expression of a number of genes coding for HSPs, and transcription factors were significantly affected by HS (Rocheta *et al.* 2014). The grapevine varieties TN and TR were also scrutinized under the same acute HS conditions for gene expression with the high-throughput transcriptomic grapevine array (Rocheta *et al.* unpublished data). To get a more complete insight on how plants acclimate to and recover after HS, in the current study, the response of the varieties TN and TR under the two HS treatments were compared at the level of photosynthesis, H₂O₂ production, the course of the antioxidant system and of cellular redox status, the expression of key antioxidant response genes and of several HSPs, as well as the expression of the genes mostly up- or down-regulated in the transcriptomic array of TN and TR under acute HS.

MATERIAL AND METHODS

Plant material and stress treatments

Cuttings from pruned wood of pre-selected plants of the varieties TN and TR collected from a production vineyard in Pegões (70 km south-west from Lisbon, Portugal) and treated with fungicide (Benlate, Syngenta Crop Protection, Greensboro, NC, USA, 2%), were kept at 4 °C for 2 months, and treated with the same fungicide before rooting. The cuttings were rooted in complete nutrient solution diluted in distilled water (10:1, v : v) in the dark. Rooted cuttings were placed in a growth room in 3 L pots filled with sterilized soil, well watered with nutrient solution whenever necessary, in controlled conditions with irradiance of 300 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$, photoperiod of 16 h light/8 h dark and temperature of 25 °C day/23 °C night.

HS treatments were applied when *circa* 4-month-old plants were 70 cm high. Two different treatments were performed, SAR, a stepwise acclimation to heat with slow recovery; and SR, with a rapid increase of temperature (acute HS) and a slow recovery (Fig. 1). In SAR, the acclimation and recovery steps were obtained by subjecting the plants to 32 °C for 3 h, termed 32u, when the temperature is increasing (or going 'up') and termed 32d, when temperature is returning to basal (or going 'down'). The HS temperatures provided by a homogenous heat source and measured at the surface of the leaf, were applied from the third hour of the light period as follows: SAR: 3 h at 32 °C, for the acclimation to temperature increase (SAR32u), 1 h at 42 °C (SAR42), 3 h at 32 °C (SAR32d), 24 h under control conditions (SAR25); SR: 1 h at 42 °C (SR42), 3 h at 32 °C (SR32d), 24 h under control conditions (SR25) as outlined in Fig. 1. Control plants (C25) were kept at 25 °C and measurements were done in the middle of the light period.

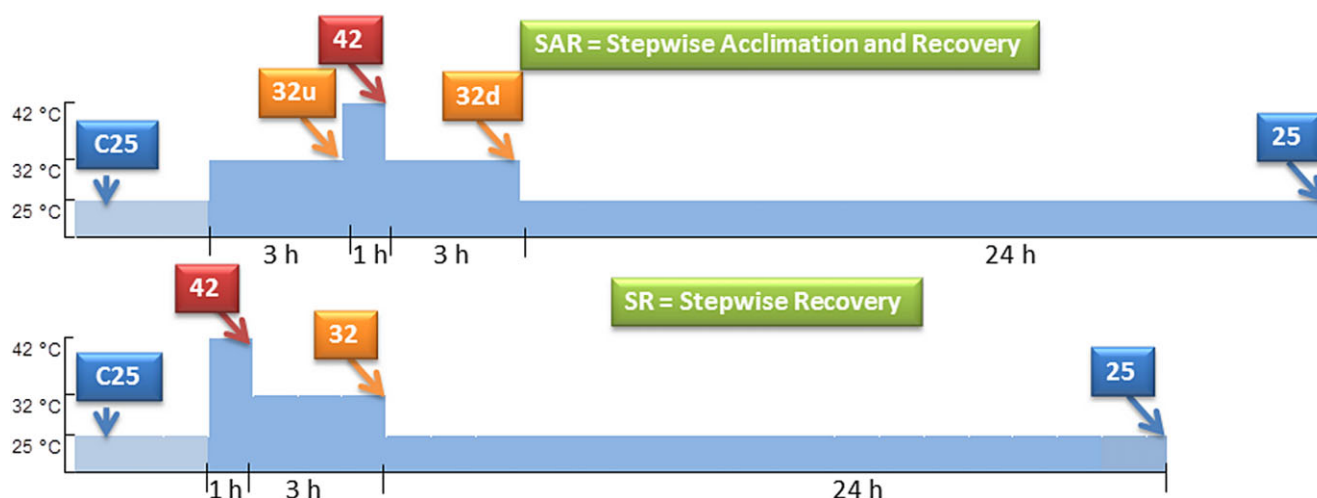


Figure 1. The experimental design used to apply two types of heat stress treatments to two grapevine varieties: Touriga Nacional (TN) and Trincadeira (TR). In stepwise acclimation and recovery (SAR), plants were subjected to a 3 h acclimation period at 32 °C (SAR32u, when temperature is increasing, or going ‘up’), followed by 1 h heat stress at 42 °C (SAR42) and a recovery of 3 h at 32 °C (SAR32d, when temperature is returning to basal, or going ‘down’). In stepwise recovery (SR), plants were subjected to a rapid 1 h heat stress at 42 °C (SR42) and a recovery of 3 h at 32 °C (SR32). C25 corresponds to plants in control conditions (temperature of 25 °C during the light period); SAR25 and SR25 correspond to a measurement taken after 24 h at control temperature in plants previously subjected to SAR and SR, respectively.

In each temperature point of both treatments and control, six plants were assessed for photosynthetic performance, and leaf samples were taken for quantification of different metabolites and gene expression analysis.

Photosynthesis and chlorophyll fluorescence

Photosynthetic rate at ambient CO₂ and light responses (A/I) curves were measured at each temperature point, on the third fully expanded leaf from six plants per treatment using an open gas-exchange system (IRGA LCPro+ da ADC Bioscientific Ltd., Herdforshire, UK). Light curves were performed at ambient CO₂ (approximately 350 μmol mol⁻¹ CO₂) and different irradiances (I) measured were recorded every 380 s or before, if the photosynthesis rate had already stabilized, at each irradiance. Light intensities rose stepwise from 0 to 1750 μmol m⁻² s⁻¹. Stomatal conductance (g_s) was measured at the same light intensities used for the photosynthesis curves. Leaf temperature was monitored along with the light response curves as shown in Supporting Information Table S1.

Using the parameters thus estimated, the further analysis of A/I curves using SPSS 17.0 software (SPSS Inc., Chicago, IL, USA) allowed the determination of the light compensation point (LCP), maximum photosynthesis rate (A_{max}) and dark respiration (R_d). R_d was the intercept of the A/I curve with the Y axis, LCP was the light intensity at which the release and consumption of oxygen were equal (intercept with the X axis) and A_{max} was given as the asymptotic A value as irradiance tends to infinity.

Simultaneously, chlorophyll *a* fluorescence was measured on the third fully expanded leaf from six plants per treatment using a Pulse Amplitude Modulation Fluorometer (mini-

PAM, Photosynthesis Yield Analyzer, Walz, Germany), with a saturation pulse intensity extending up to 18 000 μmol photons m⁻² s⁻¹ and actinic light corresponding to the photosynthetically active radiation.

Metabolites

Extraction for the quantification of glutathione, ascorbate, chlorophyll and carotenoids

Leaf material (0.5 g) was randomly collected at each time point and frozen in liquid N₂. Each sample was homogenized in 5 mL of ice cold 6% meta-phosphoric acid (pH 2.8), containing 1 mM ethylenediaminetetraacetic acid, in the presence of liquid N₂. Homogenates were centrifuged at 27 000 g for 15 min at 4 °C and the resulting acid extract was stored at -80 °C prior to analysis of glutathione, ascorbate, chlorophyll and carotenoids.

Glutathione quantification

Reduced (GSH) and oxidized (GSSG) glutathione were analysed colorimetrically by the 2-vinylpyridine method described by Anderson *et al.* (1995). GSH and GSSG concentrations were expressed in μmol g⁻¹ fresh weight. Percentage of reduction corresponds to the percentage of total glutathione pool present as GSH and is defined as GSH/(GSH + GSSG) × 100.

Ascorbate quantification

Ascorbic (AsA) and dehydroascorbic (DAsA) acids were assayed using a method adapted from Okamura (1980). To determine AsA and total ascorbate, 125 μL of the acid

extract was neutralized with 25 μL of 1.5 M triethanolamine. After thorough mixing, 150 μL of 150 mM sodium phosphate buffer pH 7.4 were added. For the quantification of total ascorbate 75 μL of 10 mM Dithiothreitol (DTT) were added. This was followed by 15 min incubation at 25 °C to reduce the DAsA present in the extract. To remove excess DTT, 75 μL of 0.5% (w/v) N-ethylmaleimide was added. The samples were then mixed and incubated 30 s at 25 °C. For the quantification of AsA, water was added instead, so that the volumes of both samples were equal. To both samples, the following reagents were added successively: 300 μL of 10% (w/v) trichloroacetic acid, 300 μL of 44% (v/v) phosphoric acid, 300 μL of 4% (w/v) 2,2'-dipyridyl in 70% ethanol and 150 μL of 3% (w/v) FeCl_3 . After mixing, the samples were incubated for 1 h at 37 °C. Absorbance was recorded at 525 nm. The concentration of DAsA was calculated by subtracting the AsA concentration measured from the total ascorbate determined. Standard curves of AsA in the range of 10–60 μM were prepared in 5% metaphosphoric acid.

Chlorophyll analysis

Chlorophyll was extracted from four leaf disks (total area 113 mm²). Disks were incubated in 3 mL dimethyl sulfoxide at 65 °C for 1 h, and absorbance was measured at 645 and 663 nm (adapted from Hiscox & Israelstam 1979). Chlorophyll concentration was calculated using the equations described by Porra *et al.* (1989): $\text{Chl } a \text{ } (\mu\text{g mL}^{-1}) = 12,00 \times A_{663} - 3,11 \times A_{645}$; $\text{Chl } b \text{ } (\mu\text{g mL}^{-1}) = 20,78 \times A_{645} - 4,88 \times A_{663}$; $\text{tot Chl } (\mu\text{g mL}^{-1}) = 17,67 \times A_{645} + 7,12 \times A_{663}$; and converted to mg Chl cm⁻² leaf area (Richardson *et al.* 2002).

H₂O₂ quantification

H₂O₂ production was detected using a fluorometric horseradish peroxidase-linked assay (Amplex Red assay kit, Invitrogen, Thermo Fisher Scientific: <https://www.lifetechnologies.com/>). Leaf material (0.1 g) was collected at each time point and ground over activated charcoal in the presence of liquid N₂ as described by Creissen *et al.* (1999). Samples were centrifuged 10 min at maximum speed and the supernatants were kept on ice until measurements. H₂O₂ concentrations in purified extracts were determined according to the manufacturer's instructions. Absorbance was then measured with a microplate reader at 570 nm. H₂O₂ concentrations were expressed in $\mu\text{mol g}^{-1}$ fresh weight.

ABA analysis

The extracts for the measurement of ABA were carried out as described by (Vilela *et al.* 2007) using [3H]-ABA (GE Healthcare, Wilmington, MA, USA) as an internal control to monitor ABA yield and recovery. ABA was quantified through immunoassay by indirect enzyme-linked immunosorbent assay with monoclonal antibodies, using a commercial kit (Olchemim Enzyme Immunoassay, Olomouc, Czech Republic), according to the manufacturer's recommendations.

Gene expression by RT-qPCR

Total RNA extraction and cDNA synthesis

Samples were ground with a mortar and pestle in the presence of liquid nitrogen. Total RNA was extracted with the RNA Plant Total RNA Kit (Sigma-Aldrich, Inc., St. Louis, MO, USA) following the manufacturer's instructions. Nucleic acid concentration of each sample was quantified by spectrophotometry using the software Gen5 1.09 (Synergy HT, Bio-Tek Instruments, Winooski, VT, USA). Total RNA quality was assessed using the A_{260}/A_{280} and A_{260}/A_{230} . Only RNA samples with A_{260}/A_{280} between 1.8 and 2.1 and A_{260}/A_{230} between 2.0 and 2.2 were used. Total RNA integrity was checked through 1% agarose gel electrophoresis under denaturing conditions.

RNA samples were treated with RQ1 RNase-Free DNase (Promega, Madison, WI, USA). cDNA was synthesized from 2 μg of total RNA using oligo(dT)₂₀ in a 20 μL reaction volume using RevertAid Reverse Transcriptase (Fermentas Life Science, Helsingborg, Sweden) according to the manufacturer's recommendations. cDNA was tested for gDNA contamination in PCRs using intron spanning primers that yield a 229 bp amplicon in cDNA and a 547 amplicon in gDNA. Amplicon sizes were compared in 2% agarose gels together with the molecular weight marker 1 Kb+ (Invitrogen) and no gDNA contamination was detected. cDNA was stored at -20 °C until further use.

RT-qPCR

Primers were designed using the software Beacon Designer (Premier Biosoft, Palo Alto, CA, USA) using a primer length of 20 ± 2 bp, melting temperature of $60 \text{ }^\circ\text{C} \pm 2 \text{ }^\circ\text{C}$, a guanine-cytosine content of *circa* 50% and an expected amplicon size of 180–280 bp (Table 1). Sequences were nine HSPs that had been significantly up-regulated in a previous experiment of acute HS (1 h at 42 °C) using the GrapeGene 520510F Array (Affymetrix, Santa Clara, CA, USA) applied to both varieties (Rocheta *et al.* unpublished data; accession: GSE57669) and nine previously tested oxidative stress response genes (Carvalho *et al.* 2006).

RT-qPCR was performed in 96-well white reaction plates (Bio-Rad, Hercules, CA, USA), using an IQ5 Real Time PCR (Bio-Rad) with three biological replicates and two technical replicates. The 20 μL reaction mixture was composed of 1 μL cDNA diluted 50-fold, 0.5 μM of each gene-specific primer and 10 μL master mix (SsoFast_EvaGreen Supermix, Bio-Rad). Amplification of PCR products was monitored via intercalation of Eva-Green (included in the master mix). The following programme was applied: initial polymerase activation, 95 °C, 3 min; then 40 cycles at 94 °C 10 s (denaturation), 60 °C 20 s (annealing), 72 °C 15 s (extension). The PCR products were run on 2% agarose gels to make sure that there was only one amplicon of the expected size. PCRs with each primer pair were also performed on samples lacking cDNA template, in triplicate (no template controls).

To generate a baseline-subtracted plot of the logarithmic increase in fluorescence signal (ΔRn) versus cycle number, baseline data were collected between the cycles 5 and 17. All

Table 1. Transcripts used for RT-qPCR analysis of Touriga Nacional (TN) and Trincadeira (TR)

	NCBI Reference	Annotation and abbreviation	Forward primer	Reverse primer	bp
HSPs	XM_002267889	Small heat shock protein 26.5 kDa, mitochondrial (HSP26.5)	5' AGGGCAGAGGAGACGAGAC 3'	5' ATCAGTCGGAGTCCATGTAATCG 3'	109
	XM_002272382	Small heat shock protein, chloroplastic HSP20 family (HSP20)	5' CCTCTGGCAACCCACAAAAC 3'	5' GGTCCATTGGTCCATCAT 3'	292
	XM_002270560	Small heat shock protein 23.6 kDa, mitochondrial (HSP23.6)	5' CCGCCTCCTCTCCTCTCC 3'	5' TCTTCCGCATCATCGTAGTTCG 3'	109
	XM_002280785	Class I heat shock protein, 18.2 kDa (HSP18.2A)	5' GAAGGAGGAAGTGAAGGTTGAG 3'	5' ACACCGTTCTCCATAGTAGCCT 3'	192
	XM_002263340	Heat shock protein 22.0 kDa (HSP22)	5' GAGGTGAAGATAGAGGTGGACG 3'	5' GAGCACCCCATTTCTCAAAGC 3'	192
	XM_002280644	Class II heat shock protein 17.9 kDa (HSP17.9A)	5' CGTCAAGGAGTACCCCAATTC 3'	5' AACTTCCCCACCCCTCCTCT 3'	177
	XM_002281249	Class I heat shock protein 18.2 kDa (HSP18.2B)	5' CCGTCCAAAGACTTCCCAIT 3'	5' CTCCTTCTCCAAACCTCTACCT 3'	170
	XM_002280449	Class II heat shock protein 17.9 kDa (HSP17.9B)	5' TTCTACGGCCTTTCATCATCG 3'	5' ACACGGCCATCTTGACAAAACC 3'	230
	XM_002281224	Class I heat shock protein 18.2 kDa (HSP18.2C)	5' CCATTCCAGGACTTCCCAIT 3'	5' CTGGTGCCACTTGTCAITC 3'	235
	XM_002282403	Translation initiation factor eIF-3 subunit 4 (TIF)	5' AAAAGCAGAAAGAAACCAAGATT 3'	5' TTGCCAGTGCCTGTAGTAGCC 3'	206
Ref. genes	XM_002271296	Translation initiation factor eIF-2B alpha subunit (TIF-GTP)	5' AGCAGCACAGAAATAAGAAACT 3'	5' CCATCAGCCCCCAACAAATACC 3'	177
	AF369525.1	Actin 2 (act)	5' TGGATTCTGTAGGTGTGAGTCC 3'	5' CAATTTCCCGTTTCAGCAGTAGTGG 3'	167
	XM_002284731	Ascorbate peroxidase (APX1), cytosol	5' GCCCCACCAATGACTTTGTACC-3'	5' ATCGCTCTGGATGTCCTTC-3'	218
	XM_002278245.1	Ascorbate peroxidase (APX3), peroxisome	5' CGCCTTGGACCGA ACTATCTGC-3'	5' AGACCTTTCACGGTTGCC-3'	186
	AF236127	Catalase (CAT), peroxisome	5' GTGCAGTCAAACCTGTGCCCTAG-3'	5' GAGCGAAGAACAGGCTACAGAT-3'	200
	XM_002285636.1	Glutathione reductase (GOR2), cytosol	5' GTATTGGCTGCGATGGCTGTTC-3'	5' ACGGCGACACAGGAGAGG-3'	274
	NM_001281138.1	Copper-Zinc SOD(CZSOD), chloroplast, cytosol	5' GATTGGGATGTTGGGTTTAC-3'	5' GTCAAAACCTCAAACGCCAAT-3'	188
	XM_002267363	Iron SOD (FeSOD), chloroplast	5' CCTTACGATTAIGGCGCAIT-3'	5' CTTTACTGGAAGGAGCAAGG-3'	542
	EU280161.1	Manganese SOD (MnSOD), mitochondria	5' CTATCCCTGAAAGTTTGTCTC-3'	5' AAGCTGTGAAGGTAGTGGC-3'	152
	NM_001281063.1	Dehydroascorbate reductase (DHAR), cytosol	5' CAACTGGTTCAAACGGTCAI-3'	5' CACAGCCTTCCCAITCTIT-3'	226
XM_002282964	Monodehydroascorbate reductase (MDHAR), cytosol, peroxisome	5' GCCCCACCAATTGACTTTGTACC-3'	5' ATCGCTCTGGATGTCCTTC-3'	140	

Transcripts coding sHSPs (obtained when using the GrapeGene 520510F array (Affymetrix) in plants subjected to 42 °C for 1 h), reference genes (Coito *et al.* 2012) and oxidative stress genes (Carvalho *et al.* 2006). Gene names, NCBI annotation, forward and reverse primers, and length of the product obtained are indicated.

amplification plots were analysed with an R_n threshold of 0.2, at the beginning of the region of exponential amplification to obtain C_q (quantification cycle) and the data obtained were exported into an MS Excel workbook (Microsoft Inc., Redmond, WA, USA) for further analysis (Livak & Schmittgen 2001). In order to compare data from different PCR runs, C_q values were normalized to the C_q values of *act*, *TIF* and *TIF-GTP* (Table 1), that are found to be stable under abiotic stress conditions (Coito *et al.* 2012).

Statistical analysis

Two-way analysis of variance (ANOVA; SAS 9 for Windows, SAS Institute, Inc., Cary, NC, USA) was used to study the effects of the stress treatments on the different physiological parameters and contents of H_2O_2 , pigments, glutathione, ascorbate and ABA in the two varieties studied. For the expressions of the genes studied, the relative quantity values were transformed into \log_2 (thus rendering them parametric) and also tested through ANOVA when the P value of the ANOVA was lower than 0.05, treatments were compared with their respective controls and each treatment was compared in both varieties, through Tukey's multiple comparison tests and statistically significant differences were accepted for a P value lower than 0.05. These analyses were performed using SAS 9 for Windows (SAS Institute, Inc.).

RESULTS

H_2O_2 , pigments and antioxidative metabolites

Leaf H_2O_2 was quantified in TR and TN in both heat treatments (Fig. 2). In TR-SAR, H_2O_2 content showed a bimodal pattern, increasing significantly in SAR32u and SAR32d as compared with the control while the values measured at the highest tem-

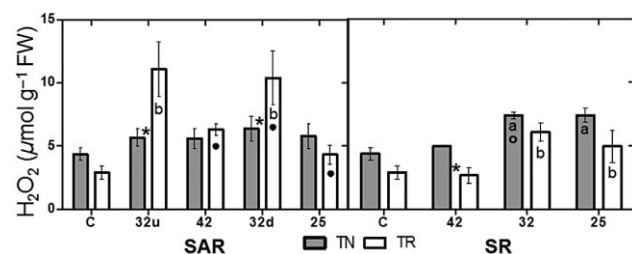


Figure 2. Hydrogen peroxide quantification in leaves of Touriga Nacional (TN) and Trincadeira (TR) in stepwise acclimation and recovery (SAR) and stepwise recovery (SR). Each value expressed in $\mu\text{mol g}^{-1}$ fresh weight (FW) is the mean of four independent samples measured in triplicate ($n = 4$). Statistically significant differences after Tukey's multiple comparison tests for a P value lower than 0.05 are the following: * Significant difference between TN and TR; ^a significant difference between TN and the respective control; ^b significant difference between TR and the respective control; [°] significant difference between a TN stress moment and the previous one; [•] significant difference between a TR stress moment and the previous one ([°] and [•] do not exist in SAR32u and SR42 because they correspond to the significant differences ^a and ^b, respectively).

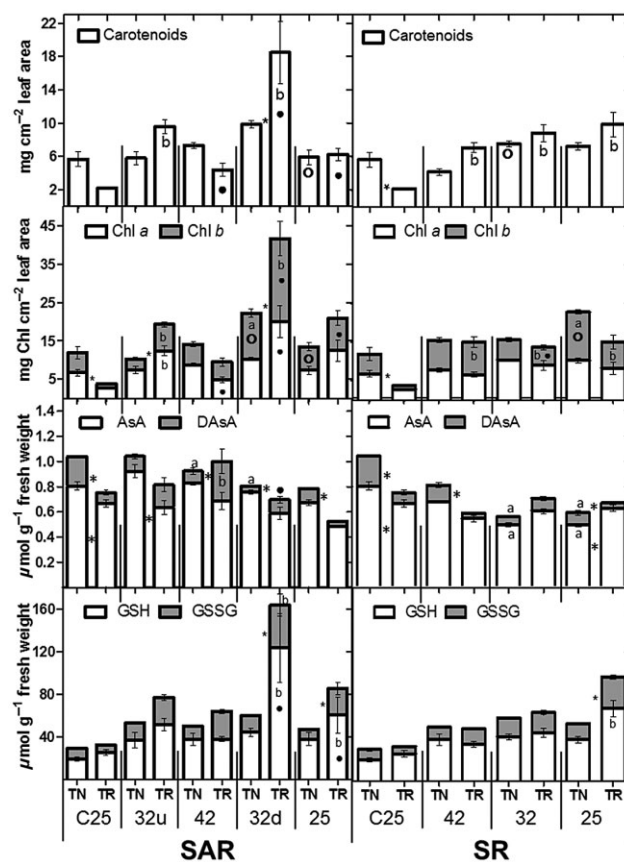


Figure 3. Carotenoid, chlorophyll *a* and *b* (Chl *a* and Chl *b*), reduced (AsA) and oxidized (DAsA) ascorbate and reduced (GSH) and oxidized (GSSG) glutathione concentration in leaves of Touriga Nacional (TN) and Trincadeira (TR) in stepwise acclimation and recovery (SAR) and stepwise recovery (SR). Each value is the mean of four independent samples measured in triplicate ($n = 4$). Statistically significant differences after Tukey's multiple comparison tests for a P value lower than 0.05 are the following: * Significant difference between TN and TR; ^a significant difference between TN and the respective control; ^b significant difference between TR and the respective control; [°] significant difference between a TN stress moment and the previous one; [•] significant difference between a TR stress moment and the previous one ([°] and [•] do not exist in SAR32u and SR42 because they correspond to the significant differences ^a and ^b, respectively).

perature (SAR42) and after 24 h recovery at 25 °C (SAR25) did not differ significantly from the control while in TN-SAR H_2O_2 content remained unchanged. In SR, H_2O_2 did not increase after 1 h at 42 °C (SR42), being delayed to SR32 and not regaining control values after the 24 h recovery (SR25).

Total chlorophyll (Chl *t*) and Chl *b* content was significantly higher in leaves of control plants of TN than in TR (Fig. 3), unsurprising, as the typical darker hue of green in the leaves already implies. In TR-SAR, Chl *t* was only significantly higher after acclimation (SAR32u), while in SAR32d, both varieties showed a dramatic increase. Chl *t* decreased after 24 h recovery (SAR25) to control values in TN and maintained significantly high values in TR. In both varieties,

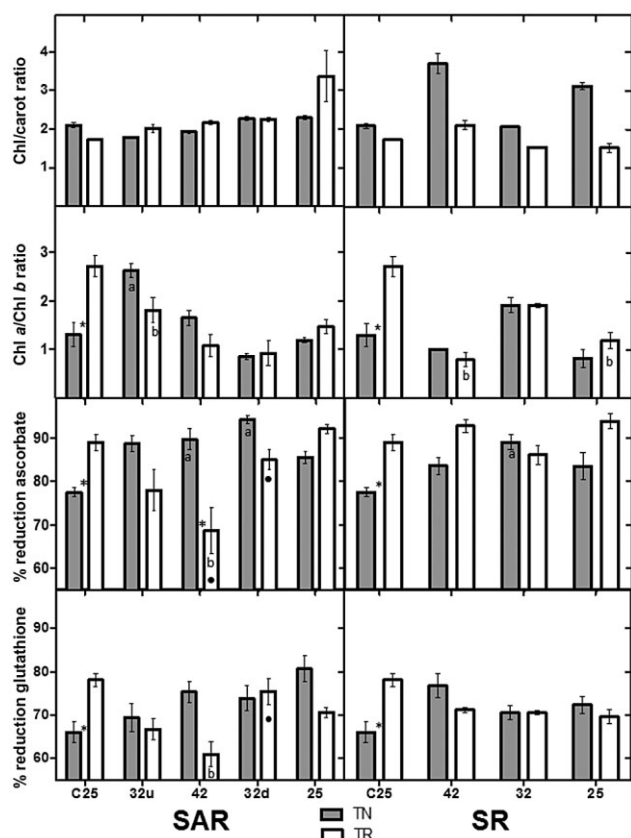


Figure 4. Total chlorophyll (Chl)/carotenoid (carot) ratio, Chl *a*/Chl *b* ratio, percentage of reduction of ascorbate and of glutathione in leaves of Touriga Nacional (TN) and Trincadeira (TR) in stepwise acclimation and recovery (SAR) and stepwise recovery (SR). Each value is the mean of four independent samples measured in triplicate ($n = 4$). Statistically significant differences after Tukey's multiple comparison tests for a P value lower than 0.05 are the following: * Significant difference between TN and TR; ^a significant difference between TN and the respective control; ^b significant difference between TR and the respective control; [○] significant difference between a TN stress moment and the previous one; [●] significant difference between a TR stress moment and the previous one ([○] and [●] do not exist in SAR32u and SR42 because they correspond to the significant differences ^a and ^b, respectively).

Chl *t* increased significantly after the recovery in SR. Carotenoid values followed the same pattern as Chl *t* in the control (TR significantly lower than TN; Fig. 3). In TR-SAR (Fig. 3), the value of SAR42 was similar to the control and to the recovery (SAR25), while the two SAR32 values and all the TR-SR values were significantly high.

The amounts of reduced and oxidized ascorbate, as well as the percentage reduction were measured in both treatments in both varieties (Figs 3 and 4). The two varieties have different basal levels of ascorbate and of percentage reduction. TR has higher percentage reduction (Fig. 4) and lower amounts of both reduced and oxidized ascorbate (Fig. 3). There were no significant increases in AsA either in TN-SAR or TR-SAR while DAsA decreased after acclimation and stress in TN (SAR32u and SAR42) and increased in TR in SAR32u

(Fig. 3). As a consequence, the percentage reduction in TN increased significantly in those moments, while it decreased significantly in TR (Fig. 4). In TN-SR, there was a significant decrease of both AsA and DAsA in SR32, which was maintained until SR25 (Fig. 3). However, and as in SAR, the stress effect was less pronounced in TN, and consequently, the percentage reduction increased significantly on SR32, attaining almost basal levels at SR25 (Fig. 4).

The percentage reduction of glutathione (Fig. 4) was significantly higher in TR. Upon both treatments, TN maintained the total amount of glutathione, but the reduced pool increased steadily and was still high on SAR/SR25. In TR-SAR, a bimodal pattern was detected in total glutathione content, with peaks on both SAR32 (u and d), and values were still high on SAR/SR25. The increase of total glutathione in TR-SAR was also accompanied by an increase in GSSG (Fig. 3), which led to a significant decrease of the percentage reduction to the lowest values measured, in SAR32u (Fig. 4).

ABA content

ABA concentration changed consistently in both varieties on both treatments (Fig. 5): SAR experienced a steady rise along the experiment, peaking on SAR32d, returning to near-control levels on SAR25 while on SR, the lowest ABA concentrations corresponded to the maximum temperature (SR42), increasing abruptly on SR32 and returning to basal levels on SR25.

State of the photosynthetic apparatus

The effect of both treatments on the plant's photosynthetic capacity was assessed through light response curves that allowed the calculation of A_{max} , g_s , LCP and R_d (Fig. 6), and through the quantification of chlorophyll fluorescence parameters (Fig. 7).

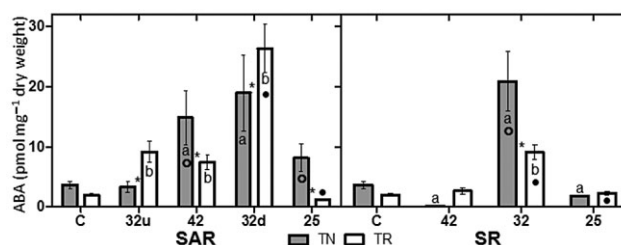


Figure 5. Abscisic acid (ABA) concentration in leaves of Touriga Nacional (TN) and Trincadeira (TR) in SAR and SR. Each value expressed in pmol mg^{-1} dry weight is the mean of four independent samples measured in triplicate ($n = 4$). Statistically significant differences after Tukey's multiple comparison tests for a P value lower than 0.05 are the following: * Significant difference between TN and TR; ^a significant difference between TN and the respective control; ^b significant difference between TR and the respective control; [○] significant difference between a TN stress moment and the previous one; [●] significant difference between a TR stress moment and the previous one ([○] and [●] do not exist in SAR32u and SR42 because they correspond to the significant differences ^a and ^b, respectively).

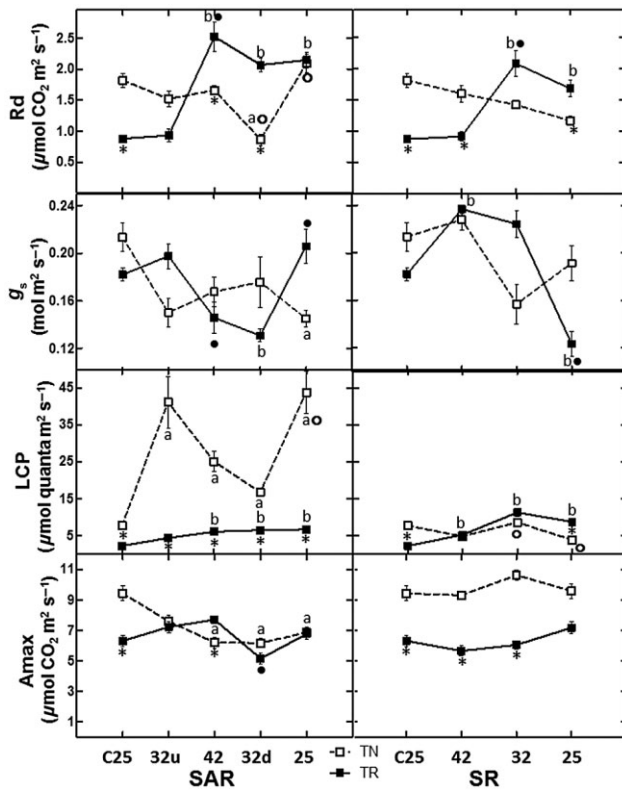


Figure 6. Photosynthetic parameters measured in plants of Touriga Nacional (TN) and Trincadeira (TR) in stepwise acclimation and recovery (SAR) and stepwise recovery (SR), using an open gas-exchange system (IRGA LCPro+ da ADC Bioscientific, Ltd.). Amax corresponds to the maximal rate of photosynthesis, g_s is the stomatal conductance at $300 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$, LCP represents the light compensation point and Rd is the dark respiration. Each value is the mean of six independent samples ($n = 6$). Statistically significant differences after Tukey's multiple comparison tests for a P value lower than 0.05 are the following: * Significant difference between TN and TR; ^a significant difference between TN and the respective control; ^b significant difference between TR and the respective control; ^o significant difference between a TN stress moment and the previous one; [•] significant difference between a TR stress moment and the previous one (^o and [•] do not exist in SAR32u and SR42 because they correspond to the significant differences ^a and ^b, respectively).

Although plants were grown at relatively low light intensities, overall photosynthesis rates were more similar to those measured in sun-type leaves than in shade leaves (Greer *et al.* 2011), and the differential response of the two varieties was put in evidence by other photosynthetic parameters (Figs 6 and 7). On SAR Amax and g_s were not significantly different in both varieties (Fig. 6), while Rd was initially lower in TR, increasing after SAR42 and reaching the same values as TN on SAR25. LCP was significantly higher in TN with the onset of stress (from SAR32u), pointing to a reduction of photosynthetic efficiency. On SR the most striking difference among varieties was measured in Amax, with TN keeping higher values than TR during the period of stress. In parallel, TR-SR had more difficulty in recovering the values of g_s after stress than TN-SR, what possibly affected photosynthesis

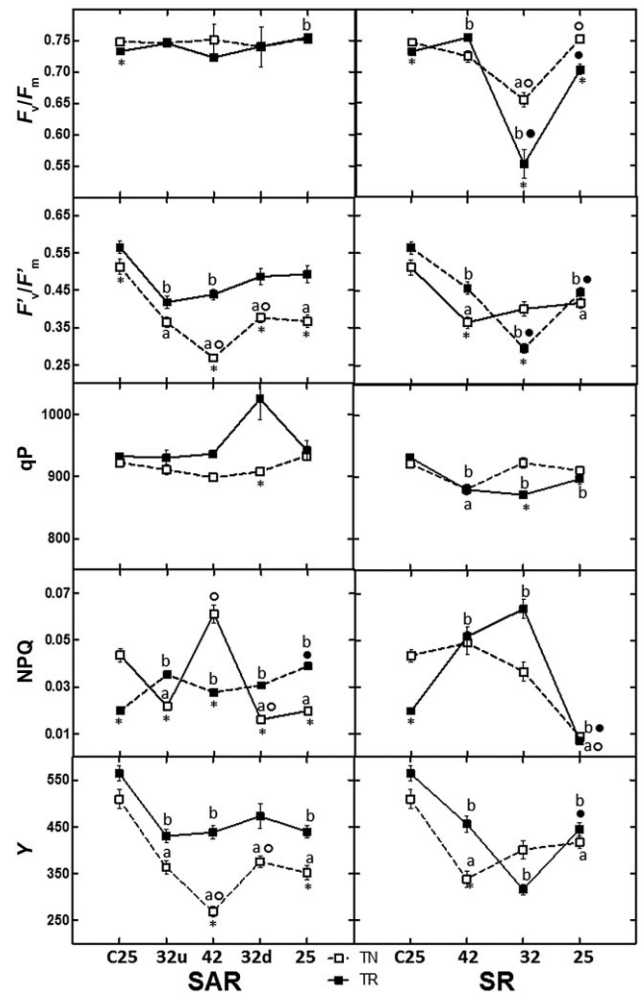


Figure 7. Chlorophyll fluorescence parameters measured in leaves of Touriga Nacional (TN) and Trincadeira (TR) in stepwise acclimation and recovery (SAR) and stepwise recovery (SR). F_v/F_m represents the maximum efficiency of photosystem II (PSII) photochemistry in dark-adapted leaves, F_v'/F_m' corresponds to the maximum quantum efficiency of PSII in light-adapted leaves, qp corresponds to the light energy used in photosynthesis, NPQ ($F_m/F_m - 1$) gives an estimate of the light energy not used in photosynthesis and functions as an indicator of photoprotective processes through the dissipation of energy as heat (Demmig-Adams and Adams 1992), Y equates to the operating quantum efficiency of PSII photochemistry. Statistically significant differences after Tukey's multiple comparison tests for a P value lower than 0.05 are the following: * significant difference between TN and TR; ^a significant difference between TN and the respective control; ^b significant difference between TR and the respective control; ^o significant difference between a TN stress moment and the previous one; [•] significant difference between a TR stress moment and the previous one (^o and [•] do not exist in SAR32u and SR42 because they correspond to the significant differences ^a and ^b, respectively).

rates because of closed stomata. Also, there was a loss of photosynthetic efficiency because of increased respiration rates in TR-SR after the beginning of stress conditions.

Maximum efficiency of photosystem II (PSII) photochemistry in dark-adapted leaves (F_v/F_m) was unaffected on SAR

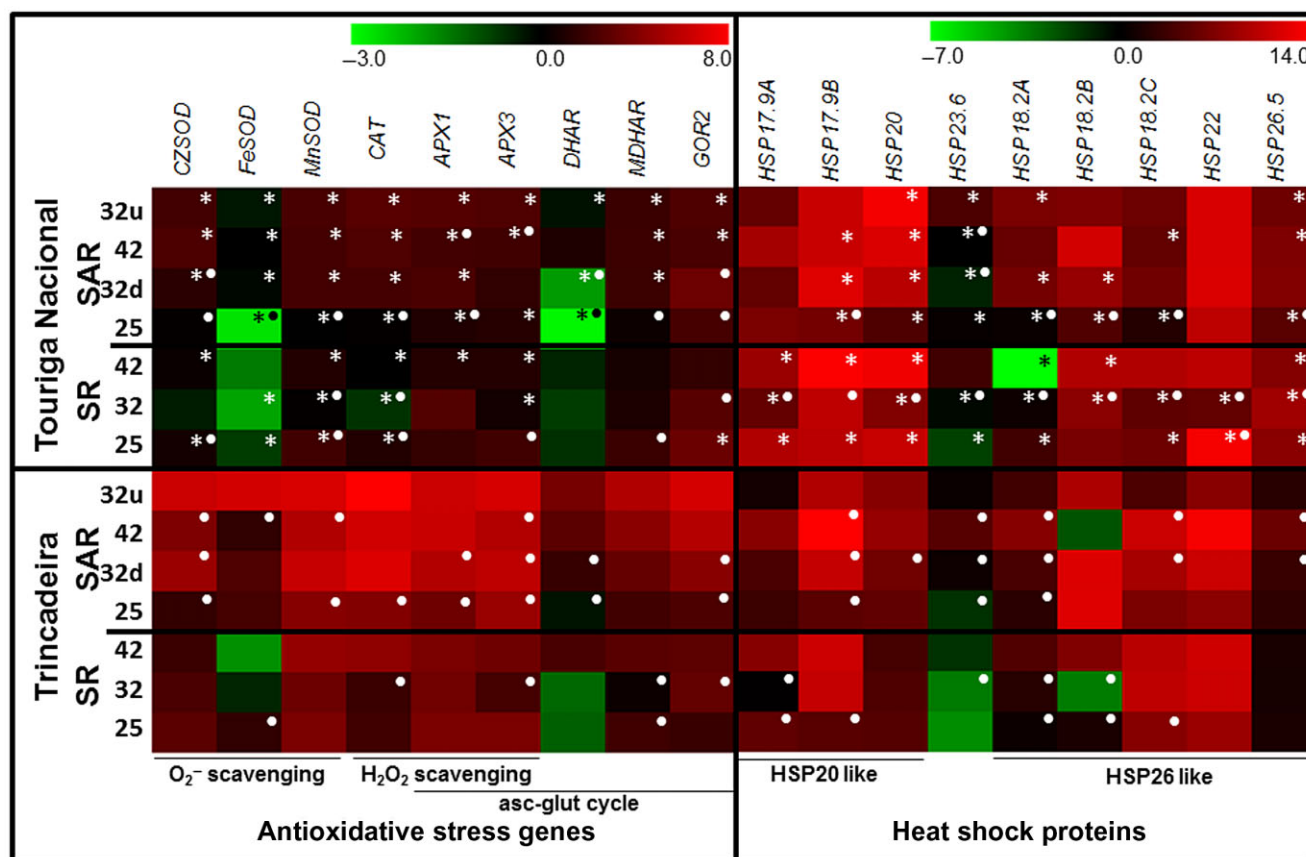


Figure 8. Relative gene expression ratios obtained by RT-qPCR of nine antioxidative stress genes (*APX1*, *APX3*, *CAT*, *GOR2*, *FeSOD*, *MnSOD*, *CZSOD*, *DHAR* and *MDHAR*) and of nine transcripts coding sHSPs (*HSP17.9A*, *HSP17.9B*, *HSP18.2A*, *HSP18.2B*, *HSP18.2C*, *HSP20*, *HSP22*, *HSP23.6* and *HSP26.5*) quantified in Touriga Nacional (TN) and Trincadeira (TR). The sequences of the primers used for amplification are presented in Table 1. Relative expressions in relation to control plants in each treatment and stress moment were calculated and values were normalized with respect to translation initiation factor eIF-3 subunit 4 (*TIF*), translation initiation factor eIF-2B alpha subunit (*TIF-GTP*) and actin 2 (*act*) mRNA. The data correspond to $\log_2(\text{fold-expression})$ of three independent samples measured in duplicate. Values within $\log_2(\text{fold-expression}) < |2|$ are not significantly different from the controls. Statistically significant differences after Tukey's multiple comparison tests for a *P* value lower than 0.05 are the following: • significant difference between a TN/TR stress point and the previous one; * significant difference between TN and TR within a stress moment and indicated in the figure only in TN. SAR, stepwise acclimation and recovery; SR, stepwise recovery.

in both varieties, while on SR, there were significant decreases on SAR32 (Fig. 7). Conversely, maximum quantum efficiency of PSII in light-adapted leaves ($F'v/F'_m$) showed a steady decrease until SAR42 in TN, while in TR, the lowest values were registered in SAR32u and a recovery thereafter to values lower than control in both varieties. In SR, this parameter was negatively affected in both varieties, TN attaining the lowest values on SR42 while the lowest value of TR was reached on SR32, both recovering to equivalent values on SR25, although lower than control. Photochemical quenching (qP), a factor indicating the amount of light energy actually channelled to photosynthesis, increased on SAR32d in TR and on SR32 in TN. On SR25, equivalent values were attained on both varieties. Non-photochemical quenching (NPQ) gives an estimate of the light energy not used in photosynthesis and functions as an indicator of photoprotective processes through the dissipation of energy as heat (Demmig-Adams & Adams 1992). TR-SAR showed

no significant changes in the values of this parameter while TN-SAR responded with a clear peak on SAR42 (Fig. 7). On SR, both varieties showed increases, TN peaking on SR42 and TR on SR32 decreasing to equivalent values on SR25, lower than control for both varieties. *Y* equates to the operating quantum efficiency of PSII photochemistry and its values steadily followed the same pattern as $F'v/F'_m$ in both varieties in SAR and SR (Fig. 7).

Gene expression signatures

Previously tested oxidative stress response genes (Carvalho *et al.* 2006) were monitored in SAR and SR in both varieties, and the level and significance of gene expression clearly separated the varieties as well as the two treatments (Fig. 8). In TN-SAR, the levels of up-regulation were just above the level of significance in all time points except 25, while the change in gene expression in TN-SR was hardly significant in

all time-points and for all genes, with the exception of *GOR2*. In TR-SAR, the levels of up-regulation (again with the exception of SAR25) were very significant in all genes, while in TR-SR, up-regulation levels were slightly lower. The most significantly up-regulated genes in TR were ROS scavengers (*CZSOD*, *MnSOD*, *CAT* and both *APXs*) while *GOR2* had the highest levels of expression in TN. The monitoring of the expression of nine HSPs yielded a slightly different pattern of response as compared with antioxidative genes (Fig. 8). The levels of up-regulation in SAR in both varieties were significantly high in all transcripts except *HSP23.6*. In SR, the responses were different in TN and TR, with high levels of up-regulation kept after 24 h recovery at 25 °C in TN for all the transcripts except *HSP18.2A* and *HSP23.6*. In fact, *HSP18.2A* was significantly down-regulated upon stress (SR42). In TR-SR *HSP26.5* suffered no significant changes from the control, *HSP18.2A* was only significantly up-regulated on SR42 and *HSP23.6* and *HSP18.2B* were down-regulated.

DISCUSSION

Plants have several response mechanisms at their disposal to cope with high temperature. These range from short-term avoidance or acclimation to long-term evolutionary adaptations (Kotak *et al.* 2007). Stress-related responses are divided into two major groups: the first comprising antioxidants and scavengers of ROS and the second responsible for signalling cascades and transcriptional control (Valliyodan & Nguyen 2006). HS is no exception to this dual behaviour, and when plants are subjected to high temperatures, the response takes place at those two main levels. Stress-responsive mechanisms are set in motion to secure cell homeostasis and the integrity of cell components. When both levels of response are not synchronized, the final result can be inadequate to counter the deleterious effects of the stress, ultimately leading to irreversible cell damage and having as the final consequence, cell death (Bohnert *et al.* 2006).

Here we determined how grapevine varieties TN and TR differ in their response to HS applied with and without acclimation and after recovery. The responses were evaluated through ROS production (H_2O_2), chlorophyll and carotenoid content, ascorbate and glutathione content and redox state, ABA production, photosynthetic capacity and expression of key genes.

Cellular redox state

H_2O_2 is a strong oxidant that targets thiol groups, but it can also mediate the cellular redox status and regulate events that control gene expression (Foyer & Noctor 2005; Mullineaux *et al.* 2006). The Mehler reaction is considered the foremost source of H_2O_2 in chloroplasts, and the rate of O_2 photoreduction, leading to H_2O_2 -generating superoxide, depends on environmental and stress conditions (Slesak *et al.* 2007). In TN-SR, this does not seem to be the case, as both plastidial SODs (*FeSOD* and *CZSOD*; Alscher *et al.* 2002) were not induced, nor was the H_2O_2 scavenging mechanism.

However, H_2O_2 scavenging is fully operational in SAR as indicated by the activation of *CAT*, *APX1* and *APX3*. L-AsA is a key antioxidant in the detoxification of ROS (Conklin & Loewus 2001), that eliminates H_2O_2 through the ascorbate–glutathione (asc–glut) cycle. Glutathione (GSH) is a multi-functional metabolite in plants that also acts in cellular defence and protection, reacting with a range of ROS, and also detoxifying H_2O_2 in the asc–glut cycle (Foyer & Noctor 2005).

When HS was applied with SAR, TR plants produced high amounts of H_2O_2 on SAR32u and SAR32d that corresponded to high levels of chlorophyll, carotenoids and glutathione. ABA increased steadily with time, returning to basal levels at SAR25 and ascorbate amounts were highest on SAR42, decreasing thereafter. The levels of expression of the H_2O_2 scavengers *APX1*, *APX3* and *CAT*, together the asc–glut cycle *MDHAR* and *GOR2* were significantly increased in TR-SAR. Carotenoids protect the photosynthetic apparatus against photooxidative damage by quenching the triplet states of chlorophyll molecules (Koyama 1991) but also by acting as scavengers of ROS, protecting pigments and unsaturated fatty acids of lipids from oxidative damage (Edge *et al.* 1997). In TR-SAR, carotenoids seem to play an important role in the scavenging of ROS in parallel with that of ascorbate and glutathione, the usual first line of antioxidative defence in plants (Noctor & Foyer 1998). In TR-SAR and -SR, mitochondrial ROS production is evident, with high levels of *MnSOD* expression. The H_2O_2 thus produced can contribute to the cytosolic pool and there be scavenged by *APX* and the asc–glut cycle (Slesak *et al.* 2007). Also, as both *CZSOD* and *FeSOD* were induced to scavenge the O_2^- present in chloroplasts as a consequence of overreduction of the electron transport chain because of stress. The resulting H_2O_2 levels appear to be scavenged by the *MDHAR*–*GOR2* branch of the asc–glut cycle.

In TN-SAR and -SR, only the mitochondrial *SOD* was activated, indicating a mitochondrial origin for most of the O_2^- in need of scavenging. As in TR, H_2O_2 levels were tuned by the *MDHAR*–*GOR2* branch of the asc–glut cycle and kept at levels that were far from dangerously high (Carvalho *et al.* 2006). In TN, the returning of antioxidative gene expression to basal levels towards the end of acclimation was very evident, especially in SAR.

While TR showed stress-related metabolites in control conditions at concentrations that indicate ‘good health’, TN had high levels of total ascorbate but lower percentage reduction of both AsA and GSH, thus indicating some level of readiness to rapidly react to both stress conditions, as was in fact verified. In these conditions, TN reacted rapidly, significantly increasing reduction pools of AsA and GSH, without the need for *de novo* synthesis of either metabolites and thus being able to boost the buffering capacity of the cell’s redox pool. Conversely, upon the same stress, TR needed to synthesize both AsA and GSH and its response was slower and insufficient to maintain the redox pool at working levels. TR was also unable to keep the asc–glut pool in the reduced state, despite the high levels of expression of

CAT and APXs, thus rendering the cycle less effective in the task of ROS removal.

Although the experimental difference between treatments is the acclimation in SAR and the acute temperature rise in SR the most striking difference in the response of the plants takes place in the recovery period. Levels of pigments and cell redox state regained control levels 24 h after stress in SAR, while in SR, most of the values were still significantly altered at the corresponding moment. This pattern was similar in both varieties, indicating that when plants are subjected to acclimation, the response to stress is more intense and efficient and that, after the stress is no longer present, the return to basal metabolism occurs more rapidly.

Photosynthesis

Photosynthesis rates were not as significantly affected by the acclimation/recovery procedure as cell redox state, although other photosynthetic parameters point to different strategies of response to HS and to acclimation. In SAR, both varieties showed decreases in photosynthetic capacity during and after stress, and 24 h after stress LCP and g_s were still significantly affected in TN, as well as the maximum quantum efficiency of PSII in light-adapted leaves (F'_v/F'_m) and the quantum efficiency of PSII photochemistry (Y). Nevertheless, the amount of light energy channelled for photosynthesis (qP) was back at control levels on both varieties. The typical LCP response to HS would be a significant increase (Berry & Björkman 1980). However, this only occurred in TN-SAR, and neither this increase nor the decrease of g_s affected the photosynthetic rate, that kept the same levels as TR. An hypothesis for this unusual response can be that the sun-type behaviour of TN (Camejo *et al.* 2005) is the sole responsible for the high LCP values, and not a response to heat. In fact, g_s only showed the typical response to heat in TR-SAR, slightly increasing at SAR32u and significantly decreasing when temperature exceeded 40 °C. This should influence photosynthesis, as it did in sweet orange, abruptly decreasing at temperatures above 40 °C (Ribeiro *et al.* 2006), but this was not the case in grapevine, where photosynthesis was slightly higher in TR than in TN on SAR42.

TR-SR had the same photosynthetic behaviour as TR-SAR, contrasting with TN, where Amax and LCP responded more efficiently during and after the recovery from stress. In theory, this should in fact be the expected behaviour, as the catalytic activity of Rubisco increases with temperature (Salvucci & Crafts-Brandner 2004). Usually under moderate HS (30–42 °C), the inhibition of photosynthesis is the responsibility of a slower regeneration of Ribulose-1,5-bisphosphate (RuBP), caused by disruption of electron transport activity (Salvucci & Crafts-Brandner 2004). Quantum yield of photosynthesis in fact decreased on SR42/35, recovering slightly on SR25.

Maximum quantum efficiency of PSII in dark-adapted leaves (F_v/F_m) suffered a significant decrease in both varieties on SR32, recovery by SR25 while the maximum quantum efficiency of PSII in light-adapted leaves (F'_v/F'_m) also

decreased on SR32, not as abruptly and only marginally recovered by SR25. Quantum efficiency of photosynthesis is known to decrease upon HS and to increase in the recovery period, while the thermal dissipation of excitation energy, measured by NPQ, follows the opposite trend (Wang *et al.* 2010). This pattern was clearly visible on SR while when acclimation to stress was added, there was a clear absence of this pattern, especially in TR where Y did not decrease significantly nor NPQ increase markedly. This indication of low-energy dissipation in the photosynthetic electron transport chain is in accordance with the levels of gene expression of SODs, which point to high levels of O₂⁻ removal in the mitochondria instead of the chloroplast.

In maize subjected to temperatures above 41 °C for 20 min, there was permanent damage unless the plants had been previously acclimated (Sinsawat *et al.* 2004). In our case, the influence of the acclimation process affected photosynthesis in the same manner as it had affected cellular redox state, although significant differences were measured between varieties, with TN less affected than TR, and recovering after 24 h.

Transcriptional signature of HS

The common and most typical element present upon HS is the expression of HSPs (Vierling 1991). Their typical expression signature is an up-regulation upon stress and during recovery. Interestingly, in grapevine's response to HS (42 °C for 1 h), the only HSPs with altered levels of expression were nine sHSPs (three *HSP20*, one *HSP23.6* and five *HSP26*) only three of which had significantly changed expression levels in TR (Supporting Information Fig. S1). sHSPs play structural roles in preserving the integrity of cell membranes during stress and are known to return to basal levels shortly after the end of the stress applied (Barua *et al.* 2003), as was the case in SAR in both varieties and as was the pattern of the similar *VvHSF30* (Liu *et al.* 2012). Although there are no conclusive studies of SHSPs in acclimation-to-stress studies, their contrasting response in SAR and SR indicates that their chief assignment might be closely connected to acclimation and that their concerted response might be triggered by acclimation itself.

In both varieties the mitochondria-located *HSP23.6* was the only exception to this clear pattern of up-regulation. In fact, in *Triticum durum* the level of mitochondrial HSP transcripts, namely *HSP23.6*, was found to be related to the ability to acquire thermotolerance (Rampino *et al.* 2009). Its response in TN is a further confirmation that this variety might not have been suffering from severe stress, neither in SAR nor in SR. The *HSP20* family is known for a strong up-regulation in response to various biotic and abiotic stresses (Swindell *et al.* 2007), so the high levels of up-regulation of *HSP17.9* in both varieties and treatments was not surprising, and had already been reported (Larkindale *et al.* 2005; Schramm *et al.* 2008; Coito *et al.* 2012). The mitochondria targeted *HSP26.5* (Desikan *et al.* 2001; Wang *et al.* 2004) was more significantly up-regulated in TN, in synchrony with the antioxidative response taking part in that plastid.

A second transcriptional signature of HS is the shutdown of housekeeping metabolism (Larkindale & Vierling 2008), which was more evident in TR than in TN (Supporting Information Fig. S1 and Rocheta *et al.* unpublished data). This is an additional indication that TN may not be suffering from such a severe stress.

CONCLUSION

In Mediterranean climate regions, grapevine leaves can attain temperatures over 40 °C in hot summer days. However, these peak temperatures are achieved after a steady increase and gradual decrease thereafter. An experimental set up with and without acclimation to increasing temperature followed by recovery was applied to TN and TR grapevine varieties. The response to the experimental conditions was clearly different in the two varieties. TR had the need to synthesize AsA and GSH and its response was slow and insufficient to maintain the redox pool at working levels. TN reacted rapidly, increasing reduction pools of AsA and GSH, and boosting the buffering capacity of the cell's redox pool. The excess amount of ROS produced came mostly from the mitochondria and not from an overflow of the photosynthetic electron transport chain, and thus photosynthesis was not significantly affected. As TR was more intensely affected by stress for a longer period of time, the levels of up-regulation of antioxidative stress genes was more significant and was kept throughout recovery.

When comparing HS with acclimation and recovery (SAR) and HS without acclimation (SR), the response of the antioxidative metabolism upon the imposition of stress was similar, but this pattern changed at the end of recovery. In fact, after 24 h of recovery at 25 °C, almost all the effects of stress had returned to basal levels when plants had been previously acclimated while almost all parameters remained altered if the plants had not been acclimated. It is thus clear that acclimation leads to a swifter and clearly to a shorter-term response that affects the plant to a much lesser extent than acute stress.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Relative gene expression ratios obtained by RT-qPCR in TN and TR in the treatments SAR and SR of the five most up-regulated and the five most down-regulated transcripts obtained with the 23 K custom-made Affymetrix *Vitis* GeneChip after acute heat stress (1 h at 42 °C).

Table S1. Leaf temperature (°C) measured along the light response curves in SAR and SR in leaves of Trincadeira and Touriga Nacional. Each column corresponds to measurements taken in the six replicate plants of each time point. Different number of temperature values result from automatic reads at different time intervals (ranging from five to 10 readings per minute). The images represent the average leaf temperatures in SAR and SR of both varieties.