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Heat shock increases mitochondrial H_2O_2 production and extends postharvest life of spinach leaves

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ABSTRACT

The effects of moderate heat shock (HS) treatments on the senescence of detached spinach leaves have been studied. At harvest, detached spinach leaves received moderate heat treatments (37, 40, 43 °C and room temperature) by immersion in water baths. The content of small HS proteins increased proportionally to the temperature applied, with undetectable levels in untreated leaves. A HS treatment at 40 °C delayed leaf senescence as indicated by higher chlorophyll content and potential quantum yield of PSII (Fv/Fm), and decreased solute leakage after storage for 7 d compared with untreated samples. A higher *in vivo* production of H_2O_2 was observed as HS temperature increased. Oxidation of 2′,7′-dichlorodihydrofluorescein diacetate observed by *in vivo* confocal microscopy revealed that mitochondria were the main site of reactive oxygen species generation under either untreated or HS-treated leaves. Although high-temperature treatments did not prevent the loss of water soluble antioxidant concentrations, the ratio of reduced/oxidised forms of ascorbic acid was higher 3 d after HS treatment, compared with control leaves. The effect of HS treatments on physiologically based protective mechanisms that delay leaf senescence is discussed.

1. Introduction

After harvest, spinach leaves can be stored without refrigeration for a short period of about 3–5 d before dehydration and senescence decrease their quality and acceptance by consumers. Storage of excised leaves in darkness causes chlorophyll and protein breakdown, typical indicators of the progress of senescence in green tissues (Noodén et al., 1997). Although light deprivation causes a decrease in the availability of photoassimilates, starvation does not seem to be the only component of the initiation of dark-induced senescence. More likely, senescence acceleration by darkness represents a response to low phytochrome far red/total phytochrome ratios (Noodén and Schneider, 2004), exacerbated by the combined stresses of wounding and isolation from the cytokinin-producing roots (Van Staden et al., 1988). In addition, an imbalance between reactive oxygen species (ROS) production and antioxidant activities leads to oxidative stress, which may accelerate senescence (Hodges and Forney, 2000).

Heat shock (HS) is a non-chemical treatment that has been recently used to ameliorate senescence-related symptoms appearing during postharvest storage (Paull and Chen, 2000; Saltveit, 2003). The rationale for the use of HS treatments is that exposure to sublethal high temperatures may trigger physiological responses that allow the plant to cope better with subsequent stress conditions. A characteristic response of plant tissues exposed to high temperatures is the increased synthesis and accumulation of heat shock proteins (HSP) of a broad range of molecular weights (Schöffl et al., 1998). These proteins act as chaperones that protect other proteins from HS-dependent modifications. Besides HSP accumulation, other physiological responses are triggered by HS that might be involved in the acquisition of thermotolerance. ROS, antioxidants, salicylic acid, abscisic acid and the transcription factor UVH6 have been postulated as signal molecules participating in metabolic pathways involved in HS tolerance (Larkindale et al., 2005; Dat et al., 1998a,b). In addition, the effect of high-temperature treatments on the time course and subcellular localisation of ROS production and antioxidant activities still remains to be studied in detail (Suzuki and Mittler, 2006).

The main objectives of this work were: first, to select a HS treatment that might inhibit the start, or slow down the rate of postharvest senescence of spinach leaves and, second, to study the

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effects of HS on physiological processes possibly involved in delaying senescence.

2. Materials and methods

2.1. Plant material

Spinach (*Spinacia oleracea* L. cv Bison) plants grown in the greenhouse were obtained from a local producer during the winter season and brought immediately to the lab. Fully expanded leaves were cut at the petiole and subjected to HS treatments by immersing them in water baths at room temperature (RT), 37, 40 and 43 °C for 3.5 min. After the HS treatment, leaves were rapidly cooled down in water at 22–23 °C, carefully blotted with paper towels, placed in low-density polyethylene bags and stored in darkness at 23 °C. Determinations were made at harvest, and 3 and 7 d after the HS treatment. All experiments consisted of five plastic bags containing three leaves each, for each treatment and sampling time.

2.2. Chlorophyll content and fluorescence

The content of chlorophyll was estimated with a chlorophyll meter (SPAD-502, Minolta). The potential quantum yield of PSII (Fv/Fm) was measured with a Fluorescence Modulated System (FMS2 Hansatech Instruments Ltd., Norfolk, UK) in leaves adapted to darkness for 30 min.

2.3. Solute leakage

Five leaf discs (fresh weight of 150–200 mg), excluding the main veins, were washed and placed in 20 mL of deionised water. Water conductivity was recorded at the beginning of the incubation (initial cond.) and after incubation for 3 h with gentle shaking (cond. 3 h). Then leaf discs were boiled for 5 min for the determination of maximum conductivity (max. cond.). Solute leakage was calculated as: [(cond. 3 h − initial cond.)/(max. cond. − initial cond.)] × 100.

2.4. Small heat shock proteins (sHSP) accumulation

The sHSP were determined in a membrane-associated protein fraction isolated using a phenol extraction procedure according to Hurkman and Tanaka (1986). Four hours after the HS treatments were applied, about 500 mg (15 discs) of spinach leaves were ground with liquid nitrogen and then added with 0.5 mL extraction buffer (100 mmol L^{-1} Tris–HCl pH 8, 1 mmol L^{-1} EDTA, 1 mmol L^{-1} PMSF, 2% (v/v) β-mercaptoethanol) plus 2 mL phenol saturated with Tris–HCl 100 mmol L^{-1} pH 8. The homogenates were centrifuged at 21,000 × *g* and 4 °C for 10 min. The phenolic phase was re-extracted with 1 vol. of extraction buffer and then mixed with 4 vol. of 100 mmol L^{-1} ammonium acetate (dissolved in methanol). Then the mixture was incubated overnight at −20 °C and proteins were precipitated by centrifugation at 21,000 × *g* and 0 °C for 20 min. The pellets were washed twice with 100 mmol L^{-1} ammonium acetate (in methanol) and once with 80% (v/v) acetone, all washes at −20 °C. Finally the pellets were dried under nitrogen and resuspended in a small volume of sample buffer for SDS-PAGE according to Laemmli (1970). After electrophoresis in a 12% (w/v) polyacrylamide gel, proteins were transferred to nitrocellulose membranes for Western blot analysis using rabbit antibodies against a sHSP from tomato (*Lycopersicon esculentum*) (Polenta, 2005).

2.5. Respiration

Leaf respiration was measured by placing leaf discs (fresh weight of about 500 mg) in an air-tight chamber fitted with a Clark type O_2 electrode (Hansatech, UK).

2.6. Hydrogen peroxide (H_2O_2) production

The effect of HS treatments on leaf H_2O_2 production was determined by placing leaf discs (fresh weight of about 50 mg), immediately after the treatment, in 1 mL of a solution containing 10,000 U L^{-1} horseradish peroxidase, 10 μmol L^{-1} Amplex Red (*N*-acetyl-3,7-dihydroxyphonxazine) and 50 mmol L^{-1} Tris–HCl buffer pH 7.4 (Amplex Red assay kit, Molecular Probes). The reaction was linear for several hours. Fluorescence was recorded 100 min after HS by setting the spectrofluorometer at 560 and 590 nm, excitation and emission wavelengths, respectively (Zhou et al., 1997).

2.7. Subcellular detection of H_2O_2

A laser scanning confocal microscope (LSM Pascal, Zeiss) was used for *in vivo* localization of H_2O_2 (Yao and Greenberg, 2005). After a HS treatment at 40 °C for 3.5 min, discs were cut and incubated in a solution containing 10 μmol L^{-1} 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA, Molecular Probes), 5 μmol L^{-1} MitoTracker Red (CMXRos, Molecular Probes), and 20 mmol L^{-1} Tris–HCl buffer pH 7.4. Control leaves were kept at room temperature. The oxidation of H_2DCFDA was detected with excitation at 488 nm and fluorescence emission between 505 and 550 nm. For MitoTracker Red, a 543/560–615 nm excitation/emission wavelength setting was used. Chlorophyll fluorescence was detected with emission above 650 nm. These observations were made in three-independent experiments, with a minimum of five leaf discs per treatment.

2.8. Ascorbic acid, glutathione and α-tocopherol content assays

Total and reduced ascorbic acid (AA) were measured with a HPLC system (Shimadzu LC-10ATvp solvent delivery module) fitted with a C-18 column (Varian Chromsep 100 mm × 4.6 mm) and detected at 265 nm (Shimadzu UV–vis SPD-10Avp detector) as described in Bartoli et al. (2006). The oxidised ascorbate, dehydroascorbic acid (DHA) was estimated as the difference between the contents of total and reduced forms.

For the measurement of glutathione, five leaf discs (about 150–200 mg, fresh weight) were ground in 0.5 mL of TCA (3%, w/v), centrifuged at 17,000 × *g* for 10 min and the supernatants used for the assay. Total and oxidised glutathione (GSSG) were determined spectrophotometrically as described in Griffith (1980). Reduced glutathione (GSH) was calculated as the difference between total and oxidised contents.

For the extraction of α-tocopherol five leaf discs were ground in 1 mL of methanol and centrifuged at 17,000 × *g* for 5 min. Seven hundred microlitres of hexane were added to 500 μL of the supernatant (methanol phase), vortexed vigorously and centrifuged at 17,000 × *g* for 1 min. Six hundred microlitres were taken from the upper phase, evaporated under vacuum and dissolved in a small volume of methanol. Then samples were loaded onto a C-18 column (BondElute, Varian) and eluted with methanol. The fraction containing α-tocopherol was evaporated until dryness and finally resuspended in a small volume of methanol. Samples were measured with the HPLC system mentioned above, but α-tocopherol was separated isocratically using methanol:H_2O (95:5, v/v) at 0.17 mL s^{-1} and detected at 292 nm.

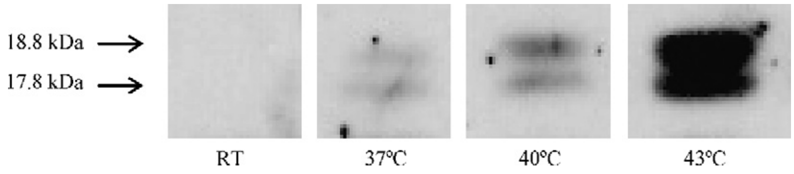

Fig. 1. Typical Western blot showing the accumulation of sHSP in spinach leaves. Leaves were treated with water baths at the indicated temperatures for 3.5 min and proteins extracted 4 h later. Two other experiments each with one replicate showed similar results.

2.9. Statistical analysis

Data are presented as the average of the results obtained from four-independent experiments and analyzed by means of ANOVA. The means were compared by the LSD test at a significance level of 0.05.

3. Results and discussion

3.1. Effects of heat shock in spinach leaves

The accumulation of sHSP, which are known to be induced by heat shock (Sommers et al., 1989), was measured to determine if the temperature and duration of the HS treatments applied in these experiments elicited a physiological response in spinach leaves. Fig. 1 shows that no sHSP were detected in control leaves, while two sHSP of about 17.8 and 18.8 kDa accumulated in HS-treated tissues. The contents of both sHSP were higher as the temperature applied to the leaves increased.

Leaf respiration was not affected at 2 h after HS, or during the following days of incubation. Control leaves showed an O_2 respiration rate of 192 mmol kg^{-1} s^{-1}. Data are expressed on the basis of dry weight.

3.2. Heat shock delays spinach senescence

A wide range of temperatures was assayed in preliminary experiments to assess the effects of HS treatments on senescence of detached spinach leaves. Temperatures lower than 35 °C had no effect, while senescence was markedly accelerated at 46 °C or higher temperatures (data not shown). Therefore, temperatures between 37 and 43 °C were chosen for subsequent experiments.

The effects of HS treatments on spinach leaf senescence were determined using chlorophyll content, potential quantum yield of PSII (Fv/Fm) and electrolyte leakage. Untreated leaves showed a 30% decrease in chlorophyll content on day 7 while HS treatments partially prevented chlorophyll loss (Fig. 2A). HS was also effective to prevent chlorophyll degradation in broccoli (Costa et al., 2005). Chlorophyll fluorescence can be used to determine Fv/Fm, which estimates the integrity of PSII reaction centers. Recently, Fv/Fm has been used for the evaluation of postharvest deterioration of different plant organs (DeEll and Toivonen, 2003) during senescence. A 67% decrease in Fv/Fm in control leaves on day 7 indicates that damaged or partially disassembled PSII centers accumulated during dark storage, but 40 °C HS-treatment delayed this detrimental process (Fig. 2B). Exposure to 43 °C caused a decline in Fv/Fm on the third day suggesting that detrimental processes may take place at 43 °C or higher temperatures. An almost 10-fold increase of solute leakage was observed in control leaves but it was significantly lower for all HS treatments on the seventh day after harvest (Fig. 2C).

Together these data indicate that exposure of spinach leaves to 37, 40 or 43 °C for 3.5 min delayed their senescence.

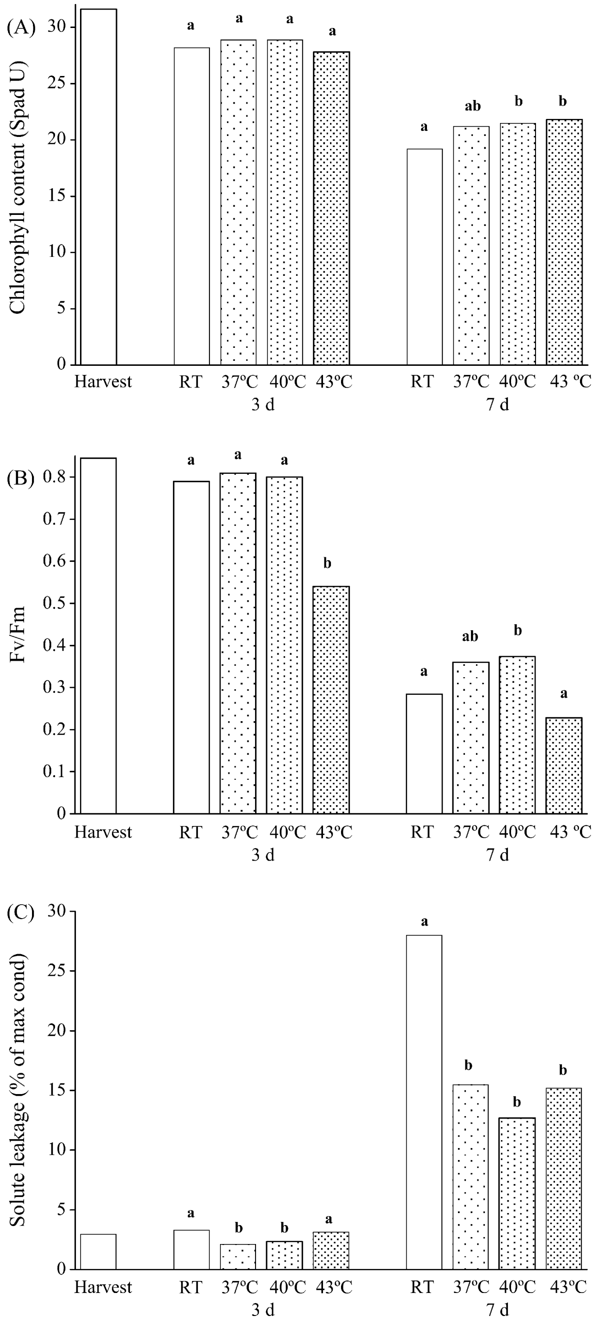

Fig. 2. Chlorophyll content (A), solute leakage (B) and Fv/Fm (C) during dark-induced senescence of HS-treated spinach leaves. Leaves were treated with water baths at the indicated temperatures for 3.5 min, placed in polyethylene bags and stored in a dark chamber at 23 °C. Values are the mean of four-independent experiments with at least three replicates each. Data with same letters represent a statistically homogenous group on the same sampling day (ANOVA, $P \leq 0.05$).

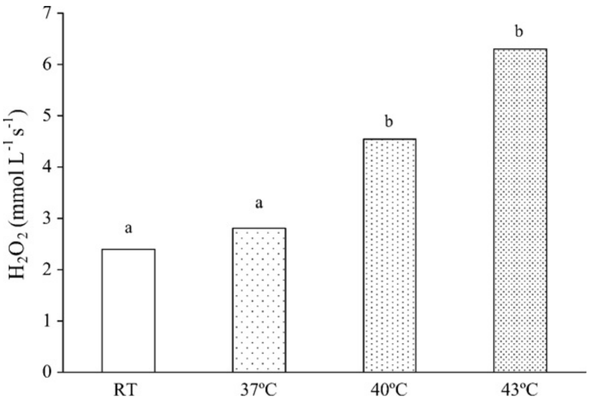

Fig. 3. H_2O_2 production by HS-treated spinach leaves. Leaves were treated with water baths at the indicated temperatures for 3.5 min and H_2O_2 production measured during the following 100 min. Values are the mean of four-independent experiments with three replicates each. Data with same letters represent a statistically homogenous group on the same sampling day (ANOVA, $P \leq 0.05$).

3.3. *Heat shock increases mitochondrial H_2O_2 production*

Heat shock caused a significant increase in H_2O_2 production 100 min after treatment with temperatures between 40 and 43 °C (Fig. 3). An important question concerns the subcellular site of HS-dependent H_2O_2 production. The distribution of H_2O_2 in the cells was visualized *in vivo* by confocal microscopy in leaves treated with HS at 40 °C for 3.5 min. H_2O_2 (i.e., oxidised H_2DCFDA) is clearly seen within the cell, concentrating in rounded organelles about 1–2 μm in diameter, and co-localizing with MitoTracker Red, a marker for mitochondria. The merged image in Fig. 4 shows that mitochondria contain most H_2O_2 in the cell, with no detection of H_2DCFDA oxidation outside the cell. The concentration of H_2O_2 in mitochondria strongly suggests that most H_2O_2 is produced in these organelles under the conditions of these treatments.

H_2O_2 production increases in plants growing under harmful environmental conditions (Mittler, 2002 and references therein). Moderate and transient increases in H_2O_2 steady state levels might be involved in acquisition of thermotolerance (Orozco-Cadenas and Ryan, 1999; Lopez-Delgado et al., 1998). Consistent with the operation of ROS as an acclimatizing physiological signal, spinach leaves receiving HS treatments showed an early increase of H_2O_2 production and an extension of postharvest life.

3.4. *Heat shock delays the increase in the oxidised state of water soluble antioxidants*

Water soluble antioxidant contents decreased during senescence of detached spinach leaves, while the lipid soluble α-tocopherol did not change during senescence, or with any of the HS treatments (Fig. 5A–C).

After 3 d of storage, all HS treatments prevented the increase in the DHA/AA ratio but not the drop in the AA content. Plants have evolved GSH-dependent or -independent pathways to keep a low DHA/AA proportion (Potters et al., 2004). HS treatments might have enhanced the operation of mechanisms for the recovery of the reduced forms of antioxidants, such as the ascorbate–glutathione cycle.

At the end of the period examined, on day 7, the antioxidant profile was different. AA content continued decreasing but HS-treated leaves reached an oxidised state similar to that of control leaves. The content of GSH was kept higher in 40 °C treated than untreated leaves and GSSG/GSH ratio, which increased in control leaves, was lower in 40 and 43 °C treated leaves. Although recovery mechanisms induced by HS still kept a low GSSG/GSH ratio, the senescence programme was already initiated.

Modifications in the contents and the redox state of soluble compounds such as AA or GSH, are considered as important sensor mechanisms providing physiological signals to the plant under changing environments (Noctor, 2006). Previous work demonstrated that dark storage of leaves causes decreases in the contents of both chlorophyll and antioxidants, and that the addition of AA may delay the initiation of senescence (Garg and Kapoor, 1972; Borraccino et al., 1994). The results presented here show that the loss of AA and its change to a more oxidised state were early events, even earlier than chlorophyll loss, and might play a role in the processes leading to the initiation of the senescence programme.

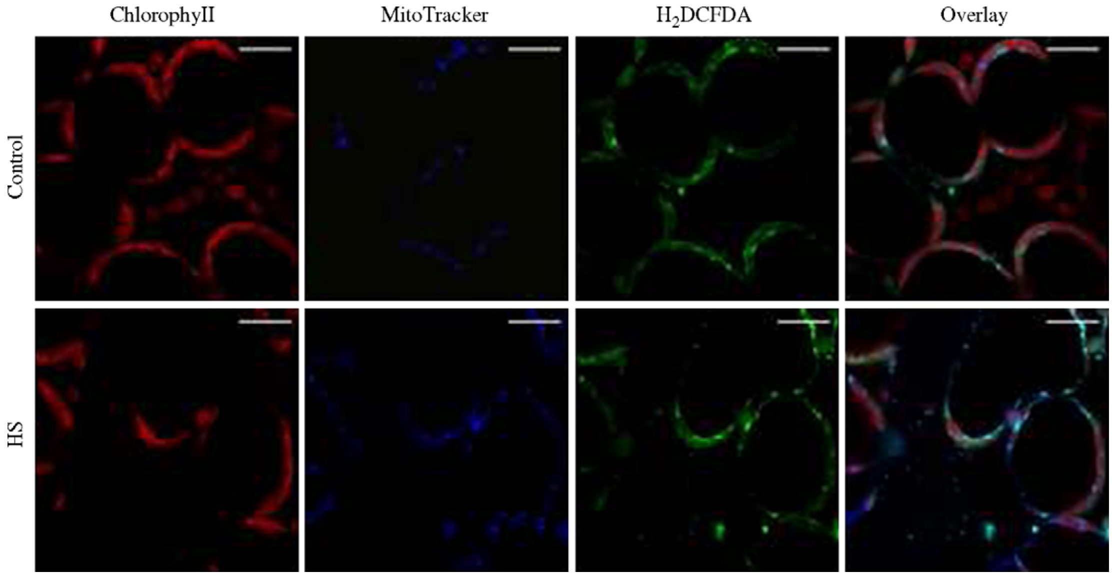

Fig. 4. *In vivo* subcellular H_2O_2 production in HS-treated spinach leaves. Leaves were immersed in water baths at RT or 40 °C for 3.5 min and leaf disks were taken for incubation in MitoTracker and H_2DCFDA. Samples were immediately observed by confocal microscopy after incubation for 5–15 min with the fluorescent probes. Bar = 15 μm.

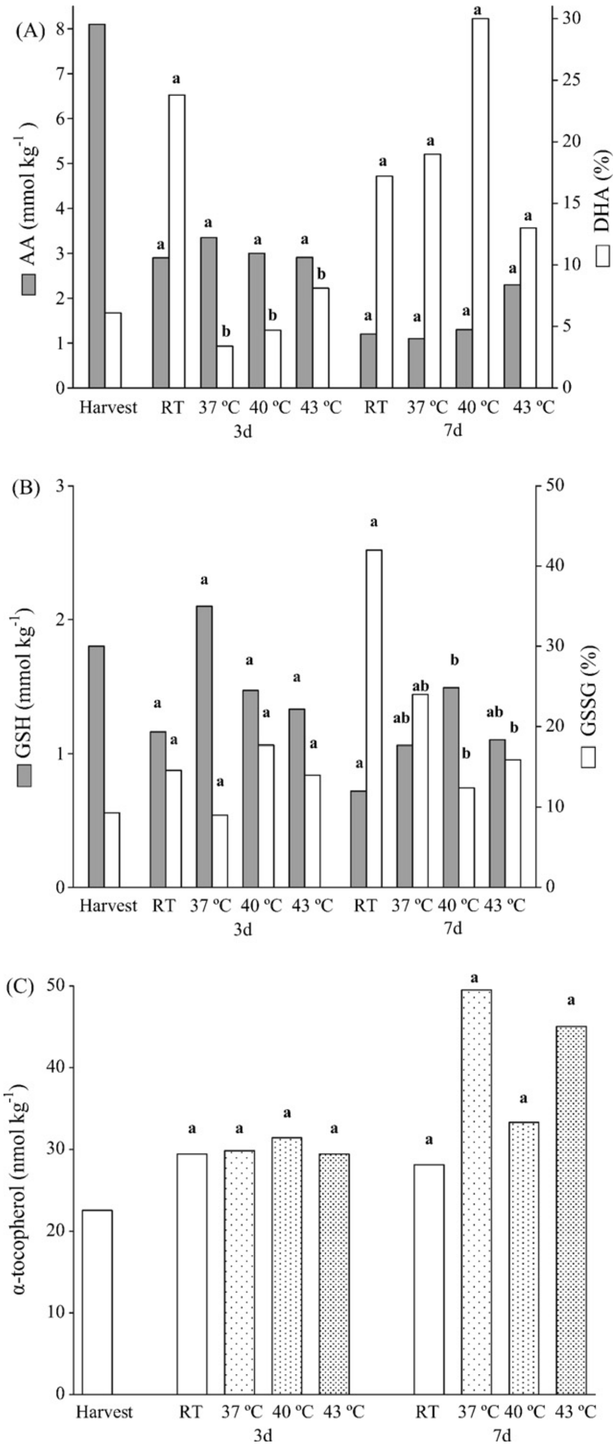

Fig. 5. Content and redox state of antioxidants during dark-induced senescence of HS-treated spinach leaves: AA and DHA (A), GSH and GSSG (B) and α-tocopherol (C). Leaves were treated with water baths at the indicated temperatures for 3.5 min, placed in polyethylene bags and stored in a dark chamber at 23 °C. Data are expressed on the basis of dry weight. Values are the mean of four-independent experiments with at least three replicates each. Data with same letters represent a statistically homogenous group on the same sampling day (ANOVA, $P \leq 0.05$).

α-Tocopherol (vitamin E) is a lipid soluble antioxidant that is mainly present in chloroplasts where it is synthesized (Foyer et al., 2006). The stability of α-tocopherol content, and the generation of ROS outside the chloroplast suggest that the oxidative metabolism of chloroplasts was not altered during dark-induced senescence.

4. Conclusions

The experiments presented here show that HS treatments between 37 and 43 °C for 3.5 min (i) delay the senescence of spinach leaves, (ii) increase mitochondrial H_2O_2 production and (iii) transiently prevent the increase of the oxidised state of water soluble antioxidants.

These results suggest that HS treatments, particularly at 40 °C, delay leaf senescence and might be used to improve postharvest storage life of spinach.

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