Industrial Crops & Products 138 (2019) 111469

Contents lists available at ScienceDirect

Industrial Crops & Products

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

Accumulation of rosmarinic acid and behaviour of ROS processing systems in *Melissa officinalis* L. under heat stress

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ARTICLE INFO

Keywords:
High temperature
Lemon balm
Soilless cultivation
Rosmarinic acid
Signalling systems
Antioxidants
Heat shock proteins

ABSTRACT

Heat stress (HS) due to increased air temperature is a major agricultural problem. On the other hand, short-term HS can represent a natural easy-to-use elicitor of bioactive compounds in plants. Similar elicitations can be induced by biotechnological approaches such as hydroponic cultures. The present study pioneering investigated the capability of using a short-term HS (38 °C, 5 h) as a tool to rapidly elicit rosmarinic acid (RA) content in leaves of *Melissa officinalis* L. (a species for which RA is the dominant active phenolic compound) hydroponic cultures, highlighting the cross-talk among antioxidant and signalling molecules involved in the heat acclimation. During HS treatment, we found an elicitation of RA biosynthesis associated with (i) an imbalance in reactive oxygen species (ROS) production and scavenging, (ii) an involvement of reduced ascorbate (AsA) in maintaining a high normal reduced state of cells, (iii) an induction of heat shock proteins (i.e. HSP101-like), and (iv) a stimulation of phytohormones. The RA biosynthesis lasted also during the recovery, although plants activated cellular processes to partially control ROS production, as confirmed by the increased activity of AsA regenerating enzymes, the accumulation of total carotenoids and the stimulation of total antioxidant capacity. The unchanged values of abscisic acid, ethylene and salicylic and jasmonic acids during the recovery phase also documented a reduced demand for protection. The present study represents a wide-ranging investigation of the potential use of HS (without drought interaction) as a technological application for improving bioactive compound production.

1. Introduction

Heat stress (HS) is defined as a condition of high air temperature (HT; i.e. 10–15 °C above ambient) for a sufficient time to induce a negative impact on plant development, growth and reproduction (Wahid et al., 2007). Plant species and genotypes have several capabilities to cope with HS, and the response depends on the intensity, duration and rate of temperature increase (Wahid et al., 2007). At very HT such as 10–15 °C above the ambient air temperature, severe cellular injury and even cell death occur within minutes, caused by a catastrophic collapse of cellular organization (e.g. protein denaturation/aggregation and increased fluidity of membrane lipids; Schöffl et al., 1999). At moderately HT, injuries or death may occur only after long-term exposure, which could be attributed to reduced cellular function and overall plant fitness (Driedonks et al., 2015).

In the plant-HS interaction, an important role is played by the accumulation of reactive oxygen species (ROS; Mittler, 2006). Usually, ROS production rapidly becomes excessive in plants subjected to HS (Pucciariello et al., 2012; Driedonks et al., 2015; Zhao et al., 2018), causing a cellular damage to membranes, organelles, DNA and denaturation/activation of proteins (Howarth, 2005). To prevent this cell damage and regain redox homeostasis, plants can trigger a heat stress response (HSR) by the hyper-activation of non-enzymatic and/or enzymatic ROS scavenging systems (Apel and Hirt, 2004; Halliwell, 2007; Foyer, 2018). The expression and protein level of genes responsible for ROS scavenging are also induced under HS in several plant species (Panchuk et al., 2002; Qiu et al., 2006; Driedonks et al., 2015), and has been associated to basal heat tolerance (Wahid et al., 2007).

Under HS, similarly to other oxidative stresses, the ROS processing system is not only a simple protection mechanism, but also represents a

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https://doi.org/10.1016/j.indcrop.2019.111469
Received 21 March 2019; Received in revised form 6 June 2019; Accepted 7 June 2019

signal for the modulation of other multiple responses (Berkowitz et al., 2016). Therefore, ROS are thought to be involved in the transduction of intra- and intercellular signals controlling gene expression and activity of anti-stress systems (Singh et al., 2019). Several studies documented that HS together with drought lead to an increase of abscisic acid (ABA) concentration that could regulate the acclimation process through the promotion of heat shock proteins (HSP; Larkindale and Knight, 2002; Liu et al., 2006). Asensi-Fabado et al. (2013) reported that prolonged HS (alone or in combination with water stress) induced the synthesis of ABA, salicylic acid (SA) and α-tocopherol in three *Labiatae* species.

Changing perspective, short-term stress conditions can also represent natural easy-to-use elicitors of bioactive compound production in plants. In the last years, many attentions have been given to enhance the production of plant secondary metabolites that are unique sources of pharmaceuticals, food additives, flavors and industrially important biochemicals (Ramakrishna and Ravishankar, 2011; Trivellini et al., 2016; Thakur et al., 2018). Among elicitors, several chemical or physical tools (i.e. signal compounds and/or abiotic factors) have been used. Recently, Khaleghnezhad et al. (2019) demonstrated that the combination of ABA application and HS treatment positively influences the accumulation of secondary metabolites in *Dracocephalum moldavica*. Biotechnological approaches (i.e. shoots, callus, cell suspension and root cultures; Petersen, 2013; D'Angiolillo et al., 2015) can also be used to trigger an array of defense or stress responses that improve the yield of secondary metabolites (Bertoli et al., 2013; Tonelli et al., 2015; Pellegrini et al., 2018; Mosadegh et al., 2018). Hydroponic cultures represent a good approach to investigate the effects of HS alone on plants, in contrast with many reports conducted with standard soil methods where HS was unavoidably combined with other related abiotic stresses (e.g. drought and salinity, Zandalinas et al., 2018).

Melissa officinalis L. (lemon balm) is an aromatic plant from the Mediterranean area, widely cultivated worldwide (Szabó et al., 2016). High quantities of secondary metabolites such as phenolic compounds, tannins and flavonoids (contained both in leaves and essential oils) were identified/quantified in *M. officinalis* and represent raw material for pharmaceutical, food, beverage, and cosmetic purposes (Moradkhani et al., 2010). Rosmarinic acid (RA), which is constitutively accumulated in field-grown plants as antimicrobial compound and as protection against herbivores (Szabo et al., 1999), is the main phenolic compound found in all organs of *M. officinalis*, with a level of about 6% of the dry weight (DW) in leaves (Petersen and Simmonds, 2003). For these reasons, as well as for its fast growth, *M. officinalis* has been exposed to abiotic stress to stimulate some bioactive compounds (e.g. RA, phenols, flavonoids, etc.). Ozone (O_3) exposure caused an alteration in leaf morphology and metabolism both *in vitro* (Tonelli et al., 2015; D'Angiolillo et al., 2015) and *in vivo* plants (Pellegrini et al., 2013) with an enhanced pattern of phenylpropanoids (e.g. phenols, anthocyanins, tannins, carotenoids and RA).

The present study pioneering investigated the capability of using a short-term HS as a tool to rapidly increase RA content in leaves of *M. officinalis* hydroponic cultures, highlighting the cross-talk among antioxidant and signalling molecules involved in the heat acclimation. The soilless cultivation allowed the determination of the heating effect, avoiding the crosstalk with drought stress. It is known that different combinations of stresses seem to influence the transcriptome analyses in *Arabidopsis thaliana*, while it cannot be predicted the response of one single stress factor (Rasmussen et al., 2013). Specifically, the purpose of this study was to answer the following questions: (i) Does HS elicit the biosynthesis of RA in *M. officinalis* hydroponic cultures? (ii) What is the behavior of ROS processing systems carrying the potential RA elicitation? (iii) What is the role of hormonal changes in the *M. officinalis*-HS interaction? We hypothesized that the short-period HS could elicit RA production as part of the heat acclimation consisting of a cross-talk among cellular processes and growth regulators tuned by a partial control of ROS production.

2. Materials and methods

2.1. Plant material, culture conditions and heat treatment

Four-week-old micropropagated shoots (Tonelli et al., 2015) were transferred to hydroponic cultivation using rockwool plug trays (Grodan® Pro Plug) with Hoagland modified nutrient solution (for further details, see supplementary material). The nutrient solution contained the following concentration of macronutrient and trace elements: NO_3^- 14 mM, NH_4^+ 0.5 mM, P 1.2 mM, K^+ 10 mM, Ca_2^+ 4.0 mM, Mg_2^+ 0.75 mM, Na^+ 10-01 mM, SO_4^{2-} 1.97 mM, Fe_2^+ 56 μM, BO_3^- 23.1 μM, Cu_2^+ 1.0 μM, Zn_2^+ 5.0 μM, Mn_2^+ 10 μM, MoO_4^- 1.0 μM. The electrical conductivity and pH of the nutrient solution were, respectively, 1.55–1.80 dS m^{-1} and 5.5–6 (adjusted with diluted H_2SO_4). Cultures were maintained in a growth chamber at 22 ± 1 °C, 60 ± 5% of relative humidity (RH) and under 16 h photoperiod of provided by cool white fluorescent tubes (Philips TLM 40 W/33RS) with 80 μmol m^{-2} s^{-1} photosynthetic active radiation (PAR).

After 14–20 days of hydroponic growing, uniformly sized plantlets were placed in a controlled environment fumigation facility under the same climatic conditions as the growth chamber, and then subjected for 5 h to HT (38 ± 1 °C). Shoot samples were collected at 0, 1, 2, 5 and 24 h from the beginning of treatment (FBT), instantly frozen in liquid nitrogen and stored at −80 °C until biochemical analyses.

2.2. Rosmarinic acid content

Rosmarinic acid content was determined following Tonelli et al. (2015). High performance liquid chromatography (HPLC) separations were performed using a PU-2089 four-solvent low-pressure gradient pump with a UV-2077 UV/Vis multichannel detector (Jasco, Easton, MD, USA). Analyses were performed using a Macherey-Nagel C18 250/4.6 Nucleosil 100-5 column, at a flow rate of 1 ml min^{-1}, equipped with a guard column, using acetonitrile (eluent A) and aqueous 0.1% H_3PO_4 (eluent B). Rosmarinic acid was detected at 325 nm and quantified on the basis of the integrated peak area, as compared with a standard curve. Further details are reported in supplementary materials.

2.3. ROS production, SOD, CAT and POD activity, lipid peroxidation and antioxidant capacity

Hydrogen peroxide production was estimated fluorimetrically using the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Molecular Probes, Invitrogen, Carlsbad, CA, USA), according to Shin et al. (2005). Spectrofluorimetric determinations were performed with a fluorescence/absorbance microplate reader (Victor3 1420 Multilabel Counter, Perkin Elmer, Waltham, MA, USA) at 530 and 590 nm (excitation and emission resofurin fluorescence, respectively). The superoxide radical ($O_2^{\cdot -}$) concentration was measured according to Tonelli et al. (2015), after extraction with K/P buffer (50 mM, pH 7.8), with a spectrophotometer (Jenway 6505 UV–vis, Cole-Parmer, Stone, UK) at 470 nm, after subtracting the background absorbance due to the buffer solution and to the assay reagents.

Enzymes were extracted from plantlet material (200 mg fresh weight, FW) with 2 ml of 50 mM sodium phosphate (Na/P) buffer (pH 7.0) containing 1.0 mM EDTA, 1.0 mM PMSF and 2.0% insoluble PVPP (w/v), according to Pistelli et al. (2017). Superoxide dismutase (SOD, EC 1.15.1.1) activity was assayed in terms of its ability to inhibit the photoreduction of NBT, according to the method of Beyer and Fridovich (1987). Spectrophotometer determinations were performed at 560 nm (Shimadzu UV-1800, Shimadzu Corporation, Milan, Italy). Catalase (CAT, EC 1.11.1.6) activity was assayed according to the method of Aebi (1984), by monitoring the decomposition of H_2O_2 for 1 min at 240 nm. Peroxidase activity was assayed according to the method of Hemeda and Klein (1990) by measuring the decomposition of H_2O_2 for

10 min at 470 nm. Ascorbate peroxidase (APX, EC 1.11.1.11) activity was assayed according to Nakano and Asada (1981), by measuring the oxidation of AsA at 290 nm for 1 min (at 25 °C).

Lipid peroxidation was determined by the thiobarbituric acid reactive substances (TBARS) method (Heath and Packer, 1968), after extraction with trichloroacetic acid (TCA; 0.1%, w/v). The malondialdehyde (MDA) concentration was determined at 532 nm corrected for nonspecific turbidity by subtracting the absorbance at 600 nm using a spectrophotometer (Jenway 6505 UV–vis, Cole-Parmer, Stone, UK).

The antioxidant properties were assessed spectrofluorimetrically by the Oxygen Radical Absorption Capacity (ORAC) and Hydroxyl Radical Antioxidant Capacity (HORAC) assays (Ou et al., 2001, 2002). Spectrofluorimetric determinations were performed with a fluorescence/absorbance microplate reader (excitation/emission = 485/527 nm and 485/520, respectively). The final ORAC values were calculated by using a regression equation between the Trolox concentration and the net area under the fluorescein (FL) decay curve. The final HORAC values were calculated using a regression equation between the standard antioxidant concentration and the net area under the curve. Further details about the investigation of ROS production, SOD activity, lipid peroxidation and antioxidant capacity are reported in supplementary materials.

2.4. Cell and membrane damage

For visualization of dead cells, Evans Blue staining was used according to the method of Keogh et al. (1980) with slight modifications. For determination of H_2O_2, fresh plantlet material was stained with DAB using a modification of the procedure described by Thordal-Christensen et al. (1997). Observations were performed under a light microscope (DM 4000 B, Leica, Wetzlar, Germany). Further details are reported in supplementary materials.

2.5. Enzymatic scavenging of ROS

Monodehydroascorbate reductase (MDHAR, EC 1.6.5.4) activity was assayed according to the method Huang et al. (2008) by measuring the oxidation of NADH for 1 min through the decrease in absorbance at 340 nm. Dehydroascorbate reductase (DHAR, EC 1.8.5.1) activity was assayed by measuring the production of AsA by dehydroascorbic acid (DHA) reduction at 265 nm for 1 min (at 25 °C), according to the method of Hossain and Asada (1984). Glutathione reductase (EC 1.6.4.2) activity was assayed according to the method of Foyer and Halliwell (1976) by monitoring the oxidation of NADPH by GSSG for 3 min (at 30 °C) through the decrease in absorbance at 340 nm. For all assays, proteins were determined according to Bradford (1976), using bovine serum albumin (BSA) as standard. Further details about the investigation of chloroplast and general enzymatic scavenging of ROS are reported in supplementary materials.

2.6. Non-enzymatic scavenging of ROS

Ascorbate and DHA content were measured spectrophotometrically, according to Kampfenkel et al. (1995), after extraction with TCA (5%, w/v). This assay is based on the reduction of ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}) with AsA in acid solution followed by formation of the red chelate between Fe^{2+} and 4,7-diphenyl-1,10-phenanthroline (bathophenanthroline) that absorbs at 525 nm.

Total and oxidized glutathione (GSSG) content were measured spectrophotometrically, according to Pellegrini et al. (2013), after extraction with TCA (5%, w/v). This assay is based on an enzymatic recycling procedure in which glutathione was sequentially oxidized by 5,50-dithiobis-2-nitrobenzoic acid and reduced by NADPH in the presence of glutathione reductase. All determinations were performed at 412 nm. Oxidized glutathione was determined after removal of reduced glutathione (GSH) from the sample extract by derivatization with 4-vinilpyridine. The amount of GSH was calculated by subtracting the GSSG amount, as GSH equivalents, from the total glutathione amount.

Proline (Pro) content was determined following Bates et al. (1973), after extraction with sulfosalicylic acid (3%, v/v). Spectrophotometric determinations were performed at 520 nm, using toluene as a blank.

Carotenoids were measured according to Cotrozzi et al. (2017) after extraction with HPLC-grade methanol. HPLC separations were performed at room temperature with a reverse-phase Dionex column (Acclaim 120, C18, 5 µm particle size, 4.6 mm internal diameter × 150 mm length). Further details about the investigation of chloroplast and general non-enzymatic scavenging of ROS are reported in supplementary materials.

2.7. Hormones and signalling molecules

Abscisic acid was determined by an indirect ELISA based on the use of DBPA1 monoclonal antibody, raised against S(+)-ABA, as described by Trivellini et al. (2011). The ELISA was performed following Walker-Simmons (1987), with minor modifications. Abscisic acid was measured after extraction in distilled water (water: tissue ratio = 10:1, v/w) overnight at 4 °C and quantified at 415 nm with an absorbance microplate reader (MDL 680, Perkin-Elmer, Waltham, MA, USA).

Fifteen min after excision, ET production was measured by enclosing one plantlet in an air-tight container (250 ml). Gas samples (2 ml) were taken from the headspace of containers after 1 h incubation at 22 °C. Ethylene concentration in the sample was measured by a gas chromatograph (HP5890, Hewlett-Packard, Ramsey, MN, USA) using a flame ionization detector, a stainless steel column (150 × 0.4 cm internal diameter packed with Hysep T). Analytical conditions were as follows: injector and transfer line temperature at 70 and 350 °C, respectively, and carrier gas nitrogen at a flow rate of 30 ml min^{-1} (Mensuali Sodi et al., 1992). Quantification was performed against an external standard.

Conjugated and free SA were determined according to Zawoznik et al. (2007), with minor modifications. HPLC separation was performed at room temperature with a Dionex column described above. SA was quantified fluorometrically (RF 2000 Fluorescence Detector, Dionex, USA), with excitation at 305 nm and emission at 407 nm and it was eluted using the mobile phase described above. The flow rate was 0.8 ml min^{-1}. Further details are reported in supplementary material.

For JA extraction, plantlets were added to 3 ml methanol and incubated overnight at 4 °C. HPLC separations were performed with the Dionex system and column described above. Analytical conditions were as follows: absorbance at 210 nm, mobile phase containing 0.2% (v/v) acidified water, and the flow rate was 1 ml min^{-1} (Kramell et al., 1999). Further details are reported in supplementary material.

2.8. Immunoblotting of hsp 101

Proteins were fractionated on a NuPAGE 10% Bis-Tris gel. Blotting was performed on a PVDF membrane, using a Trans-Blot Turbo Transfer System (Biorad, Milan, Italy). The chemiluminescent signal was detected Enhanced ChemiLuminuescence reagent (LiteAblot TURBO) and Biospectrum Imaging System (UVP, Analytik Jena, Upland, CA). Amido Black staining of total proteins on the PVDF membrane was performed using standard procedure. Proteomic analyses were performed at 0, 1, 2, 3, 4, and 5 h FBT. The immunoblotting was performed in duplicate showing similar results.

2.9. Statistics

Normality of data was preliminarily tested by the Shapiro-Wilk W test. The effects of high temperature and time were tested using two-way analysis of variance (ANOVA) and comparisons among means were determined by the Tukey's HSD post hoc test. Analyses were performed

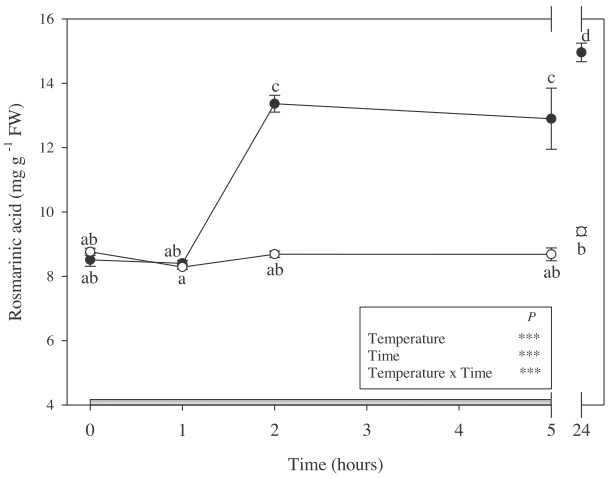

Fig. 1. Time course of rosmarinic acid content in leaves of *Melissa officinalis* exposed to high temperature (38 °C, 5 h, closed circle) or maintained at 22 °C (open circle). Data are shown as mean ± standard error. Measurements were carried out at 0, 1, 2, 5 and 24 h from the beginning of treatment. Boxes show the results of the full-factorial two-way ANOVA with temperature and time as variability factors (***: $P \leq 0.001$). According to the Tukey's HSD Post Hoc test, different letters indicate significant differences ($P \leq 0.05$). The grey bar indicates the temperature treatment (5 h). Abbreviations: FW, fresh weight.

by NCSS 2000 Statistical Analysis Systems Software (Kaysville, UT, USA).

3. Results

3.1. Rosmarinic acid content

High temperature significantly increased rosmarinic acid content starting to 2 h FBT and reaching a high value at 24 h FBT (+59% in comparison to controls; Fig. 1).

3.2. ROS production, SOD activity, lipid peroxidation and antioxidant capacity

The content of H_2O_2 and $O_2^{\cdot -}$ significantly increased under HT throughout the whole experiment. Both parameters reached their maximum already at 1 h FBT (about 2- and 4-fold higher than controls, respectively; Fig. 2 A–B). The activities of SOD, CAT and POD significantly increased under HT already at 1 h FBT (about 2-fold higher than controls, respectively; Fig. 2 C–E), and maintained similar increased levels throughout the whole period of the experiment (except for POD activity at 5 h FBT; Fig. 2 E). Membrane integrity was significantly affected by high temperature as confirmed by the MDA concentrations that were always higher in treated shoots than in controls, starting from 1 h FBT (+100%; Fig. 2 F). The ORAC values showed a similar trend than SOD, already increasing at 1 and 2 h FBT, and even more at 5 and 24 h FBT, when reached levels around 100% higher than controls (Fig. 2 G). Compared with controls, HORAC of treated plants significantly decreased at 1 h FBT (-63%), did not show differences at 2 and 5 h FBT, and decreased again at 24 h FBT (Fig. 2 H).

3.3. Cell and membrane damage

Throughout the whole experiment, leaves appeared macroscopically symptomless. However, HT-injuries were already detectable at the microscopic level just after 1 h FBT, as confirmed by the appearance of dead cells (Fig. 3 A–E). Histological staining showed local accumulation of H_2O_2 in HT-treated material at 5 h FBT, evidenced by reddish-brown areas (Fig. 3 F–J).

3.4. Enzymatic scavenging of ROS

High temperature strongly decreased APX activity already at 1 h FBT (about 2-fold lower than control), as well as at the other times of analysis (Fig. 4 A). Differently to APX activity, MDHAR and DHAR ones increased throughout the whole experiment under HT, except for DHAR activity at 5 h FBT. MDHAR activity especially peaked at 1 h FBT (+281%, compared with controls), and again at 24 h FBT (+49%; Fig. 4 B–C). The activity of GR increased under HT only at 5 h FBT (+65%, compared with controls), but resulted lower in treated material than in control at the end of the recovery period (i.e., 24 h FBT, -66%; Fig. 4 D).

3.5. Non-enzymatic scavenging of ROS

High temperature significantly increased AsA/DHA ratio and Pro content: AsA/DHA ratio started to increase at 1 h FBT and peaked at 2 h FBT (about 2-fold higher than controls; Fig. 5 A); Pro started to increase at 2 h FBT and reached its maximum at 5 h FBT (about 3-fold higher than control; Fig. 5 D). Both AsA/DHA ratio and Pro in treated plants came back to control levels at the end of the recovery period (i.e. 24 h FBT). Differently to AsA/DHA and Pro, GSH/GSSG ratio and total carotenoids (Tot Car) decreased under HT at 1 and 2 h FBT (around -15 and -20% for GSH/GSSG and Tot Car, respectively; Fig. 5 B–C). GSH/GSSG in treated plants recovered at the end of the treatment and remained at control levels at 24 h FBT (Fig. 5 B). Tot Car did not recover at the end of the treatment, but markedly increased at 24 h FBT (+19%, Fig. 5 C).

3.6. Hormones and signalling molecules

High temperature significantly increased all the examined hormones and signalling molecules only during the treatment period, although with a different timing among these molecules (Fig. 6). Abscisic acid reached its maximum at 1 h FBT (10-fold higher than control), slightly decreased at 2 and 5 h FBT, and reached control levels at 24 h FBT (Fig. 6 A). Ethylene started to increase at 2 h FBT, reached its maximum at 5 h FBT (2-fold higher than control) and dropped to control levels at 24 h FBT (Fig. 6 B). Salicylic acid peaked at 1 h FBT (+690%) and dropped later, reaching control levels at 5 and 24 h FBT (Fig. 6 C); JA slightly increased at 1 h FBT, reached its maximum at 2 h FBT (more than 3-fold higher than control), and came back to control values at 5 and 24 h FBT (Fig. 6 D).

3.7. Induction of heat shock protein Hsp101 under high temperature

It has been established that HSP101 plays a major role in thermotolerance (Queitsch et al., 2000), preventing deleterious effects of heat at the cellular levels. In this context, the level of Hsp101 protein reached a high level between 2 and 4 h FBT. Subsequently, the *M. officinalis* HSP101-like level decreased (Fig. 7).

4. Discussion

4.1. Heat stress elicits the biosynthesis of rosmarinic acid in Melissa officinalis hydroponic cultures

Plant tissue cultures can be considered a convenient and useful experimental system for (i) examining various factors influencing the biosynthesis of desired products and (ii) exploring effective biotechnologies to enhance their production without interference with pathogens and other microbes (Chattopadhyay et al., 2002). They are considered an alternative to the whole plant in relation to their capacity to produce homogeneous quality and quantity of secondary metabolites, independent of seasonal and geographical limitations (Xu et al., 2011). To enhance the yield of high-value secondary metabolites, plant

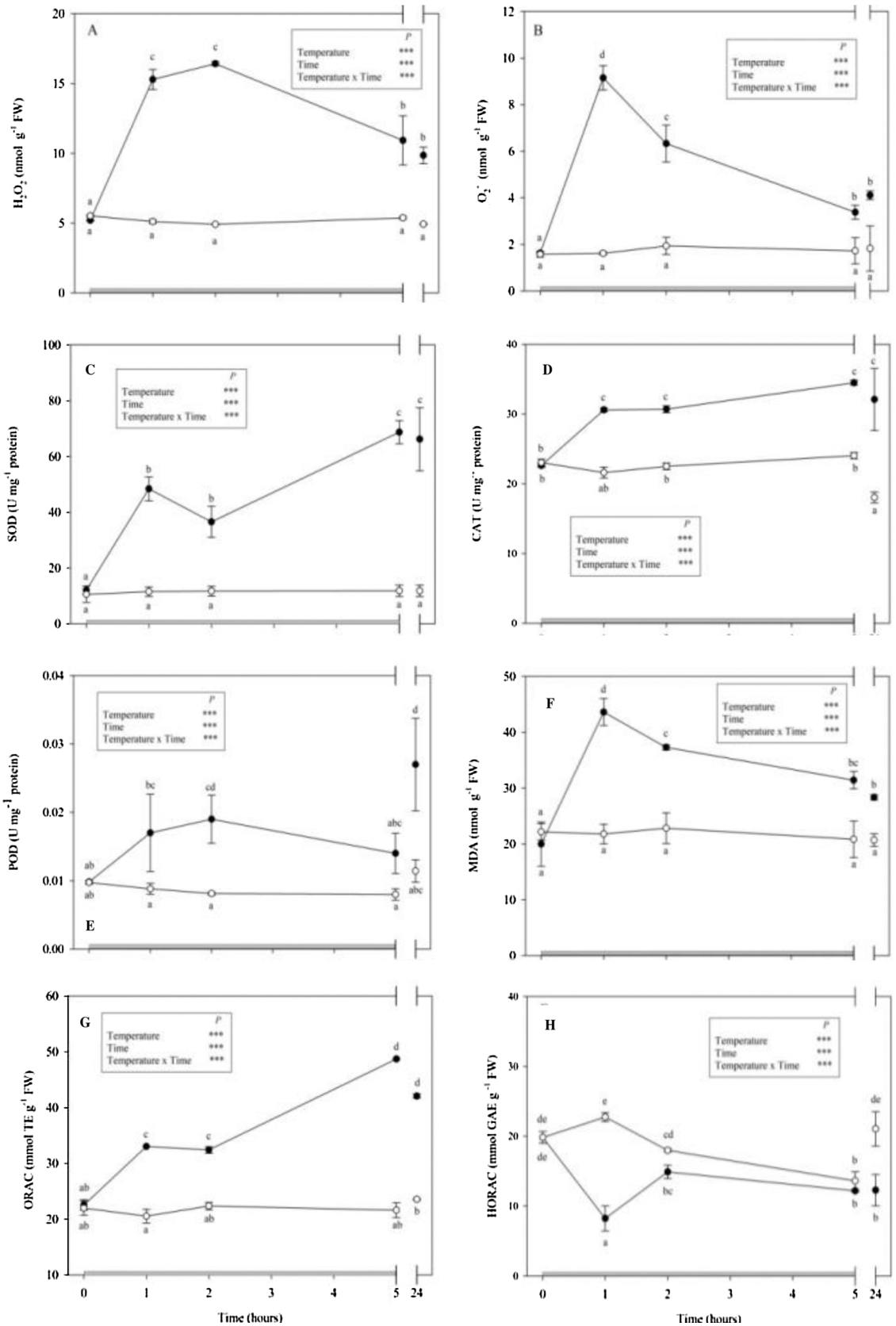

Fig. 2. Time course of hydrogen peroxide (H_2O_2, A), superoxide anion radical ($O_2^{\cdot -}$, B), superoxide dismutase activity (SOD, C), catalase (CAT, D), peroxidase (POD, E), malondialdehyde (MDA, F), antioxidant capacity expressed as oxygen radical absorbance capacity (ORAC, G) and hydroxyl radical antioxidant capacity (HORAC, H) in leaves of *Melissa officinalis* exposed to high temperature (38 °C, 5 h, closed circle) or maintained at 22 °C (open circle). Data are shown as mean ± standard error. Measurements were carried out at 0, 1, 2, 5 and 24 h from the beginning of treatment. Boxes show the results of the full-factorial two-way ANOVA with temperature and time as variability factors (***: $P \leq 0.001$). According to the Tukey's HSD Post Hoc test, different letters indicate significant differences ($P \leq 0.05$). The grey bar indicates the temperature treatment (5 h). Abbreviations: FW, fresh weight; GAE, gallic acid equivalents; TE, trolox equivalents.

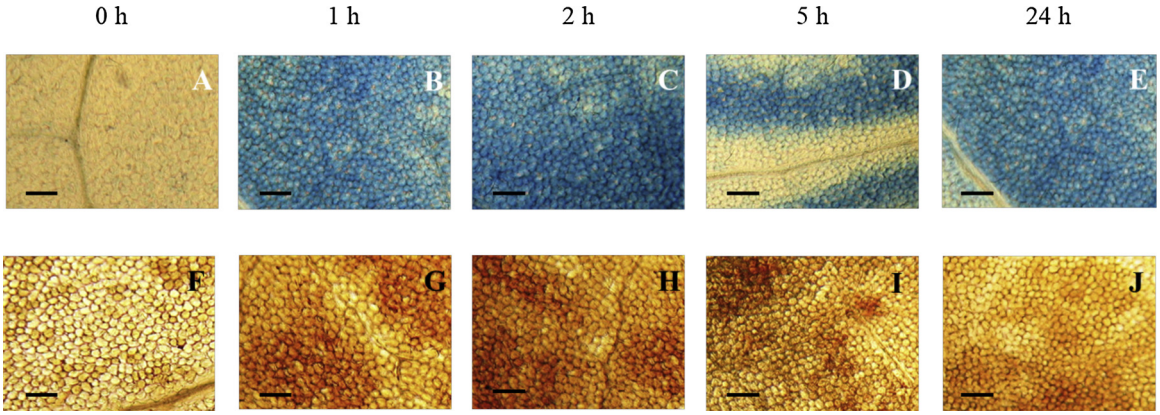

Fig. 3. Localization of dead cells visualized with Evans blue staining (A–E) and of hydrogen peroxide (H_2O_2) visualized with the 3,3′-diaminobenzidine (DAB) uptake method (F–J) in leaves of *Melissa officinalis* exposed to high temperature (38 °C, 5 h). The assays were performed at 0 (i.e. before starting the treatment), 1, 2, 5 and 24 h from the beginning of treatment. Bars: 50 µm.

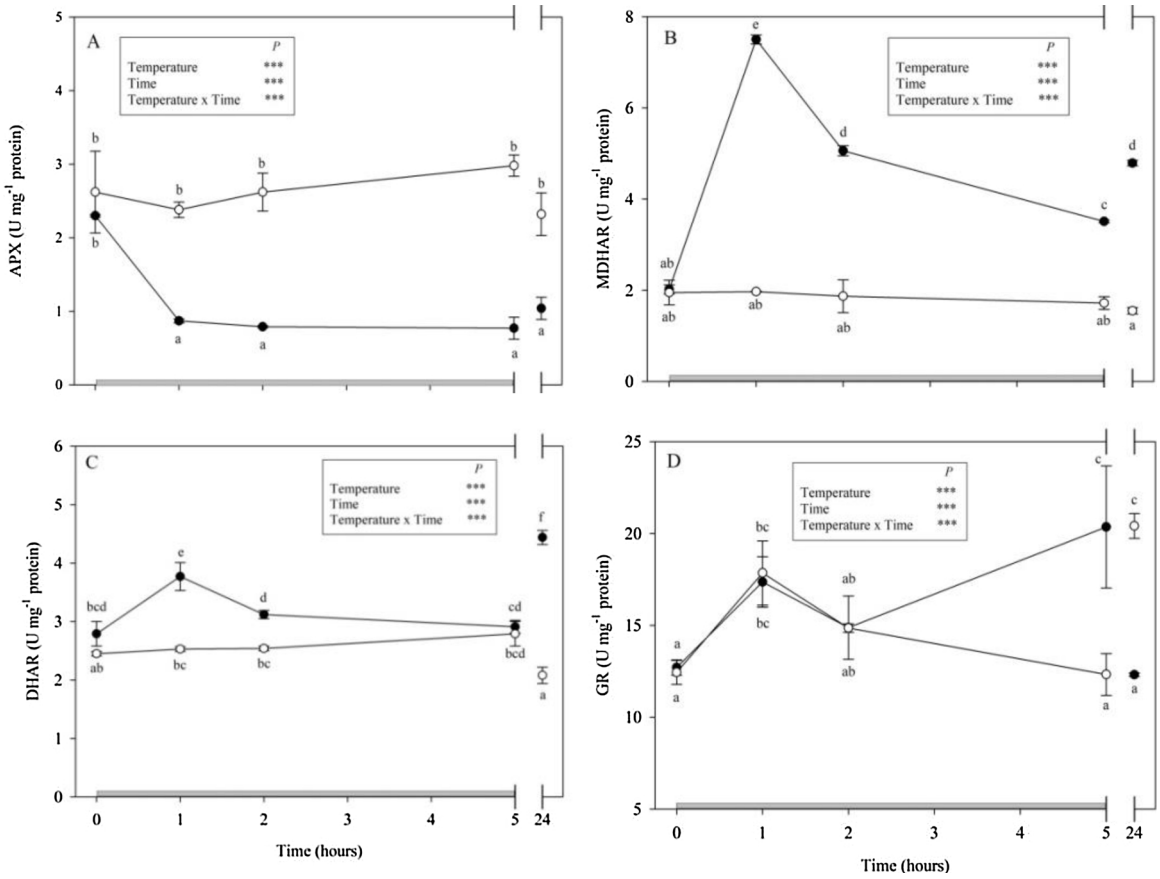

Fig. 4. Time course of ascorbate peroxidase (APX, A), monodehydroascorbate reductase (MDHAR, B), dehydroascorbate reductase (DHAR, C) and glutathione reductase (GR, D) activities in leaves of *Melissa officinalis* exposed to high temperature (38 °C, 5 h, closed circle) or maintained at 22 °C (open circle). Data are shown as mean ± standard error. Measurements were carried out at 0, 1, 2, 5 and 24 h from the beginning of treatment. Boxes show the results of the full-factorial two-way ANOVA with temperature and time as variability factors (***: $P \leq 0.001$). According to the Tukey's HSD Post Hoc test, different letters indicate significant differences ($P \leq 0.05$). The grey bar indicates the temperature treatment (5 h).

tissue cultures are manipulated using different strategies, such as the use of physical and chemical elicitors (Khan et al., 2018).

Among abiotic stress, it is well known that HS induces the biosynthesis of phenolic compounds (e.g. phenylpropanoids and flavonoids) and suppresses their oxidation (Rivero et al., 2001; Wahid et al., 2007). However, few reports have evaluated the impact of HS on individual metabolites (Fletcher et al., 2005; Khaleghnezhad et al., 2019). Here, our study shows a significant increase of RA content in *M. officinalis* hydroponic cultures under HT, from 2 h FBT until the end of the experiment (i.e. 24 h FBT; Fig. 1), identifying HT as a RA elicitor. Although Fletcher et al. (2005) reported that prolonged HS (30 °C day/night for 4 weeks) negatively regulated RA accumulation by causing a potential rapid biological breakdown of RA in *Mentha spicata* leaves, our outcome is in agreement with a number of previous studies focused on other stressors: the stimulation of RA by biotic (such as yeast elicitor) and abiotic elicitors (e.g. silver ions, methyl jasmonate and O_3) has been previously observed in cell cultures of several plants [e.g. *Lithospermum erythrorhizon* (Ogata et al., 2004), *Coleus blumei* (Petersen

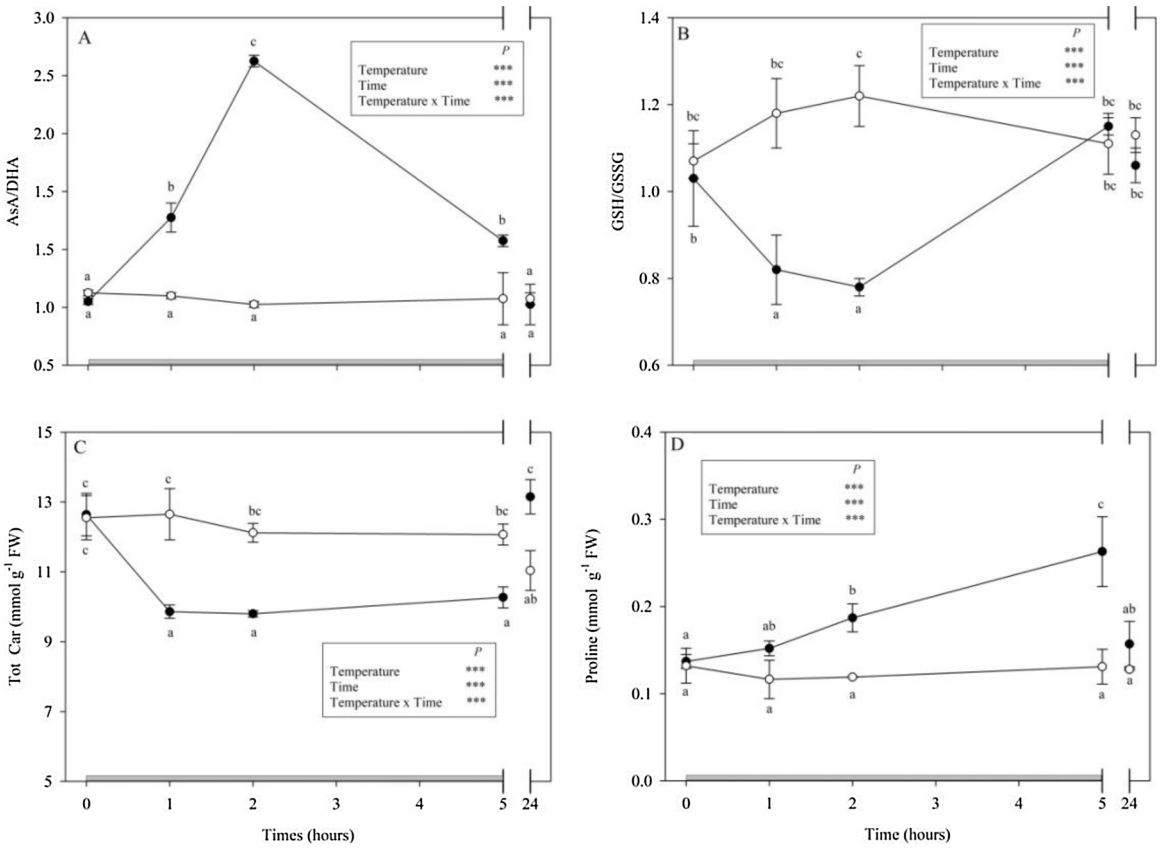

Fig. 5. Time course of reduced/oxidized ascorbate (AsA/DHA, A) and glutathione (GSH/GSSG, B) ratio, total carotenoids (Tot Car, C) and proline (D) in leaves of *Melissa officinalis* exposed to high temperature (38 °C, 5 h, closed circle) or maintained at 22 °C (open circle). Data are shown as mean ± standard error. The measurements were carried out at 0, 1, 2, 5 and 24 h from the beginning of treatment. Measurements were carried out at 0, 1, 2, 5 and 24 h from the beginning of treatment. Boxes show the results of the full-factorial two-way ANOVA with temperature and time as variability factors (***: $P \leq 0.001$). According to the Tukey's HSD Post Hoc test, different letters indicate significant differences ($P \leq 0.05$). The grey bar indicates the temperature treatment (5 h). Abbreviations: FW, fresh weight.

et al., 1994; Szabo et al., 1999), and *Salvia miltiorrhiza* (Yan et al., 2006; Zhao et al., 2010)], including *M. officinalis* (Tonelli et al., 2015).

4.2. ROS generation and scavenging during rosmarinic acid elicitation

One of the events occurring in response to HSs is ROS production (for a review see Suzuki and Mittler, 2006; Suzuki et al., 2014) and oxidative stress is involved directly or indirectly in the RA accumulation observed in our study. Although visible symptoms of oxidative stress were absent in plants subjected to HT, DAB staining and Evan's blue incorporation indicated that H_2O_2 deposition and cell death events occurred starting at 1 h FBT. The loss of membrane integrity was also confirmed by the significant build-up of MDA by-products observed starting at 1 h FBT, revealing that membrane lipid peroxidation occurred (Zhao et al., 2018). Both H_2O_2 and $O_2^{\bullet -}$ contents increased at the same time, confirming that an imbalance in ROS production and scavenging occurred (Demidchik, 2015). To deal with oxidative damage, plants have evolved different ROS processing systems that are able to respond to ROS production and to transmit the stress signals (Foyer, 2018). Chloroplasts contain several pathways that limit H_2O_2 accumulation and maintain cellular redox potential, including the Halliwell-Asada cycle (Foyer and Shigeoka, 2011). In our study, AsA/DHA ratio significantly and quickly increased under HT-treatment (Fig. 5), confirming the involvement of AsA in maintaining a high normal reduced state of cells, as well as in signalling and/or limiting the inhibitory effects of ROS-induced oxidative stress (Meyer, 2008; Pellegrini et al., 2013; Zou et al., 2016). The reduced state of AsA observed under HT treatment might be ascribable to (i) the activity of AsA regenerating enzymes, as confirmed by the increased activity of MDHAR and DHAR during the initial and recovery phases (Fig. 4) and (ii) the capability of converting GSSG in GSH, via the Halliwell-Asada cycle (Gill and Tuteja, 2010). The GSH/GSSG ratio (an indicator of general cellular redox balance) showed a marked decrease during the first two hours of HT treatment (Fig. 5; although GR activity remain unchanged), indicating that GSH plays a role in mediating the defense responses of plant cells to HS (Zou et al., 2016). Concomitantly, SOD, CAT and POD activities increase was observed in HT-treated shoots throughout the whole experiment, suggesting the subsequent reduction of superoxide to water. This response has been already detected in other plants to counteract the ROS production during HS condition (Suzuki et al., 2014; Zhao et al., 2018). Indeed, the levels of the oxidative-damage markers (i.e. ROS and MDA contents) decreased at the end of the treatment as well as after recovery, reaching values slightly higher than controls (similarly to SOD activity). These outcomes suggest that *M. officinalis* hydroponic cultures activated cellular processes to partially control ROS production (e.g. ROS contents never reached levels to cause visible symptoms) and to induce a heat acclimation. In this context, the protective function of HSP might occur only under the first hours of HS (i.e. from 2 to 4 h FBT), as suggested by the transient induction of HSP101-like in treated plants (Fig. 7). The activation of HSPs plays an essential role in preventing or minimizing the harmful effect of heat at the molecular level (Gullì et al., 2007). A signal cascade for HS response activated by HS Transcription Factors (HSF) has been extensively examined in promoting the transcription of HSP genes in abiotic stress conditions where ROS are produced (Guo et al., 2016). The subsequent heat acclimation included the prolonged RA elicitation

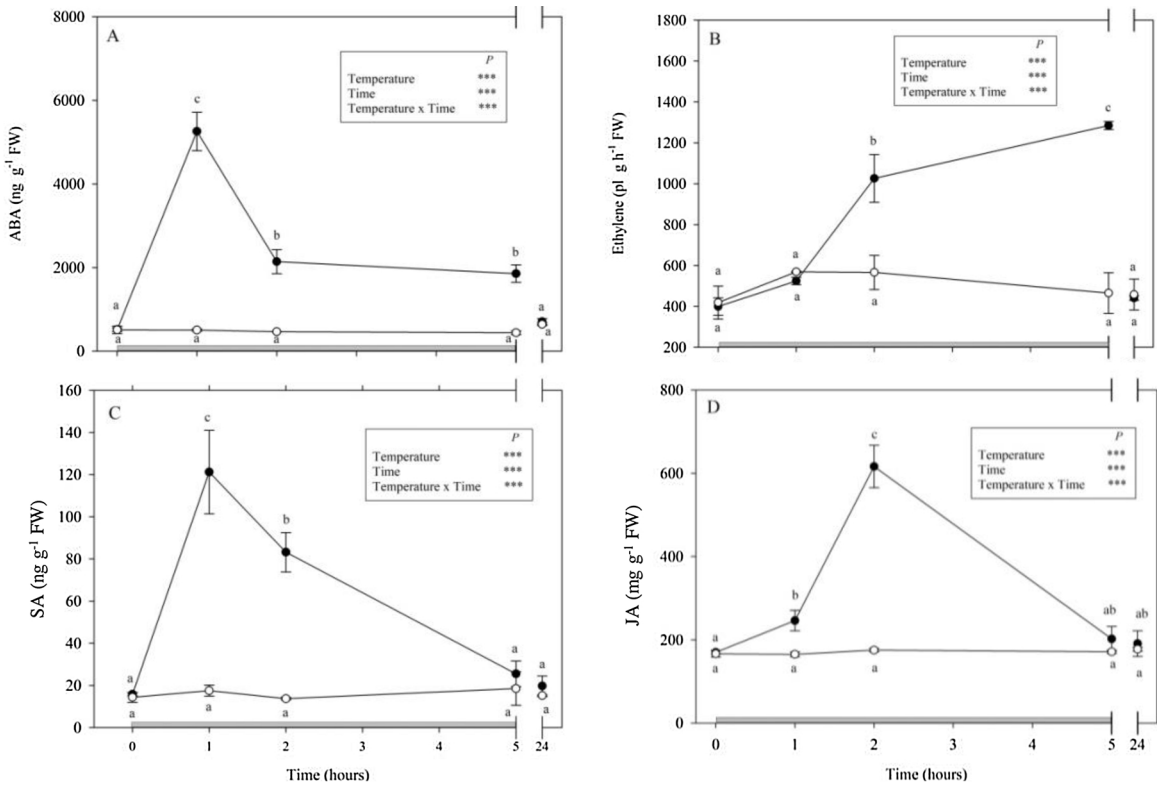

Fig. 6. Time course of abscisic acid (ABA, A) and ethylene (B), salicylic (SA, C) and jasmonic (JA, D) acids content in leaves of *Melissa officinalis* exposed to high temperature (38 °C, 5 h, closed circle) or maintained at 22 °C (open circle). Data are shown as mean ± standard error. Measurements were carried out at 0, 1, 2, 5 and 24 h from the beginning of treatment. Boxes show the results of the full-factorial two-way ANOVA with temperature and time as variability factors (***: $P \leq 0.001$). According to the Tukey's HSD Post Hoc test, different letters indicate significant differences ($P \leq 0.05$). The grey bar indicates the temperature treatment (5 h). Abbreviations: FW, fresh weight.

that was maintained also during the recovery phase.

Among other non-enzymatic processing systems, carotenoids are pigments that play a multitude of functions in plant metabolism including photoprotection, prevention of peroxidative damage to the membrane lipids, and ROS scavenging (Havaux, 1998). In our study, Tot Car content decreased starting from 1 h FBT, but it significantly increased at the recovery time, suggesting that these metabolites could be consumed by the cell to minimize the possible ROS generation and stabilize the lipid phase of the thylakoid membranes (as confirmed by H_2O_2, $O_2^{\bullet -}$ and MDA levels returned slightly higher than controls after the HT treatment; Sharma et al., 2012).

A key adaptive mechanism in many plants grown under abiotic stress is the production of organic compounds of low molecular mass involved in osmotic adjustments (Hare et al., 1998). Proline not only facilitates water uptake, but also protects cells against ROS accumulation under stress conditions by recycling of NADPH via its synthesis from glutamate and acting as a free radical scavenger (Soares et al., 2018). In our study, Pro accumulation was observed at 2 and 5 h FBT, indicating that also this compound likely participated in the reduction of oxidative damage by buffering cellular redox potential (Verbruggen and Hermans, 2008) and enhancing photochemical electron transport activity (Zhao et al., 2018).

To further evaluate the ability of ROS processing systems to provide defense and regenerate the active reduced forms, we also analyzed the total antioxidant capacity of *M. officinalis* hydroponic cultures. According to the ORAC assay, the antioxidant capacity was significantly enhanced under HT throughout the entire period of the experiment, confirming the marked free-radical scavenging ability of antioxidants (in particular against peroxyl radical) in treated shoots (Pellegrini et al., 2013). According to the HORAC method, instead, the antioxidant capacity initially decreased at 1 h FBT, did not change at 2 and 5 h FBT, and decreased again at the recovery time. This outcome indicates the reduced radical prevention ability of treated shoots (expressed as metal-chelating properties of antioxidants; Ou et al., 2002; Marchica et al., 2019). It is worth to note that ORAC and HORAC assays measure two different, but equally important, aspects of antioxidant properties

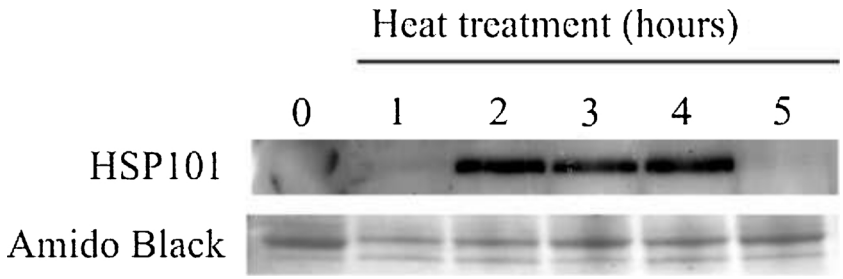

Fig. 7. Immunoblotting of proteins extracted from hydroponic cultured of *Melissa officinalis* exposed to high temperature (38 °C, 5 h). The antibodies used recognized HSP101. Blots were stained with Amido Black to confirm loading and transfer.

(radical chain breaking and radical prevention, respectively). Consequently, it is expected that the samples with high ORAC values do not necessarily have high HORAC values, and vice versa (Ou et al., 2002).

4.3. Hormonal changes in M. officinalis under heat stress

Several studies have documented that HS can induce an alteration of hormonal homeostasis by modifying the biosynthesis and/or the compartmentalization of the main signalling molecules such as ABA, ET, SA and JA (Maestri et al., 2002; Wahid et al., 2007). Abscisic acid is a ubiquitous phytohormone with a key role in the tolerance to stressful conditions that quickly affect plant water balance through the regulation of stomatal closure (Basu and Rabara, 2017). In the present study, the variation of ABA, which severely raised at 1 h FBT and then decreased at 2 and 5 h FBT, and finally reached control levels at the recovery time, confirms the involvement of this phytohormone in biochemical pathways essential for triggering HS-acclimation during the initial phase of *M. officinalis* response to HT treatment (Kurepin et al., 2008; Asensi-Fabado et al., 2013). Interesting recent results demonstrated that the combination of HS treatment and exogenous ABA application positively influences the accumulation of RA in dragonhead plants (Khaleghnezhad et al., 2019). Conversely, ET production increased under HT-treatment only at 2 and 5 h FBT, indicating that this gaseous hormone likely took part in consecutive events to regulate HT-tolerance and to protect against HT-induced oxidative stress (Suzuki et al., 2005; Asensi-Fabado et al., 2013). Ethylene, SA and JA are important components of signalling pathways involved in response to abiotic stresses (UV, O_3 and heat; Kohli et al., 2013; Pellegrini et al., 2013, 2016; Cotrozzi et al., 2017; Landi et al., 2019). Salicylic acid has been found to mediate heat tolerance through increases in antioxidant enzyme activities and heat-induced oxidative stress alleviation (Larkindale and Knight, 2002; Pan et al., 2006; Liu et al., 2006). Few studies to date have investigated the implications of JA in heat tolerance; however, some lines of evidence suggest that this compound is involved in the regulation of HS tolerance in *Arabidopsis* (Kazan, 2015). In our study, the concomitant increase of SA and JA levels observed during the first two hours of HT (SA peaked at 1 h FBT, whereas JA peaked at 2 h FBT) confirms that a multifactorial regulation of shoot HT-acclimation occurred (Clarke et al., 2009). Interestingly, the unchanged values of ABA, ET, SA and JA at the recovery time might be related to a reduced demand for protection, suggesting a key role for these hormones in HT-acclimation signalling causing the above-described ROS regulation and RA elicitation (Clarke et al., 2004).

In conclusion, our study shows that short-period HT is a valuable tool to improve the production of high-value secondary metabolites in *M. officinalis* shoots. At the timing and level utilized in this study, HT induced a cross-talk between cellular processes and growth regulators which induced a partial control of ROS production, and leaded to an HT-acclimation involving a RA elicitation, without causing macroscopic damage to the plants. The present study represents a pioneering and wide-ranging investigation of the potential use of HT (without drought interaction) as a technological application for improving bioactive compound production.

Acknowledgments

We gratefully acknowledge Dr. Rita Maggini for the RA determinations. Mr. Andrea Parrini supervised the growth chamber.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.indcrop.2019.111469.

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