EFFECTS OF ABIOTIC FACTORS ON INTERNAL HOMEOSTASIS OF *MENTHA SPICATA* LEAVES

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Abstract. *Mentha spicata* plants were subjected to various abiotic stresses in order to evaluate the triggering of signals and the phenolic compound levels of leaves. Total phenolic compound levels exhibited specific responses to a particular abiotic stress factor that responded rapidly to drought exposure, light intensity, and with a slight change in response to salt stress and heat stress. A parallel change was found in an elevation in hydrogen peroxide (H_2O_2) contents. Change in anthocyanin and carotenoid contents in response to drought, different light intensities, salinity and temperature stress was encountered with a change in H_2O_2. A change in the malondialdehyde level was also exhibited, depending on the levels of oxidant and antioxidant availability during particular abiotic stress factors. This risk was accomplished by the regulation of phenylalanine ammonia lyase activity. Prolonged exposure showed a reduction in chlorophyll content. Higher anthocyanin, carotenoid, H_2O_2 and total phenolic compound levels were expressed in the plasticity of plant acclimation at different levels. The tolerance of abiotic stress factors is apparently closely associated with the non-enzymatic antioxidant system via metabolome readjustment. We suggest that biosynthesis of these compounds is tightly regulated, reflecting plant plasticity when acclimating to different types of abiotic stress.

Keywords: *acclimation, climate changes, phenylalanine ammonia lyase, signaling molecules, stresses*

Introduction

Spearmint (*Mentha spicata* L.) belongs to the Lamiaceae family. It is an aromatic plant cultivated as a crop for their secondary metabolites products. Hence, is widely used in the food products, cosmetics and pharmaceuticals industries (Elmastas et al., 2005; Snoussi et al., 2015). In addition, spearmint leaf and oil are used for folk medicine (Nozhat et al., 2014; Snoussi et al., 2015).

Spearmint leaf contains high contents of phenolic compounds and hence could possess strong antioxidant activities among other different antioxidant compounds such as anthocyanin, carotenoids, and ascorbic acids (Kaur and Kapoor, 2002; Qader et al., 2011; Chon et al., 2012; Cao et al., 2015). Moreover, the high phenolic contents consider a promising source of bioactive secondary metabolites in the food industry and in human health (Kaur and Kapoor, 2002; Mimica-Dukic and Bozin, 2008; Soni et al., 2015).

Environmental factors including abiotic and biotic are key to spearmint growth due to the perennial nature of the crop. These variables have a direct impact on biochemical pathways, accordingly affecting the metabolism of secondary metabolites products (Muniram et al., 1992; Edwards et al., 2000; Fletcher et al., 2010). Furthermore, plant tolerance and minimizing the damage caused by stress are based upon stress perception,

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activation of complex signaling pathways in order to respond in a rapid and efficient manner for coping effectively with abiotic and biotic stimuli (Jones and Dangl, 2006; Rejeb et al., 2014). Signaling pathways induced by stress involve the accumulation of metabolites and positively affects a plant's response to stress. These metabolites include reactive oxygen species (ROS), phytohormones, phenolic compounds, ion flux (like $Ca^{2+)}$, activation of mitogen-activated protein kinases (MAPKs), protein phosphorylation and others (Dixon and Paiva, 1995; Lattanzio et al., 2006; Arbona et al., 2013; Baxter et al., 2014; Rejeb et al., 2014).

Review of literature

Abiotic environmental stresses activate a multiple response resulting in changes in primary and secondary metabolites production. Accumulation levels of secondary metabolites including phenolic compounds are controlled in response to environmental cues (Dixon and Paiva, 1995; Payyavula et al., 2012). These compounds are synthesized through derivatives of the pentose phosphate, shikimate, and phenylpropanoid pathways in plants and are stress induced (Randhir et al., 2004). Phenylalanine ammonia lyase (PAL) is the key enzyme in the biosynthesis of phenylpropanoid compounds (Solecka, 1997). These include wide ranges of secondary metabolites (Dixon and Paiva, 1995; LaCamera et al., 2004). Phenylpropanoids have important functions in several different pathways in: plant defense against pathogens and predators; protection from UV irradiation; signal transduction and communication with other organisms; and as regulatory molecules (Dixon and Paiva, 1995; Ferrer et al., 2008; Vogt, 2010). Phenylalanine ammonia lyase regulates entry into the phenylpropanoid pathway and is influenced by environmental stimuli, including pathogens, UV radiation, light intensity, temperature and drought, among others (Dixon and Paiva, 1995; Yoshioka et al., 1996; Rivero et al., 2001; MacDonald and D'Cunha, 2007).

Abiotic environmental stresses particularly drought and salinity have the most effect on spearmint growth and production. Moreover, it had shown is sensitive to water stress (Delfine et al., 2005; Telci et al., 2010; Okwany et al., 2012). Furthermore, this sensitivity was experienced through photosynthetic performance changes included stomatal conductance, chlorophyll fluorescence, and chlorophyll content. In addition, all of which affects photosynthetic capacity and eventually productivity in general (Delfine et al., 2005). Hence, the biosynthesis of secondary plant metabolites significantly increases within a plant species under abiotic stressful conditions (Selmar and Kleinwachter, 2013; Stavroula and Rahul, 2017). For that, spearmint has significant antioxidant activity (Elmastas et al., 2005).

M. spicata has been reported to have high phenolic contents among other studied vegetables (Kaur and Kapoor, 2002). The aim of the present work was the examine the biochemical changes of spearmint leaf tissue due to environmental effects. To achieve this aim, the assessment of the alterations in anthocyanins, carotenoids, H_2O_2, MDA, total phenolic compounds, chlorophyll contents and PAL activity of *M. spicata* leaves due to the insults of abiotic environmental factors (drought, salinity, heat shock and light quantity) in time dependent manner to visualize the attempt of these biochemical changes to protect leaves tissues against these insults.

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Materials and methods

Plant materials and growth conditions

Spearmint (*M. spicata* L.) was grown under controlled conditions (14 h light with~ 54 μE, and 21 °C/10 h dark and 20 °C, 55-60% relative humidity) in 2:1:1 peat moss, perlite and vermiculite. Trays filled with cultured pots were transferred to a growth chamber with controlled conditions and after 6 weeks of the process of germination, the plants were exposed to one of the following types of abiotic stress, i.e., light, salinity, temperature, and drought, with different lengths of exposure, relative to the abiotic stress type, or kept in growth chamber conditions without any changes. After the specified time periods of exposure to specific abiotic stress conditions the samples were analyzed in order to investigate the physiological changes in response to the type, duration and intensity of abiotic stress.

Abiotic stress treatments

For drought treatment, plants were irrigated with tap water three times a week for a period of six weeks of growth under controlled conditions. Then the plants were introduced to a water deficit continuing on for three time periods (3 days, 6 days or 9 days), withholding water. At the end of each specific drought exposure plant sample leaves were collected directly in liquid nitrogen in plant growth chamber and stored at -80 °C until further analysis (Egert et al., 2013). Controlled samples were irrigated continually three times a week for a further period (3, 6 or 9 days) and again after the plant reached a period of six weeks' growth under controlled conditions. Salinity treatment was of 6-week-old plants were irrigated with NaCl solution (100 mM) three times per week for up to 14 days. Samples collected in interleaves started at 2 days, 6 days, 10 days and 14 days in a growth chamber (Nishizawa et al., 2008). Controlled samples continued to be irrigated three times a week for a further 2 days, 6 days, 10 days and 14 days with tap water under controlled growth conditions. Heat shock was imposed by transferring 6-week-old plants to grow at 4 °C (cold), 25 °C, 30 °C and 35 °C. Samples collected in interleaves started after 2 h, 4 h and 6 h of incubation at the desired temperature (Egert et al., 2013). Finally, 6-week-old plants were grown at light quantity ≈54 μmol photon/m^2s (control), then light quantity treatment plants were then transferred to grow under 10 μmol photon/m^2s, 100 μmol photon/m^2s, or 1000 μmol photon/m^2s. Samples collected in interleaves started after 2 h, 6 h, 12 h and 24 h of growth under the desired light quantity described by Oelze et al. (2011).

Quantification of chlorophylls, anthocyanins and carotenoids

Chlorophylls quantification was performed according to Porra (2002). In briefly, 20 mg of the tissue samples were ground in 1 ml cold 80% acetone followed by 1 h darkness incubation then centrifugation at 13,000 rpm, 4 °C for 10 min. The supernatant was read on 646.6 and 663.6 nm.

Cold methanol/HCl/water (90:1:1, vol:vol:vol) was used as extraction solution of anthocyanins and carotenoids. Sims and Gamon (2002) was used for quantification of anthocyanins and carotenoids contents.

Measurement of H_2O_2

The leaf hydrogen peroxide content was analyzed as described by Christou et al. (2013) with minor modification. 0.1 g frozen leaf material was thoroughly mixed on ice with 0.1%

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(w/v) trichloroacetic acid. The homogenate was then spinning at 15,000 *g* for 15 min at 4 °C. In a new tube, 500 µl of the supernatant was collected to 500 µl of 10 mM potassium phosphate buffer (pH 7.0) and 1 ml of 1 M KI and mixed gently. The absorbance of the assay mixture was read at 390 nm and the content of H_2O_2 was calculated based on a standard curve of known concentrations of H_2O_2.

Lipid peroxidation

The level of lipid peroxidation was analyzed to determine malondialdehyde (Hodges et al., 1999). Briefly, 50 mg of aerial tissue was homogenized in 1 mL of 80% (v/v) ethanol on ice. The homogenate was centrifuged at 16,000 g for 20 min at 4 °C and 0.5 mL of supernatant was mixed together with 0.5 mL of 20% (w/v) trichloroacetic acid containing 0.65% (w/v) thiobarbituric acid. The mixture was incubated at 95 °C for 30 min and then immediately cooled in an ice bath. After centrifugation at 10,000 g for 10 min, the absorbance of the supernatant was measured at 532 nm, subtracting the value for nonspecific absorption at 600 nm. The MDA concentration was calculated from the extinction coefficient 155 mM^{-1} cm^{-1}.

Protein quantification

Protein extracts were determined based on the Bradford method (1976). Briefly, 50 mg of aerial tissue was homogenized in 1 mL of 100 mM HEPS buffer (pH 7.6) on ice. The homogenate was centrifuged at 13,000 rpm for 10 min at 4 °C. 0.2 mL aliquot of supernatant was mixed with 1 ml Bradford reagent, then the absorbance of the supernatant being measured at 595 nm. The content of protein was calculated based on a standard curve of known concentrations of BSA.

Determination of total phenolic content

Total phenolics were determined using the Folin-Ciocalteu (Singleton and Rossi, 1965) reagent and as described by Kaur and Kapoor (2002). 2 g Tissue samples were homogenized in 80% aqueous ethanol at room temperature and centrifuged in cold at 10,000 g for 15 min after which the supernatant was saved. The residue was re-extracted twice with 80% ethanol and the supernatants were pooled, put into evaporating dishes, and evaporated to dryness at room temperature. The residue was dissolved in 5 mL of distilled water. One-hundred micro liters of this extract were diluted to 3 mL with water and 0.5 mL of Folin-Ciocalteu reagent was added.

After 3 min, 2 mL of 20% of sodium carbonate was added and the contents were mixed thoroughly. The absorbance measured at 650 nm with UV/VIS split beam spectrophotometer (SPUV-16, SCO, Germany) for 60 min using catechol as a standard. The results were expressed as mg catechol/100 g of fresh weight material.

Assay of PAL activity

PAL activity in the leaf extract was determined according to Campos et al. (2004). Briefly, 0.2 ml of the protein extract was incubated at 40 °C for 30 min with 2 ml of 50 mM borate buffer (pH 8.5) and 110 µl of 100 mM l-phenylalanine (pre-dissolved in 50 mM borate buffer pH 8.5). Before doing the absorbance readings, the proteins were precipitated by adding TCA at 4% (w/v). The samples with TCA were incubated for 5 min at room temperature and then centrifuged at 10,000 g for 5 min. The absorbance

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of the supernatant was read at 290 nm at the beginning and after the incubation time. PAL activity was calculated as µmol of cinnamic acid per min and mg protein under the specified conditions.

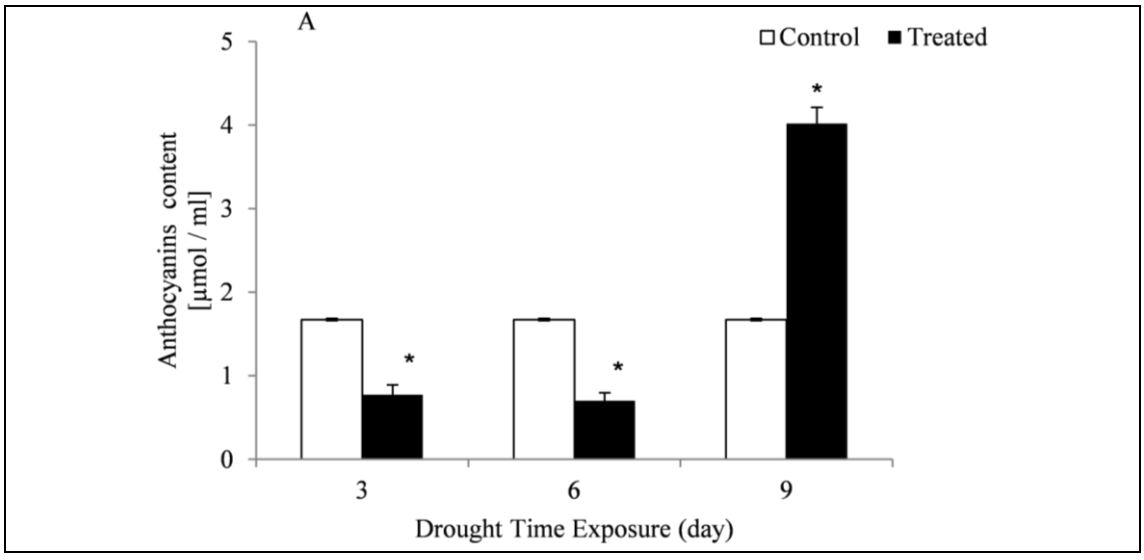
Statistical analysis

For all experiment, samples were analyzed and all the assays were carried out in triplicate. Results are expressed as mean± SD. The comparison between two samples was performed by student's *t*-test and the *P* value of ≤ 0.05 was considered as significant.

Results

Effects on anthocyanin and carotenoid contents

Anthocyanin contents increased significantly 2.3 fold over the control in plants harvested only after nine days of drought treatment. The contents increased significantly in plants exposed to different light regimes (10 µE, 100 µE and 1000 µE) compared with the control (54 µE). However, the rising in content depends on the light quantity and duration of exposure. A significant 4 folds increase in contents was detected in plants exposed to 1000 µE among all time points. 100 µE light exposure showed two points of increments; first after 2 h and the second rising which was mostly constant after the next time points of detections. The behavior of anthocyanins content after 10 µE light exposure showed high elevation after 2 h reaching 7 folds of increment then the rest time points showed around 3 folds of increment. The highest contribution to salinity treatment showed significantly increment reached 1.7 and 1.5 fold after ten and fourteen days of exposure, respectively when compared with the control. Anthocyanin contents were significantly increased after 4 °C treatment at $t = 4$ and after 35 °C at $t = 2$ when compared with the control (25 °C; *Fig. 1*).



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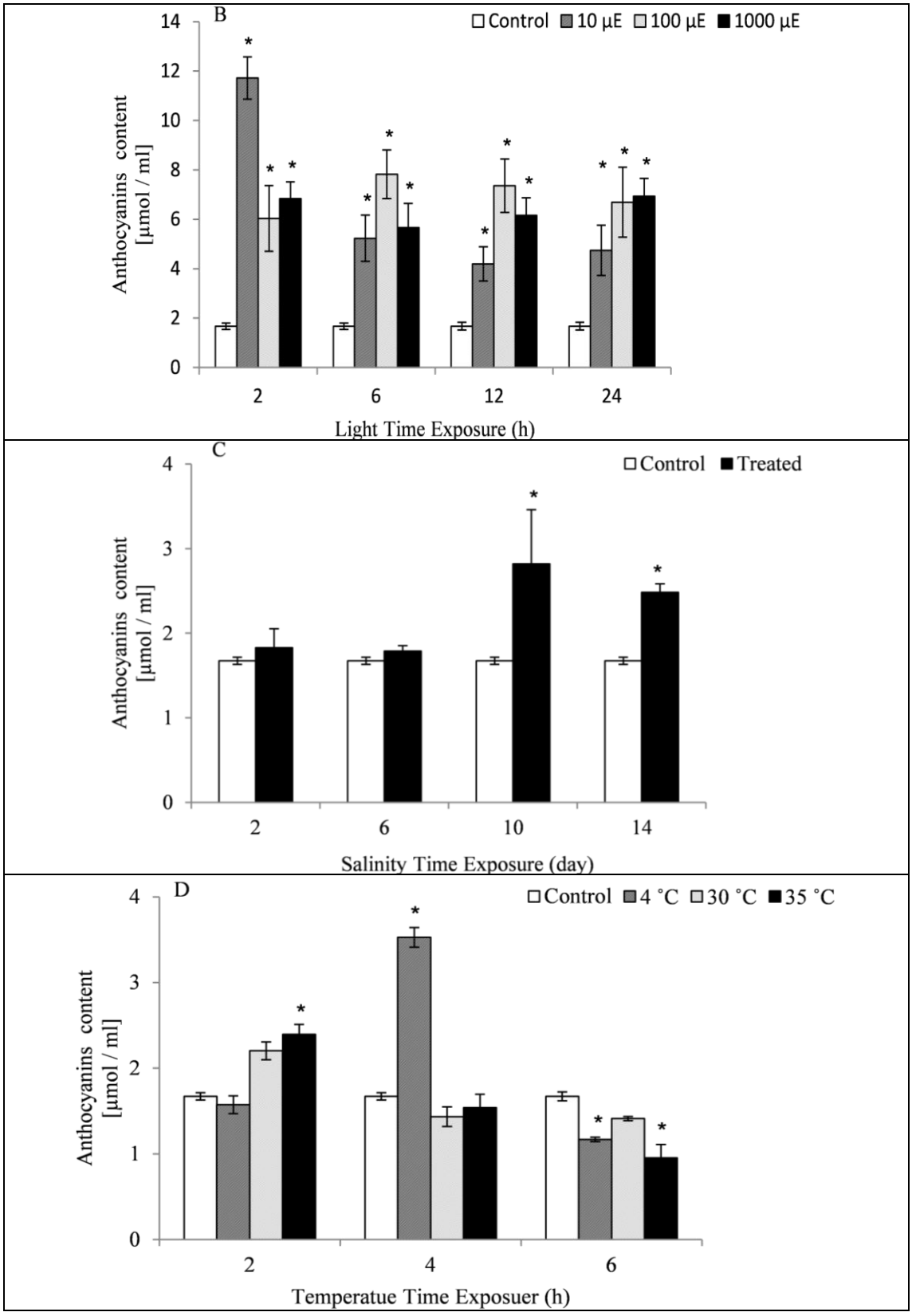

Figure 1. *Anthocyanins contents in leaves of M. spicata subjected to various abiotic stresses. A- Drought. B- Light intensity. C- Salinity. D- Heat. The effect with different time points in comparison with control. Data represent mean values ± SD of n = 3. The significance of difference was calculated using the Student's t test with P ≤ 0.05*

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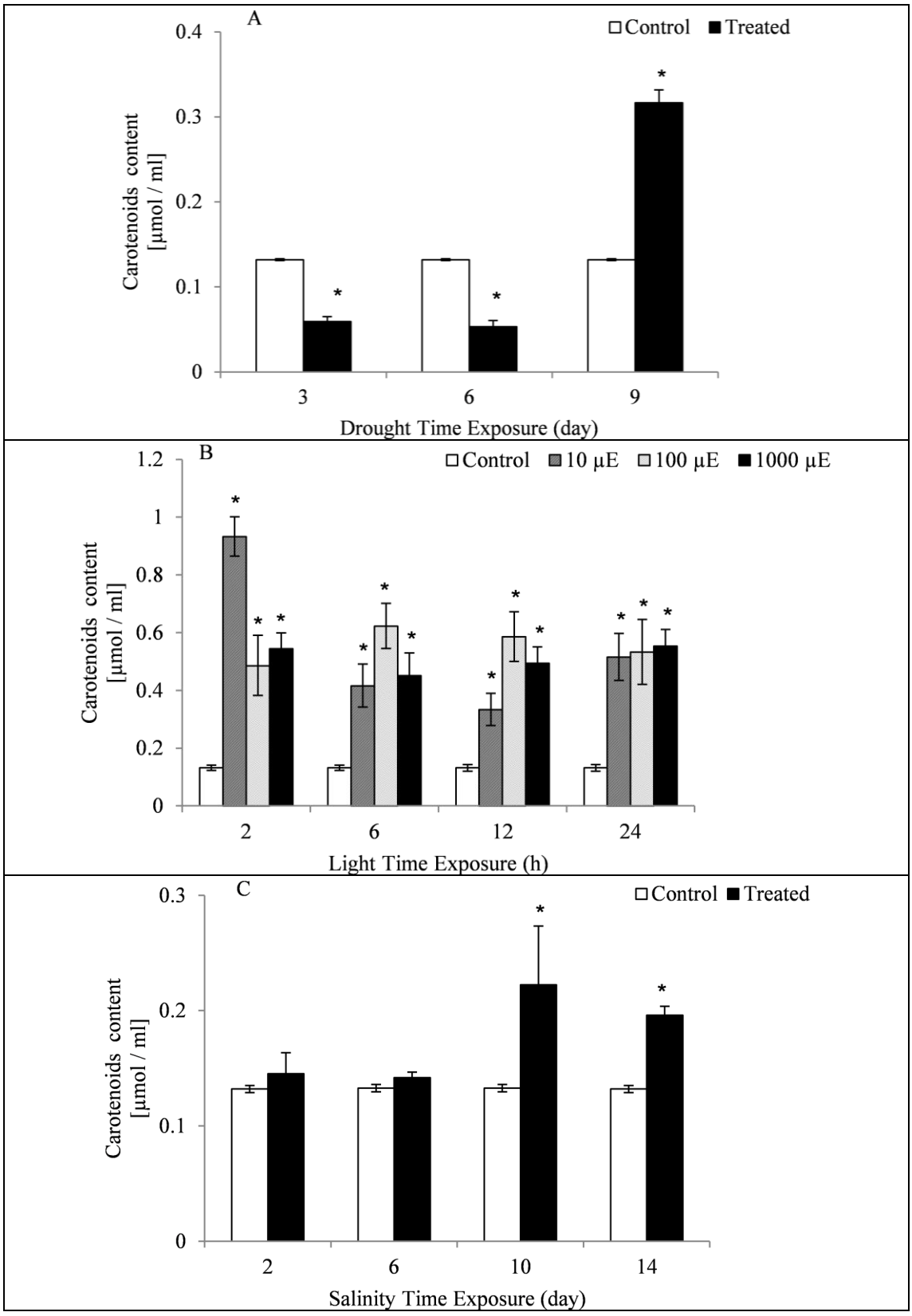
Carotenoid content elevations exhibited the same pattern of change as that of anthocyanin to various abiotic stresses for a different duration as shown in *Figure 2*.



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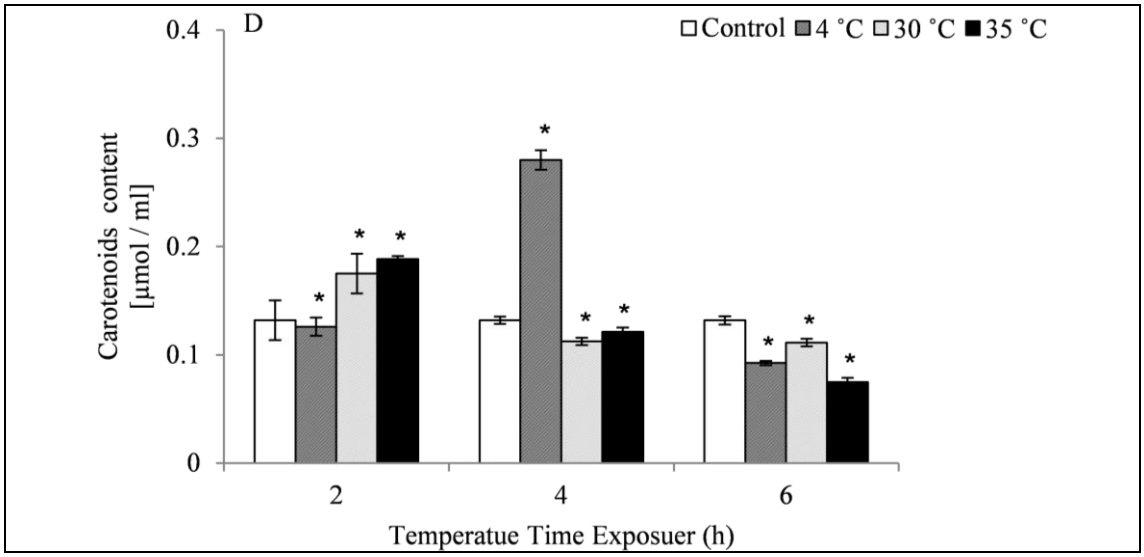
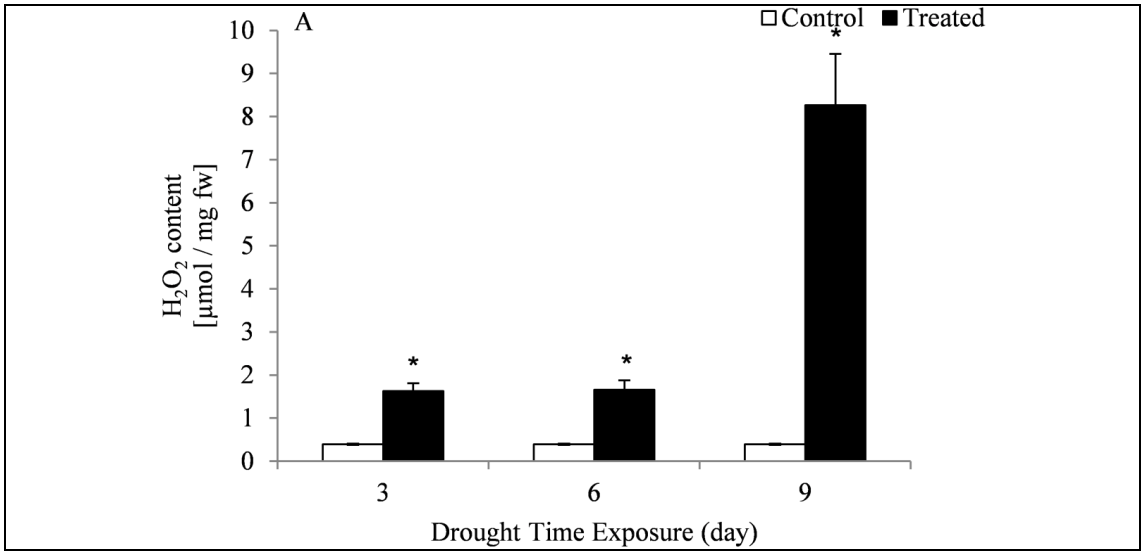


Figure 2. *Carotenoids contents in leaves of M. spicata subjected to various abiotic stresses. A- Drought. B- Light intensity. C- Salinity. D- Heat. The effect with different time points in comparison with control. Data represent mean values ± SD of n = 3. The significance of difference was calculated using the Student's t test with P ≤ 0.05*

Prolonged exposure to drought also salinity and at whatever specific light amount deviation starting with those ordinary light. Furthermore, affectability for temperature variances developed then afterward short or moderate-term exposure (*Fig. 2*).

H_2O_2 levels

H_2O_2 levels were increased significantly and linearly in all treated plant leaves according to duration of exposure compared with plants maintained in control conditions (*Fig. 3*).



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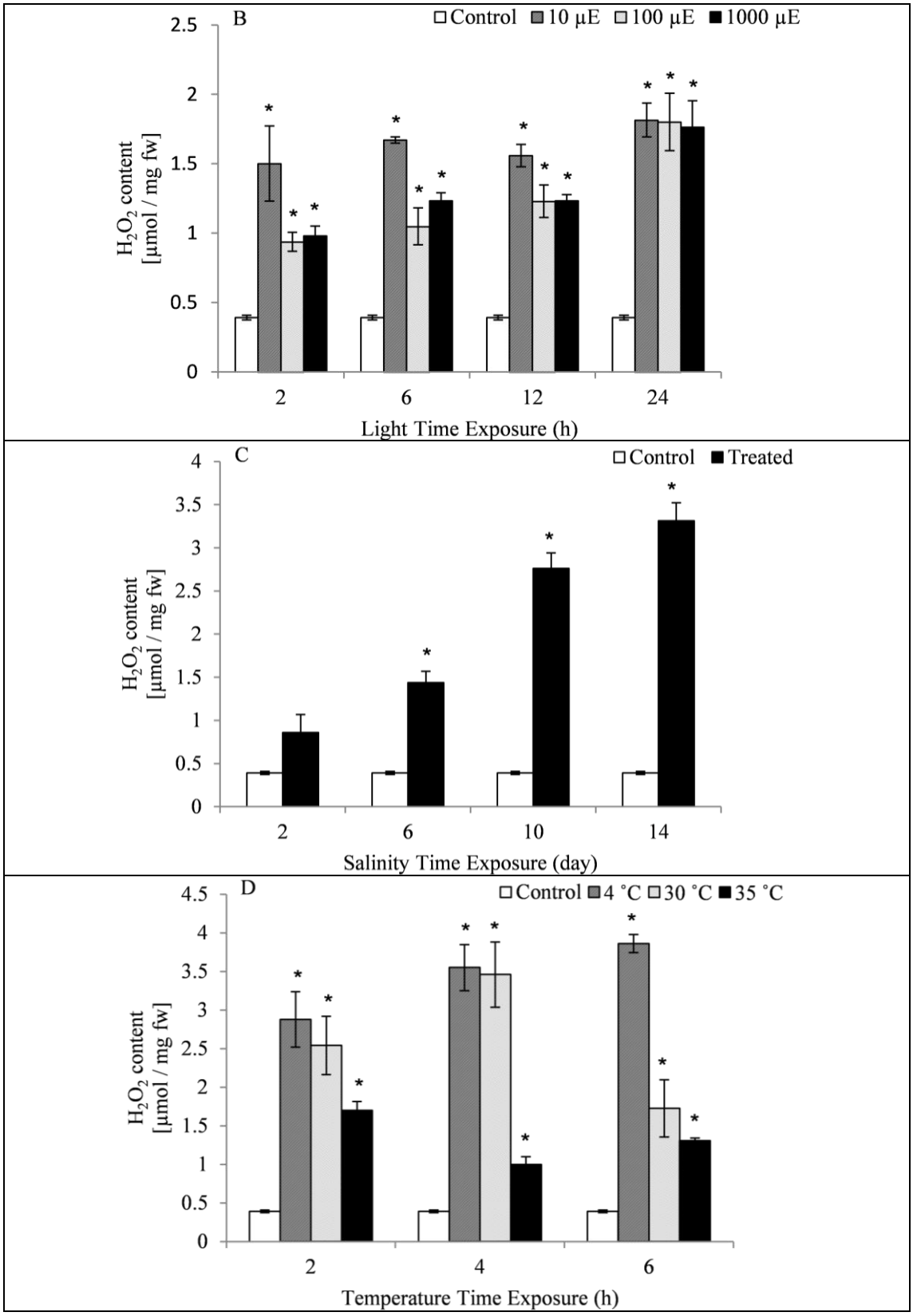

Figure 3. *H_2O_2 contents in leaves of M. spicata subjected to various abiotic stresses. A- Drought. B- Light intensity. C- Salinity. D- Heat. The effect with different time points in comparison with control. Data represent mean values ± SD of n = 6. The significance of difference was calculated using the Student's t test with $P \leq 0.05$*

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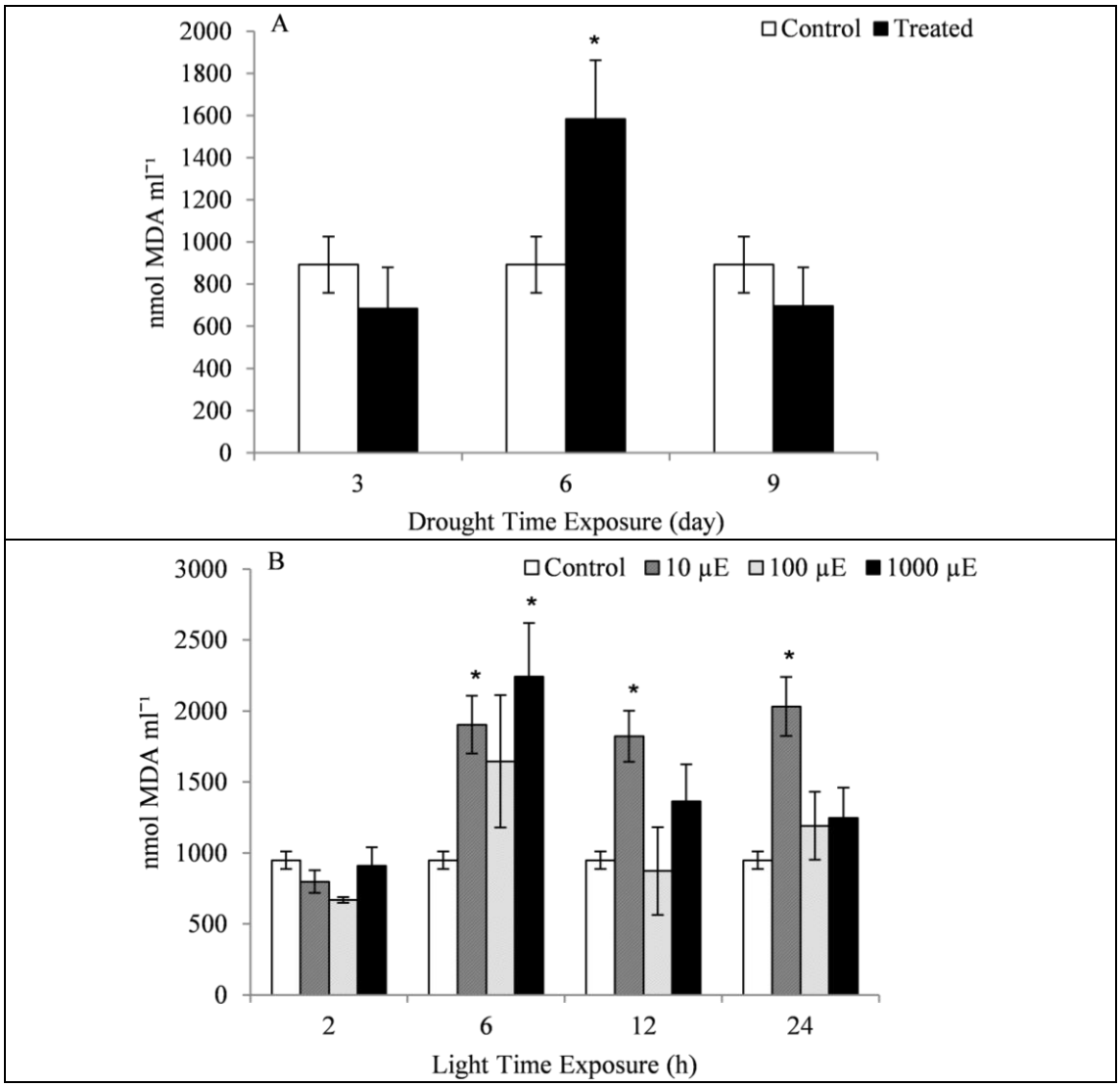
Continuous for long duration of drought (9 d) and salinity (14 d) showed the highest H_2O_2 levels reaching 21 and 9 folds when compared with the control, respectively. The basal steady state level of H_2O_2 was 0.4 μmol/g FW instantly raised after short-term (2 h) of different light intensities as well as temperature fluctuations exposure. After 24 h of sustained light intensity exposure, the content was constant despite the light dose (10 μE, 100 μE and 1000 μE compared to growth condition) which reached the maximal level of 4.6 folds compared with the control. The maximal level of H_2O_2 at 4 °C, 30 °C and 35 °C was 10, 9 and 4 folds after 6 h, 4 h and 2 h of exposure, respectively.

Lipid peroxidation changes with abiotic stressors

MDA was analyzed as measuring the end products of lipid peroxidation caused by ROS triggered by various abiotic stresses. The results showed that the lipid peroxidation level significantly increased and reached 1.7 fold after six days of drought treatment, around 2 folds after 6 h of 10 μE exposure and remain constant, 1.7 folds after 6 h of 1000 μE exposure and 2.2 folds after 4 h of 4 °C exposure when compared with the control conditions, as shown in *Figure 4*.



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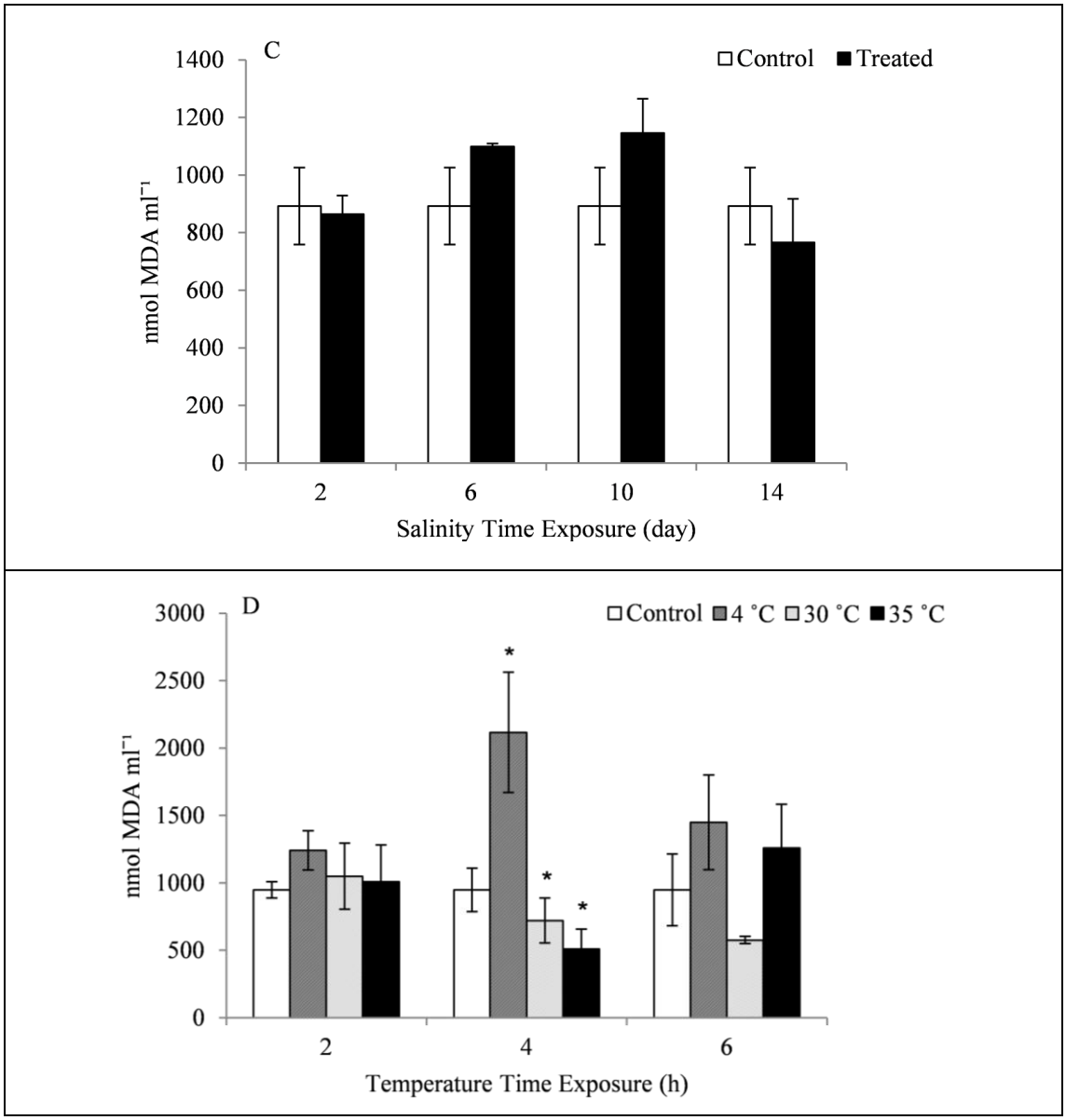

Figure 4. *Lipid peroxidation level (MDA) in leaves of M. spicata subjected to various abiotic stresses. A- Drought. B- Light intensity. C- Salinity. D- Heat. The effect with different time points in comparison with control. Data represent mean values ± SD of n = 3. The significance of difference was calculated using the Student's t test with P ≤ 0.05*

Total phenolic contents

In this study, the amount of phenolic compounds (TPC) as example of nonenzymatic antioxidants (*Fig. 5*). A significant increase in TPC levels was detected in plants of moderate duration drought exposure reached 1.9 fold, strongly related to any change in light intensity regardless to the period of exposure (> 7 folds); the shifting from normal light to low light, moderate light and high light intensities and finally for moderately high temperatures intensity regardless to the period of exposure; 30 °C reached more than 1.7 fold versus the control conditions.

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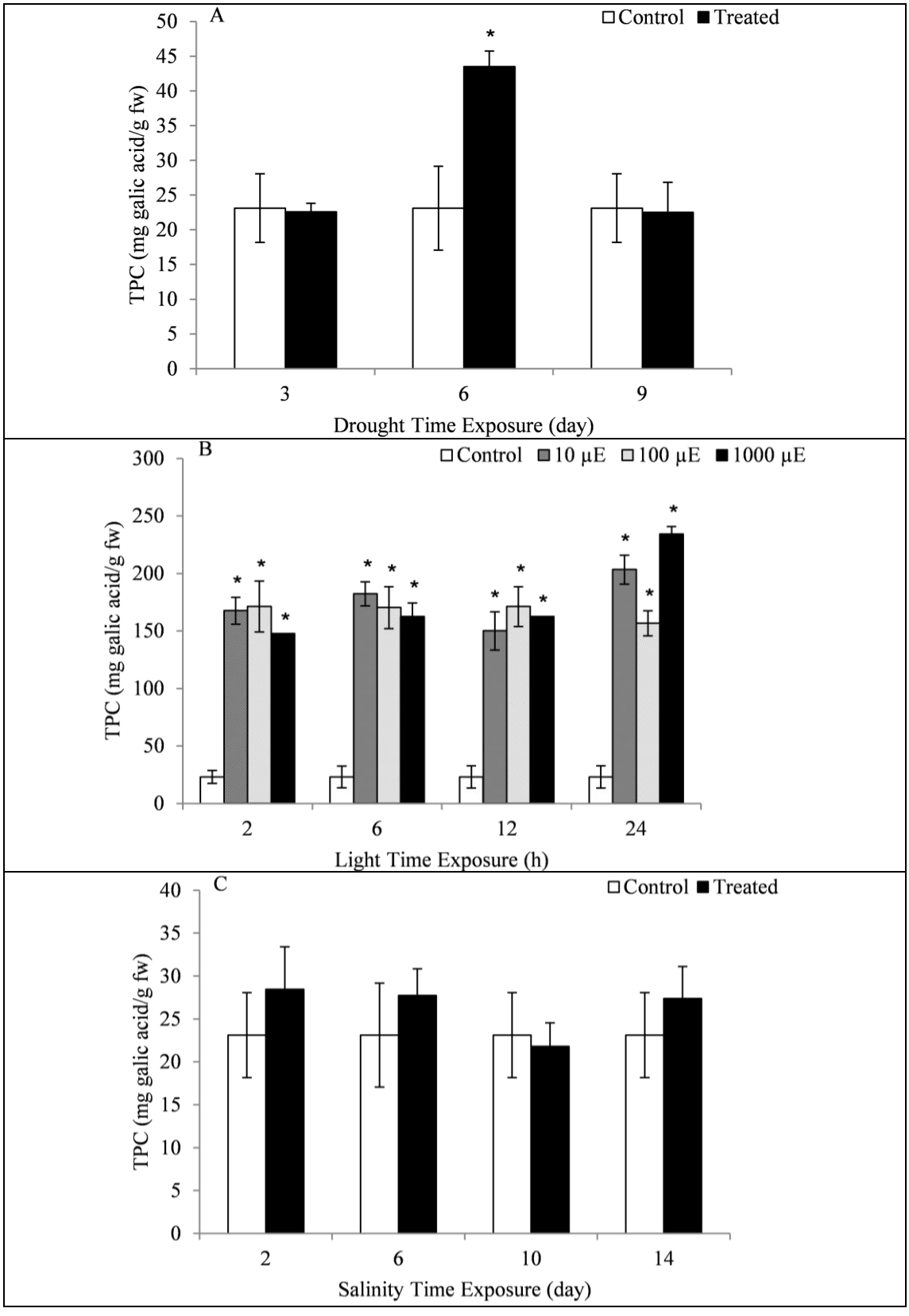

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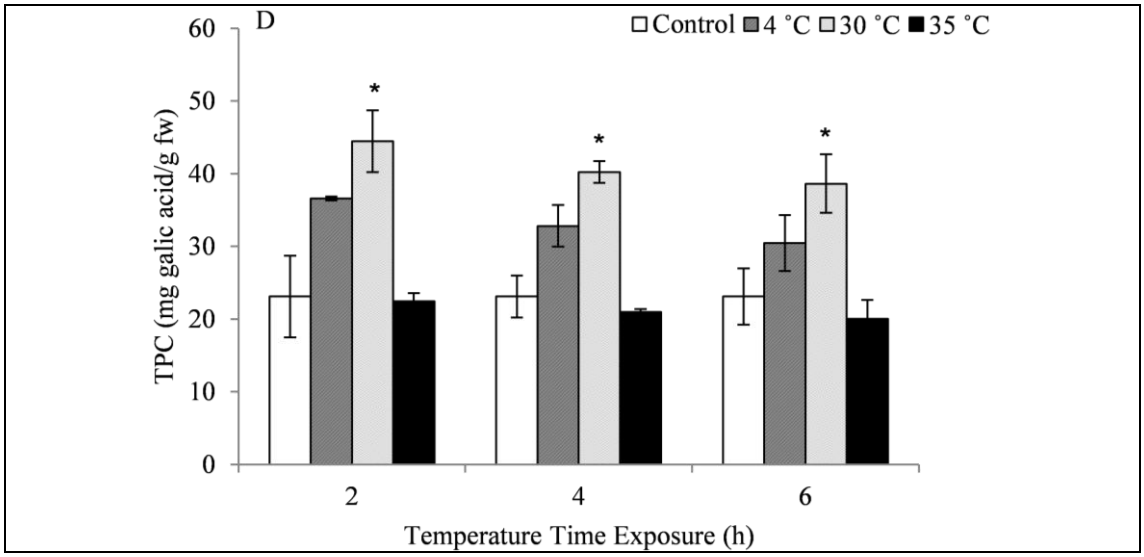
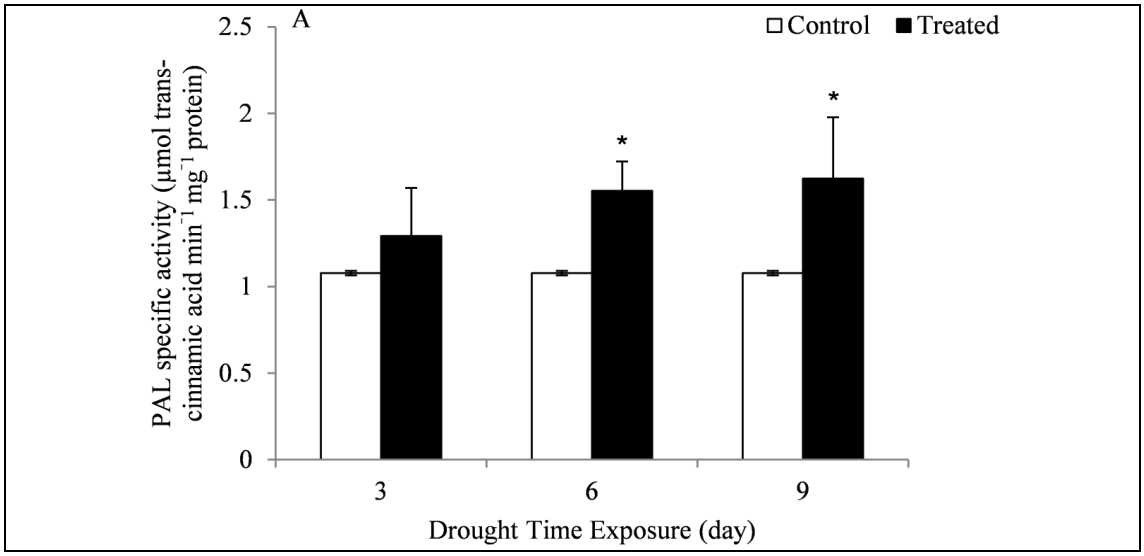


Figure 5. *Total phenol content (TPC) contents in leaves of M. spicata subjected to various abiotic stresses. A- Drought. B- Light intensity. C- Salinity. D- Heat. The effect with different time points in comparison with control. Data represent mean values ± SD of n = 3. The significance of difference was calculated using the Student's t test with P ≤ 0.05*

Effects on PAL-specific activity

Activation of PAL enzyme is also a key component of the antioxidative system of abiotic-challenged spearmint leaves. The results showed that activity of PAL was induced significantly to prolonged exposure to drought, light intensity, salinity and high temperature stresses (*Fig. 6*). PAL-specific activity in leaves of *M. spicata* significantly increased more than 1.4 fold after 6 d of drought treatment, moderate and high light caused more than 1.5 fold activity increments after 24 h of treatment, salinity was caused 1.5 fold increments and 35 °C elevated the activity around 1.4 fold after 6 h of exposure when compared with the control conditions. The overall pattern in the PAL enzyme activity showed similarity to the anthocyanin, H_2O_2 and TPC pattern in plants under prolonged abiotic stress conditional shift.



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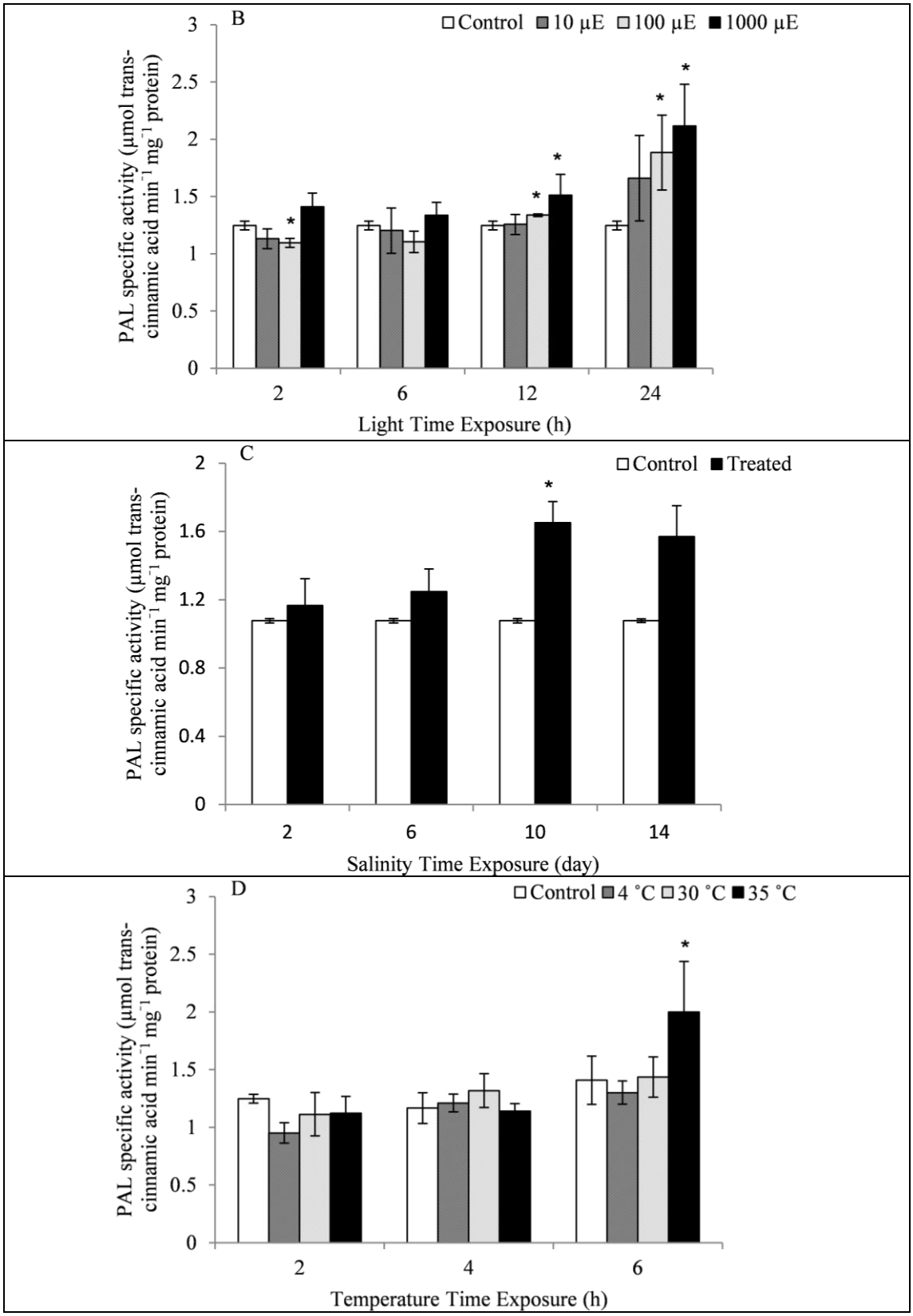

Figure 6. *Phenylalanine Ammonia Lyase (PAL) specific activity of leaves of M. spicata subjected to various abiotic stresses. A- Drought. B- Light intensity. C- Salinity. D- Heat. The effect with different time points in comparison with control. Data represent mean values ± SD of n = 3. The significance of difference was calculated using the Student's t test with P ≤ 0.05*

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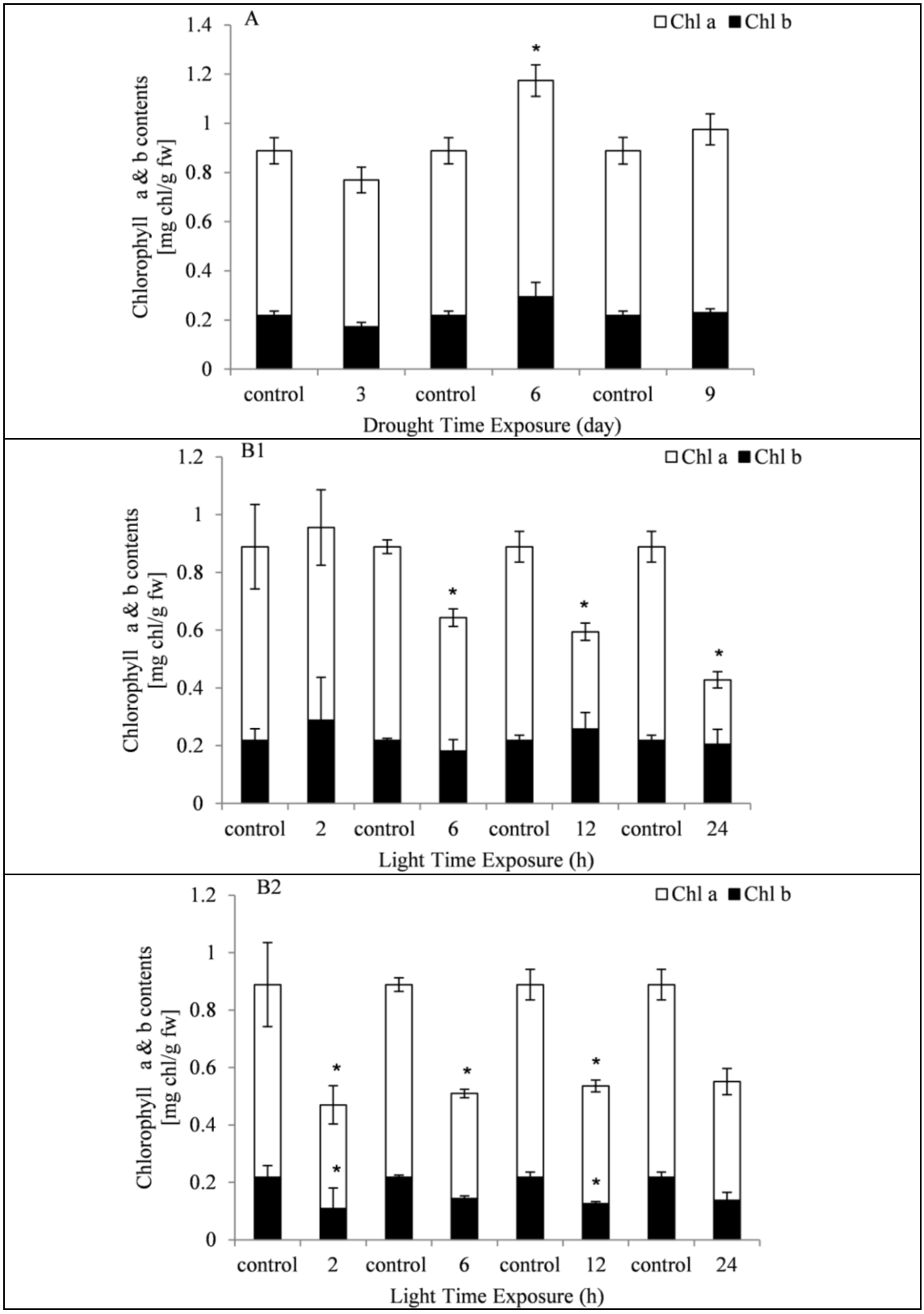
Effects on chlorophyll contents

Abiotic stresses imposed at chlorophyll *a*, chlorophyll *b* and total chlorophyll contents of *M. spicata* leaves (*Fig. 7*).



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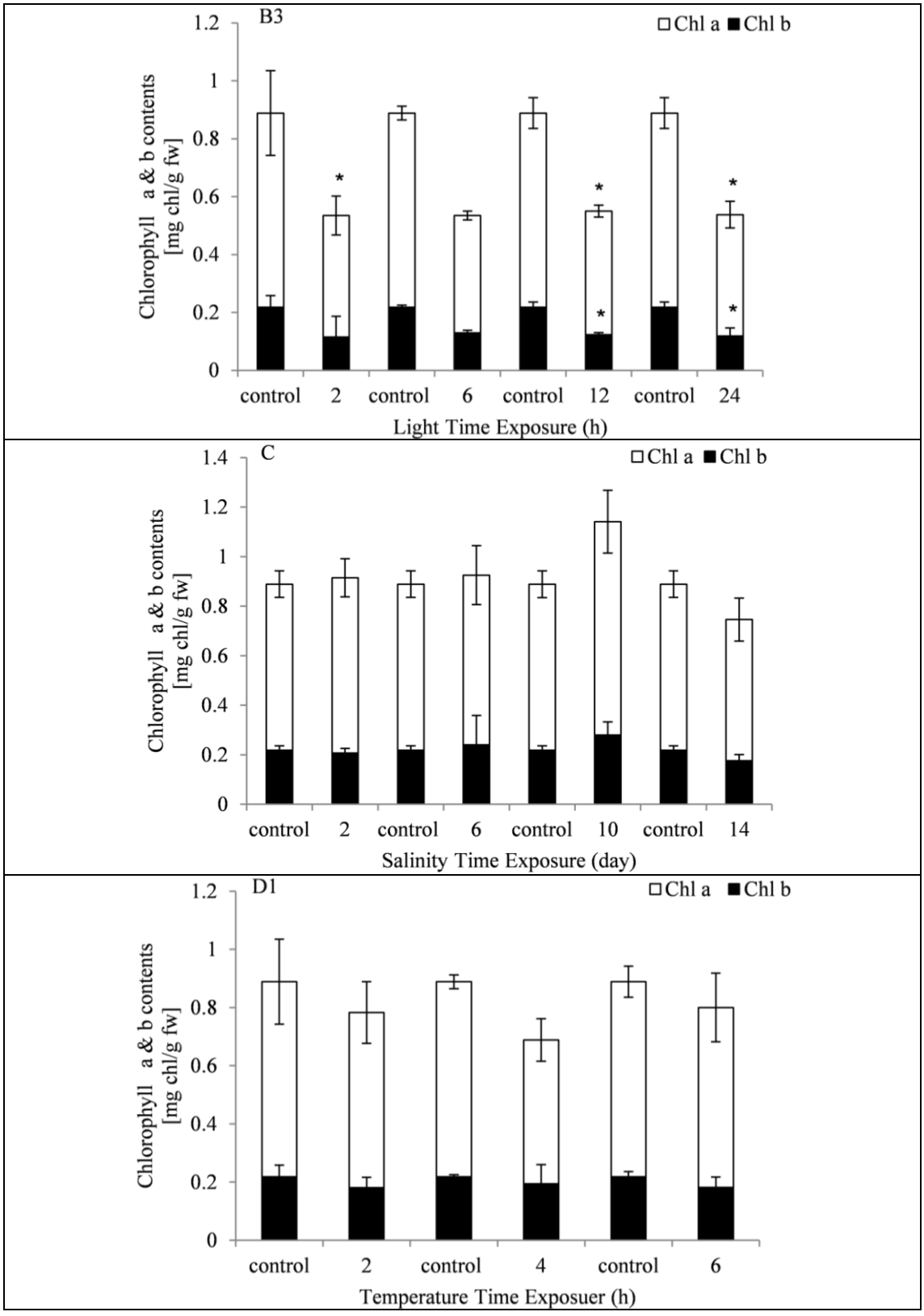

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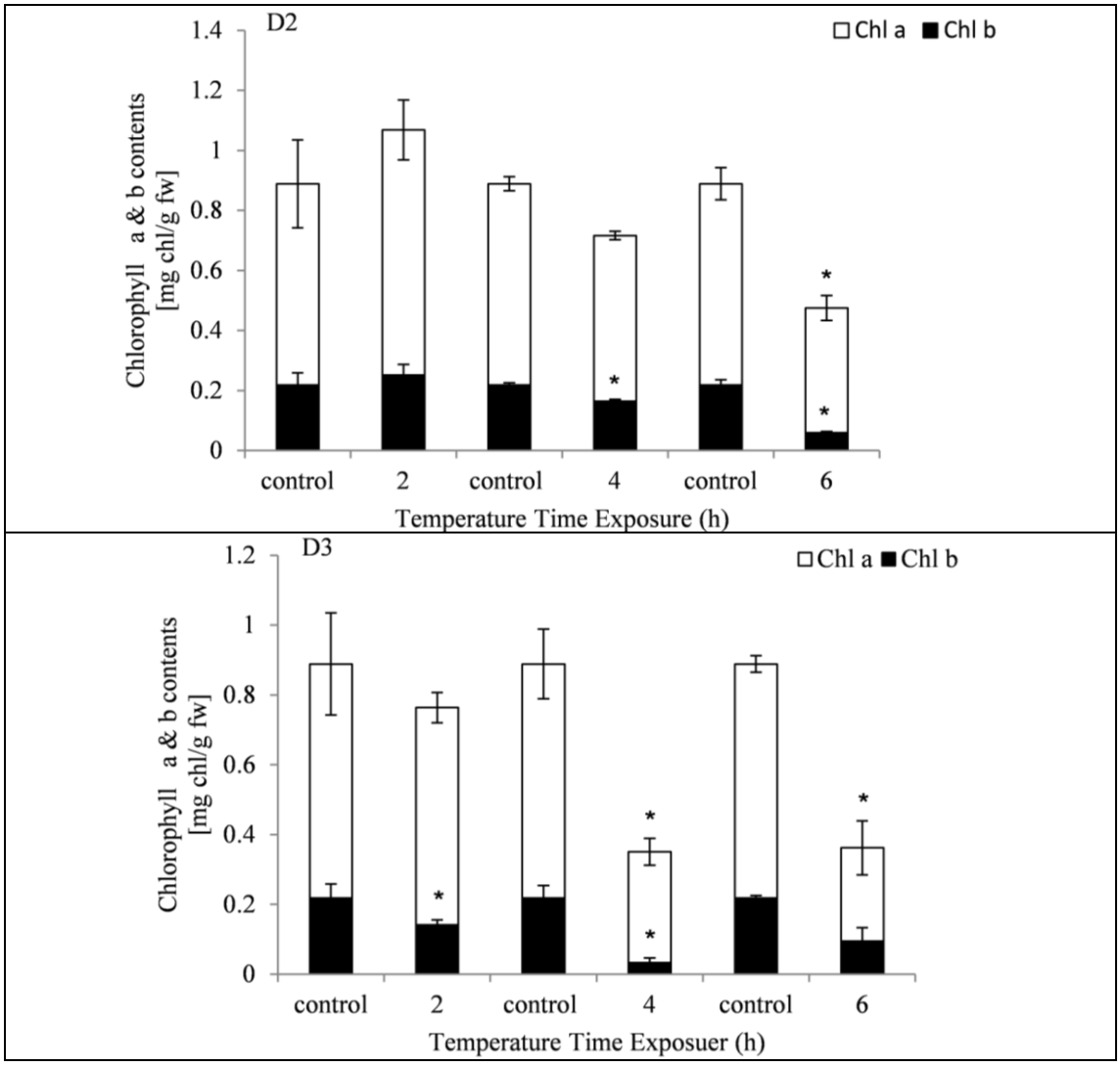

Figure 7. *Chlorophyll a and b contents in leaves M. spicata subjected to various abiotic stresses. A- Drought. B- Light intensity (B1- 10 µE, B2- 100 µE and B3- 1000 µE). C- Salinity. D- Heat (D1- 4 °C, D2- 30 °C and D3- 35 °C). The effect with different time points in comparison with control. Data represent mean values ± SD of n = 3. The significance of difference was calculated using the Student's t test with P ≤ 0.05*

Chlorophyll *a* contents decreased significantly after different light intensities and moderate and high temperatures exposure. While chlorophyll *b* contents decreased significantly after moderate and high light intensities and moderate and high temperatures exposure. These reductions caused strongly reduction in total chlorophyll content.

Discussion

The accumulation of secondary metabolites occurs in plants naturally and under adverse kinds of environmental stress (Akula and Ravishankar, 2011). However, such metabolites play a major role in the adaptation of plants to the environment and in

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acclimatizing to stress conditions. Such accumulation is dependent upon the type, severity and the period of exposure to abiotic stress factors.

Anthocyanin in foliage is stimulated by different abiotic and biotic factors (Chalker-Scott, 1999; Winkel-Shirley, 2001). Accumulation there is influenced by the type of abiotic stress and greatly increased in light (Chalker-Scott, 1999) in order to ensure that the leaf tissue has acclimated to the experimental conditions.

Anthocyanin accumulation, known as signalling, is marked by or linked to the browsing of plant stress (Close and Beadle, 2003; Eryılmaz, 2006) and supposedly has a role in the screening of underlying photosynthetic tissues, increasing light saturation, and light compensation points (Albert et al., 2009) protecting against photoinhibition, and serving as an osmotically active solute and an antioxidant of free-radicals, in addition to functioning as a UV screen (Close and Beadle, 2003; Neill and Gould, 2003; Hughes et al., 2005). However, it must be said that stress tolerance may be a consequence of an improved resistance to oxidative stress (Eryılmaz, 2006). The evidence for these changes in anthocyanin accumulation occurred in line with H_2O_2 accumulation during exposure to different light intensities, to drought for long periods, and to low temperature for short periods of exposure. Consequentially, PAL-specific activity increased with drought and long term light and salinity exposure. In addition, its protection and tolerance roles were apparent when the chlorophyll content was affected by prolonged exposure to abiotic stress factors, such as different light intensities, and different temperatures exposure.

Carotenoids act as light-harvesting pigments and protect photosynthetic systems like nonenzymatic antioxidant compounds against reactive oxygen species generated during stress (Loggini et al., 1999; Chu et al., 2010). Thus, maintaining a higher or constant level of total carotenoids during a stressful condition allows the plant to tolerate this and to cope with oxidative stress (Munné-Bosch and Alegre, 2000). Among the key biological functions of carotenoids are the sensing and signalling of oxidative stress conditions through nonenzymatic oxidation by ROS (Ramel et al., 2012).

Our results are in agreement with previous studies related to plant acclimation to stress (Loggini et al., 1999; Ruban and Horton, 1999; Chu et al., 2010). An elevation of endogenous carotenoid content was strongly apparent with different light intensities, under prolonged drought and salinity exposure. Carotenoids are considered to be the first line of defense for plants against Singlet oxygen ($^1O_2$) toxicity (Knox and Dodge, 1985). Light stress generates the oxidation of the carotenoid β-carotene, leading to the accumulation of different volatile derivatives. They were found to cause changes in the expression of a large set of genes identified as $^1O_2$ responsive genes. Therefore, next to their antioxidant and light-harvesting functions, carotenoids, through their oxidation by ROS, play a role in the sensing and signaling of oxidative stress conditions as well (Ramel et al., 2012). In addition, carotenoid content induction under salt stress is an attempt to protect the cell against harm and to detoxify plants from the effects of ROS (Verma and Mishra, 2005; Kachout et al., 2013). This is a consequence of H_2O_2 accumulation in spearmint leaves under the same treatment. Results of the present study showed that H_2O_2 accumulation in spearmint leaves was slight in the short term but great under long term drought stress exposure, that sensitivity to the quantity of light was dependent upon the time period of exposure and that salinity was greater after long periods of exposure. Damage from temperature stress was greater from a shock of cold and increased markedly with time. Being subjected to a moderately high temperature caused greater damage than being subjected to a high temperature. Under abiotic stress,

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the production of ROS clearly increased as one of the earliest responses of plant cells to stress (Jajic et al., 2015). H_2O_2 accumulation was found to precede the activation of signaling and to be a consequence of signaling, whereas, H_2O_2 acted as a central player in stress signal transduction pathways, for its features were freely diffusible and relatively long-lived (Hossain et al., 2015). However, H_2O_2 accumulation depends on the balance between the rates of its production and its enzymatic and non-enzymatic utilization.

ROS levels can rise significantly in plants subjected to various types of abiotic stress. Drought induction of H_2O_2 accumulation was in line with the manner of the severity of drought stress since several organelles are the seats, as well as the first target, of reactive oxygen species produced under drought stress (Sharma and Dubey, 2005; Farooq et al., 2009). The accumulation of H_2O_2 is considered to be one of the main features of a plant leaf's response to light stress (Mullineaux et al., 2006; Gondim et al., 2013). In addition, salinity stress has an impact on the H_2O_2 content in leaves, which is in agreement with previous reporting (Gondim et al., 2013). All of these abiotic stress factors rising of H_2O_2 accumulation; result from its role as a signalling molecule for initiating intracellular and systemic signalling or promoting oxidative stress, triggering the signalling process associated with cell death (Mullineaux et al., 2006; Upadhyaya et al., 2007; Oelze et al., 2011). In addition, induction might be associated with the ability of H_2O_2 to generate antioxidant enzymatic defenses in order to reduce abiotic stress deleterious effects, since H_2O_2 is a probable signalling molecule that mediates crosstalk between signalling pathways and contributes to the protection against other types of stress (Neill et al., 2002). However, among the ROS, a variety of plant responses has been found to be triggered by H_2O_2, such as the acclimation to stress and developmental stages (Ślesak et al., 2007; Jajic et al., 2015). Those transcriptome responses include photosynthetic light reaction genes, genes in the phenylpropanoid pathway, ROS scavenging, photorespiration, the reductive pentose phosphate pathway, and hormone biosynthesis and response (Cheeseman, 2007). This was in line with PAL-specific activity changing in response to the same abiotic stress factors (*Fig. 6*).

Non-enzymatically formed RES resulting from ROS accumulation measured by MDA levels tends to correlate with oxidative stress (Pintó-Marijuan and Munné-Bosch, 2014). However, MDA levels do not correlate with the intensity of the stress applied, and supposedly are a latent RES signal synthesized in the plant cells involved in fast-response gene activation (Farmer and Mueller, 2013). Despite its relationship to oxidative conditions, MDA accumulated under abiotic stress and has an activity biased towards abiotic stress gene expression (Weber et al., 2004). For that reason, induced lipid peroxidation is involved in diverse signalling processes in order to protect plants from oxidative stress by scavenging ROS (Mène-Saffrané et al., 2009). This thorough production of lipid fragmentation, including carotenoid fragmentation as well as radical amplification, generates a process by which defense genes protect against oxidative stress (Ramel et al., 2012; Zoeller et al., 2012).

Drought increases the generation of the MDA level particularly after moderate time exposure and exposure to light quantities, enhancing the raising of the MDA level after exposure for a moderate period of time, and at all times, after exposure to low light intensity. Salt stress causes a slight increase in the MDA level after a particular period of exposure. Heat stress raises the MDA level in response to a shock of cold. These changes in the MDA levels in response to different abiotic stress factors are in line with H_2O_2 content changes under the same abiotic stress factors. ROS causes oxidative stress

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in plants (Farooq et al., 2009). ROS can directly attack membrane lipids and increase lipid peroxidation (Mittler et al., 2004). Overproduction of ROS increases the content of MDA and is considered to be an indicator of oxidative damage (Moller et al., 2007).

The production of phenolic compounds in plant tissues rises under abiotic stress conditions (Król et al., 2014). Phenolic compounds include several classes that enhance and prod plant metabolic plasticity, enabling plants to survive under different types of environmental stress. Indeed, plant exposure to environmental constraints results in the harmful impact on plant growth and yield. The impact of severe drought stress on the accumulation of phenolic compounds showed a considerable increase in their protective action against the oxidative stress and dehydration of the leaves (Gregorová et al., 2015). Phenolic accumulation is generated by different light intensities and the UV-B activation of the phenolic biosynthetic pathway. Scavenging role of ROS results in hampering UV-B penetration, thus protecting the photosynthetically active tissues (Mazza et al., 2000; Airaki et al., 2012). Low temperatures alter membrane structure and also affect the activity of membrane-bound enzymes, as the deleterious effects of production of ROS can be associated with chilling. Moreover, low temperatures impair the whole antioxidant plant response. Hence, plants activate ROS scavenging systems (Oh et al., 2009; Airaki et al., 2012).

Secondary metabolites provide resistance that is both passive (continuously available) and active (in response to specific stressors). Phenolic compounds have been shown to be more effective antioxidants *in vitro* and *in vivo* than Vitamin A and Vitamin C, even when synergistically amplifying their biological effects (Blokhina et al., 2003; Kasote et al., 2015). Their antioxidant effectiveness is due to their high reactivity as hydrogen or electron donors, and they stabilize and delocalize unpaired electrons, chelate transition with metal ions, and alter peroxidation kinetics (action of flavonoids) and hydrogen peroxide scavenging (Rice-Evans et al., 1997; Blokhina et al., 2003).

The deleterious effect of high H_2O_2 accumulation was encountered with the rise in anthocyanin and carotenoid content for the same period of drought exposure. At the same time, total phenolic compound accumulation at moderate drought exposure was in line with increased lipid peroxidation levels and with PAL-specific activity changing in response to drought stress at all times of exposure.

The highest total phenolic compound accumulation for abiotic stress factors was in response to the exposure to different light intensities after being transferred from normal light growth intensity. This was done to protect against the highest H_2O_2 accumulation, lipid peroxidation levels and what might be the action of anthocyanin and carotenoid accumulation, including at the highest level and the increase in PAL-specific activity in response to different light intensities, particularly when increasing the time exposure. The change in total phenolic compounds did not reflect being subjected to a high level of salinity, which might be due to the moderate salt concentration (100 mM NaCl). However, prolonged exposure raised both the H_2O_2 accumulation and the MDA levels. This internal imbalance was modulated with a strong accumulation in anthocyanin and carotenoid content by greatly increasing the PAL activity by the same factor. Exposing plants to a sudden temperature shift showed the accumulation of phenolic compounds due to low and moderate temperatures. This results in an elevation in H_2O_2 content, an increase in PAL activity, and affects chlorophyll content. Low temperature impairs the whole antioxidant plant response. Hence, plants activate ROS scavenging systems when

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heat stress has been reported in order to reduce Chl content (Oh et al., 2009; Aien et al., 2011; Airaki et al., 2012).

Phenylalanine ammonia lyase activity was shown to be regulated when exposed to stress (MacDonald and D'Cunha, 2007). Thus, PAL has been recognized as a marker of environmental stress. Long-term drought stress increases PAL activity with consistent effects on lignin content since drought stress causes disruption of lignin deposits in leaves (Terzi et al., 2013). Different light intensities revealed that PAL activity was induced in leaves (Ali et al., 2006). PAL activity increased linearly and positively correlated with increased NaCl concentrations (Gao et al., 2008). High temperatures can mediate changes in secondary cell wall metabolism and possibly regulate the lignin biosynthetic pathway through a differential expression of proteins involved in cell wall biosynthesis (Han et al., 2009). In addition, low temperature had an effect on PAL activity, as well as on the rate of accumulation of different phenolic compounds in leaves (Solecka et al., 1999; Solecka and Kacperska, 2003). Moreover, our results showed that PAL activity increased in response to a prolonged exposure to different abiotic stress factors (*Fig. 6*).

Increased PAL activity could be a response to the cellular damage provoked by either the severity of abiotic stress or the duration of the stress. It seems that in spearmint leaves the enhancement of PAL activity could be related to the involvement of this enzyme as the plant response to abiotic stress factors. Phenylpropanoid compounds act as protectants against adverse environmental stress. The overall pattern of PAL activity was consistent with the change in anthocyanin, carotenoid, H_2O_2, MDA and total phenolic compound levels in response to drought stress (Gholizadeh, 2009).

PAL activity was higher in plants exposed to bright light (1000 μE) than when compared to those in normal light. The enzyme involved in lignin biosynthesis that increased lignification, may be caused by the light intensity level due to the enhancement of phenylpropanoid enzymes as an acclimation response (Ali et al., 2006). Increased PAL activity was linearly and positively correlated with the duration of increased salt stress. This elevation could be a response to the cellular damage caused by the duration of salt stress exposure. Moreover, anthocyanin and carotenoid contents were elevated in line with the change in H_2O_2 content and the increase in PAL activity (Gao et al., 2008)

Concentrations of chlorophyll components are normally used to quantify leaf senescence in stressed plants. Leaf maturation and senescence exhibit chlorophyll degradation and can be induced by stress. However, both are processes involving the regulation of plant hormones (Zhang et al., 2016). Drought can lead to a decrease in chlorophyll content, depending on the duration and severity of drought (Terzi and Kadioglu, 2006). Exposure to a higher light intensity induced a loss of chlorophyll (Jagtap et al., 1998). Salinity adversely affects Chl content (Meloni et al., 2003). Chlorophyll pigments decreased under high temperature treatments (Aien et al., 2011). Our results of Chl content showing a decrease in the early period of drought stress, and with an increase in later periods are in agreement with the findings of Terzi and Kadioglu (2006). Different light intensities exhibited that prolonged exposure to low, moderate and bright light can cause greater degradation, and consequently, a reduction in the levels of total chlorophyll as a result of the limitation of the concentration of carotenoids, which can prevent chlorophyll photo-destruction (Gonçalves et al., 2005; Zervoudakis et al., 2012). Salinity stress (100 mM NaCl) showed slight effects on Chl content, though starting in later periods, it might not be a high concentration.

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Nevertheless, the observed decrease in Chl content may be attributed to both an inhibited synthesis of that pigment and a damaged PS antenna system. Low temperature stress showed a slight gradual decrease in Chl content with the increased time duration of exposure. Low temperature can influence plant photosynthesis and lower the utilization of light Chl biosynthesis (Wu et al., 1997; Tewari and Tripathy, 1998). Moderate and high temperatures also reduced Chl content, which might be an effect of Chl biosynthesis (Tewari and Tripathy, 1998). Temperature stress may have caused a reduction or increase in the synthesis of enzymes or the corresponding mRNA responsible for Chl biosynthesis.

Conclusion

In conclusion, among the several markers used to evaluate the oxidative status of plant tissues in response to abiotic stress are the photosynthetic pigments chlorophyll and carotenoid, Reactive oxygen species, Reactive carbonyl species, Antioxidant systems and Transcriptomics and Proteomics (Pintó-Marijuan and Munné-Bosch, 2014).

The present results lead us to conclude that under different levels of abiotic stress anthocyanin, carotenoid and total phenolic compound content are enhanced in an attempt to protect leaf tissues against any damage. Signals such as ROS, and particularly in H_2O_2, might, due to its features, be the major trigger. The PAL activity and the chlorophyll content changes were used to explore the crosstalk and complexity of the responses.

The present study introduces new data about the *M. spicata* leaves responses to different abiotic stress factors that were focused on some biochemical changing. For that, study data recommend further investigation need to cover other partner and major players in plant responses such as flavonoids, glutathione, ascorbate contents and antioxidant enzymes activities, in particular, total ascorbate peroxidase, glutathione reductase, and polyphenol oxidase.

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