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Salicylic acid effects on some physio-chemical properties and secondary metabolite accumulation in *Mentha piperita* L. under water deficit stress

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Abstract: Salicylic acid (SA) play important roles in different physiological processes of plants such as plant growth, stress response, plant adaptation and secondary metabolite accumulation. This study was conducted to evaluate the effect of exogenous SA applications on the growth measurements such as fresh and dry weight of aerial part and lead dry weight, biochemical properties (membrane permeability, lipid peroxidation, Proline content and ROS scavenger enzymes) and secondary metabolite accumulation (total phenolic and the flavonoid and essential oil content) in peppermint (*Mentha piperita* L.) plants grown at different levels of water deficit stress (Field capacity: FC). For this aim, three different water deficit stress [no stress (100% FC), mild stress (75% FC) and moderate stress (50% FC)] and four different SA concentrations (0, 1, 2 and 2.5 mM) were applied to peppermint plants. Results showed that all of the measured parameters were affected by the water deficit stress and SA application. By elevating the level of water deficit stress, fresh and dry weights of aerial parts and dry leaf weight decreased. Increasing in the water deficit stress level from mild to moderate stress resulted to reduce the essential oil content while proline, lipid peroxidation, total phenolic contents, flavonoid content, and antioxidant enzyme activities increased depending on water deficit stress. Exogenous application of SA obstructed the negative effects of water deficit stress by decreasing the lipid peroxidation and membrane permeability and improving the antioxidant enzymes activities. Essential oil content increased significantly in plant treated with SA grown under water deficit stress conditions. Application of 2 or 2.5 mM of SA enhanced the plant growth and development without any toxic effects and increased significantly the total phenolic content, dry leaf weight, and the essential oil content in stressed and even in control (100% FC) peppermint plants.

1. Introduction

Peppermint (*Mentha piperita* L.) is considered as one of the most important medicinal and aromatic herb in the world cultivated in many

countries such as India, Italy, France, China, Hungary and United States among others (Lawrence, 2007). During the last two decades peppermint cultivation has experienced a remarkable increase, mainly due to the rediscovery of medicinal properties of peppermint essential oil, which has a lower risk of side effects compared to synthetic drugs (Kumar and Kumar-Gupta, 2008; Herro and Jacob, 2010). Furthermore, *M. piperita* is used as flavoring agent for beverages (phenolic and flavonoids compounds group, including eriocitrin, hesperidin and luteolin 7-O-rutinoside) (McKay and Blumberg, 2006; Hossain *et al*., 2010) and food industry (Essential oil and different type of plant exteract), as a fragrance (higher amounts of high-volatile monoterpenes such as menthone and isomenthone (Rohloff, 1999), as insecticide (Karamaouna *et al*., 2013) and fungicide in many industrial products. Essential oil of peppermint contains very important monoterpenes (Essential oil of peppermint contain menthofuran, menthol, menthone, isomenthone, pulegonecaryophyllene, betacaryophyllene, neomenthol, 1,8-cineole, sabinene and limonene) with antibacterial, antifungal (Edris and Farrag, 2003), antioxidant and cytotoxic activities (Mimica-Dukic *et al.*, 2003; Hussain *et al*., 2010). Due to markedly importance of peppermint, maintaining a high and constant essential oil production for industries requirements and supplying market demands (Silva, 2002) under unfavorable conditions such as water deficit stress is very important.

Water deficit stress is the major yield limiting factor for many agriculture crop plants affecting many biochemical, morphological and physiological parameters. Many research studies indicated that drought stress influenced the growth, yield, secondary metabolite production and composition in different aromatic and medicinal plants. Furthermore, between the abiotic stresses, drought stress can exacerbates the effect of the other stresses in plants and increase the effects of other stresses such as salinity, cold or hot stress (Khalid, 2006; Azhar *et al*., 2011; Verma and Shukla, 2015). In order to reduce the harmful effects and to increase the resistance of plants to the drought stress, some exogenous plant growth regulator applications such as salicylic acid (SA) can be used (Lee *et al*., 2019). Salicylic acid, as a plant messenger molecule, plays a non-enzymatic antioxidant role, regulates many physiological and biochemical mechanisms as cell expansion, vascular differentiation, vegetative and reproductive development, seed germination, flowering, fruit set and secondary metabolite accumulations during stress occurrence (Arfan *et al*., 2007; Hayat and Ahmad, 2007). This compound belongs to the group of the phytochemicals with beneficial effects on human health (Hayat and Ahmad, 2007). In addition, SA is a phenolic compound that has a crucial role in plant defense against pathogenic agents (Dempsey and Klessing, 1994; Hayat *et al*., 2010). Apart from growth-promoting and health related effects of SA in different plants for instance in *Artemisia annua* L. (Aftab *et al*., 2010), *Cuminumcy minum* (Rahimi *et al*., 2013) sweet basil (*Ocimum basilicum* L.) and marjoram (*Majorana hortensis*) (Gharib, 2007) and *Salvia macrosiphon* (Rowshan *et al*., 2010), SA is also reported to confer resistance to plants against various stresses e.g. in *Matricaria chamomilla* (Kovácik *et al*., 2009), and *Vigna radiate* (Khan *et al*., 2014 a).

This experiment was conducted to determine the effect of different concentrations of exogenous SA applications on some physiochemical properties and secondary metabolite accumulation in *M. piperita* plants grown under different level of water deficit stress.

2. Materials and Methods

Plant materials, SA and water deficit stress treatments

Plants were initiated from rhizome segments of *M. piperita* obtained from a commercial nursery located in Alborz province, Karaj. The plants were grown in a greenhouse at the University of Persian Gulf in pots with a diameter of 10 cm and irrigated every 2 days during the first 30 days. The daily temperature inside the greenhouse was within optimal ranges for peppermint growth (20-25°C). Fertilization was carried out at 15 and 25 days after planting. Each pot was fertilized with a solution containing $Ca(NO_3)_2$ (1.12 g/L), $MgSO_4$ (0.45 g/L), KNO_3 (0.35 g/L), KH_2PO_4 (0.30 g/L), Ferric EDTA (0.06 g/L), and M_nSO_4 (0.01 g/L). Thirty-day old seedlings were transferred to plastic pots with a diameter of 30 cm filled with soil, cocopeat and perlite (1:1:1; v/v) and kept in an incubation room where the temperature was 25°C ± 1; the relative humidity was 60-80% and photoperiod of 16 h/d. The first SA (Synonym: 2-Hydroxybenzoic acid; Linear Formula: $C_7H_6O_3$ (CAS 69-72-7), Sigma-Aldrich) treatments (0, 1, 2 and 2.5 mM) was applied to 35 days old plants by spraying aerial parts of the plants. The stock solutions were prepared by dissolving SA in ETOH and final volume was maintained by

distilled water containing 0.02% of Tween 20 as surfactant. Control plants were sprayed with distilled water including 0.02% of Tween 20 and EtOH (100%) as in SA solutions. The 2th, 3th and 4th SA application were repeated at days 50 and 65 and 80 days after transplanting on peppermint plants exposed in three different irrigation (100, 75 and 50 FC) treatments as FC (100% field capacity - FC), mild stress (75% FC), and moderate stress (50% FC). Throughout cultivation period, moisture levels in the growth media were controlled by daily weighting following the procedure of Yadav *et al*. (2014). Briefly, to calculate the amount of water necessary to bring each soil to determined FC, a 50 g soil sample from randomly chosen pots were collected and the water content was determined by drying at 100°C at 24 h after the pots were watered. The percentage of soil water content was calculated according to Yadav *et al*. (2014) method. Nutrient and water leaching from pots was captured in dish placed under each pot and the leachate was returned to the soil before the addition of any water. At the end of the experiment, all plants within each pot were harvested and then analyzed.

Determination of biochemical and physical properties

The growth response of the plants to elicitor treatments and water deficit stress were determined by measuring the fresh and dry weights of aerial parts per plant.

Lutts *et al.* (1996) method was used for determining the membrane permeability of the excised leaves at the end of the experiment by using a conductivity meter. After harvesting (from three plants per treatment), 5 leaves (randomly chosen full developed leaves from upper part of the plant) were cut into 1 cm segments. Leaf segments were washed with three changes of deionized water to remove surface adhered electrolytes. Samples were placed in 20 ml vials contain deionized water and incubated at 25°C on a rotary shaker (100 rpm). After 24 h incubated at 25°C, the electrical conductivity of the bathing solution (L_1) was determined by using a conductivity meter. Then samples were autoclaved at 120°C for 20 min and the electrical conductivity (L_2) was obtained. The membrane permeability was obtained using L_1/L_2. All measurements were made in triplicate.

Lipid peroxidation in leaves was determined by estimating the malondialdehyde (MDA). For malondialdehyde (MDA) determination, 1 g leaf sample (full developed leaves were collected from the upper part of each plant) was homogenized in 5 ml 1% trichloroacetic acid and centrifuged at 10000 *g* for 10 min. The amount of MDA in the supernatant was estimated by the thiobarbituric reaction as described by Madhava *et al.* (2000). MDA concentration was calculated from the absorbance at 532 nm by using the extinction coefficient of 155 Mm cm^{-1}. All measurements were made in triplicate.

Proline content of treated plant was determined as described by Bates *et al*. (1973). A 1:1:1 solution of proline, ninhydrin acid and glacial acetic acid was incubated at 100°C for 1 hour. For proline colorimetric determinations, the reaction was arrested in an iced bath and the cromophore was extracted with 4 ml toluene and its absorbance at 520 nm was determined in a BioMate spectrophotometer (Thermo-Spectronic). After the analyses, following equation: (g proline in extract/115.5) g^{-1} sample = mol g^{-1} FW) was used for calculation the proline concentration from a standard curve.

Enzyme assays

At the end of experiment, leaves were collected and frozen in liquid nitrogen and stored at -80°C before enzyme extraction. ROS scavenger enzymes were extracted as described by Zhang and Kirkham (1996) with some modifications. All operations were carried out at 4°C. Intact leaves were ground using mortar and pestle under liquid nitrogen in cold 50 mM sodium phosphate solution (pH 7.5) containing 250 mM Sucrose, 10 mM KCl, 1 mM $MgCl_2$, 1.0 mM EDTA, 0.5 mM 0.1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, and 1% (w/v) polyvinylpolypyrrolidone in a 6:1 proportion (w/v). The homogenate was then filtered and centrifuged at 25,000 *g* for 20 min at 4°C. Then solid ammonium sulfate $(NH_4)_2SO_4$ added to the supernatant to make up 80% saturated solution and allowed to stir gently for several hours at 4°C. After centrifugation (28,000 *g* for 45 min at 4°C), pellets, were resuspended in a small volume of 50 mM of sodium phosphate (pH 7.5) and used for enzyme assays.

The superoxide dismutase (SOD) activity was assayed by observing the inhibition of photochemical reduction of nitro blue tetrazolium according to Krishnan *et al*. (2002) protocol. One mL of assay mixture consisted of 75 µM nitro blue tetrazolium, 2 µM riboflavin, 50 mM Na-P buffer (pH 7.8) 0.1 mM EDTA, 13 mM Methionine, and enzyme extract. The samples were kept 30 cm under a light source (4000 lux) for 15 min and the reaction started during this time. The reaction was stopped by switching off the light. A non-irradiated reaction mixture, served as a control which was run in parallel. The absorbance was read at 560 nm.

The Ascorbate peroxidase (APX) activity (ε= 2.8 mM^{-1} cm^{-1}) was determined from the decrease in A290, due to the H_2O_2-dependent oxidation of ascorbate using procedure of Zhang and Kirkham (1996). One mL reaction mixture contained 50 mM Na-P (pH 7.0), 0.5 mM ascorbic acid, 0.1 mM EDTA, 0.1 mM H_2O_2, and enzyme.

The catalase (CAT) activity (ε H_2O_2 = 39.4 mM^{-1} cm^{-1}) were assayed spectrophotometrically according to Zhang and Kirkham (1996) method by monitoring the change in A240 due to the decreased absorption of H_2O_2. The reaction mixture contained enzyme extract, 50 mM Na-P, pH 7.0, and 15 mM H_2O_2 (in 1 mL final volume). The reaction was initiated by addition of H_2O_2.

Secondary metabolites determination

At the end of the experiment, in all plants within each pot leaves (1 g full developed leaves were collected from the upper part of the each plant) were collected and frozen in liquid N2 immediately and stored at -80°C before analysis. Folin-Ciocalteu colourimetric method was used for determining the total phenolic content of the peppermint extract (Singleton and Rossi, 1965). Samples were transferred into the different test tube and mixed thoroughly with Folin-Ciocalteu reagent (5 ml). After 5 mins, 4 ml of 7.5% sodium carbonate (Na_2CO_3) was added and allowed at room temperature to react for 2 hrs. The absorbance was measured using spectrophotometer at 765 nm. Samples were measured in three replicates. The flavonoid content was determined according to Liu *et al.* (2002) by aluminium trichloride method using Catechin. A volume of 125 µL of extract is added to 75 µL of a 5% $NaNO_2$ solution. The mixture was allowed to stand for 6 min, and then 150 µL of aluminium trichloride (10%) was added and incubated for 5 min, followed by the addition of 750 µL of NaOH (1M). The final volume of the solution was adjusted to 2500 µL with distilled water. After 15 min of incubation the mixture turned to pink and the absorbance was measured at 510 nm.

Total essential oil content

Hydro distillation method was used.In this method, at least 10 g of dried *M. Piperita* shoot related to the treatment immersed in 150 cc water were submitted to hydro-distillation with a Clevenger-type apparatus for 3 h (until no more essential oil was obtained). The essential oil was collected, dried with anhydrous sodium sulfate, and stored at 4°C until used.

Statistical analysis

This experiment was performed in factorial arrangement based a completely randomized design with 3 replicates per treatment and one plant per replicates in which pots were placed on the benches in the incubation room. The factors included different SA concentration (0, 1, 2 and 2.5 mM) and water deficit stress in 3 levels. Data were analyzed by SPSS 19 software, and differences among treatments were determined using Tukey's test ($p<0.05$).

3. Results and Discussion

Growth measurement

Foliar application of salicylic acid (SA) and water deficit stress affected peppermint plants in different ways, including physiological and biochemical properties. Some physiological disorders such as leaf yellowing and abscission were observed in plants at sever water deficit irrigation. Also, the high concentration of SA caused burning and drying the edge of some mature leaves in plants (data not shown). The Limited water supply is one of the major abiotic factors that adversely affect agricultural crop production worldwide. Water deficit stress significantly reduced the growth measurements (fresh and dry weights of aerial parts of plants and leaf dry weight) in peppermint plants at 50% FC (Table 1). However, SA treatments were not caused any growth reduction but in plants under water deficit stress, prevented the growth reduction rate. Highest growth values were

Table 1 - Effect of foliar spray of SA on some growth parameters

Irrigation treatment	SA (mM)	Fresh weight of aerial parts (g)	Dry weight of aerial parts (g)	Dry weight of leaf (g)
Control	0	30.74 b	5.47 b	2.61 bc
	1	31.31 b	7.15 a	3.45 b
	2	42.28 a	8.40 a	4.33 a
	2.5	41.24 a	8.34 a	4.08 a
FC (75%)	0	20.92 cd	4.56 c	1.93 d
	1	22.25 cd	4.75 bc	2.13 cd
	2	26.66 cde	4.84 bc	2.83 d
	2.5	27.71 c	4.65 c	1.91 d
FC (50%)	0	19.25 cde	4.44 c	1.58 d
	1	19.92 cde	4.26 c	1.62 d
	2	18.32 e	3.95 c	1.92 d
	2.5	19.21 de	3.91 c	1.64 d

* Differences between means indicated by the same letters are not statistically significant ($p\leq0.05$).

obtained from the plants treated only with 2 mM of SA. Khorasaninejad *et al*. (2011) also observed significant reduction in growth parameters of peppermint (*M. piperita* L.) under water stress. SA treatment in non-stress and even in stress conditions showed positive effect on the growth measurements in comparison to the control (Table 1). Different concentrations of SA (from 10^{-5} to 10^{-3} M) improved growth parameters (plant height, number of branches, nodes, and leaves per plant, as well as leaf area) in basil (*Ocimum basilicum*) and marjoram (*Origanum majorana*) plants (Gharib, 2007) and these authors suggested that such effects could be related to increased photosynthetic activity. The beneficial effects of SA on the growth measurements especially in term of dry weight of mint in this study paralleled with the results of Khodary (2004) and Hayat and Ahmad (2007). This growth enhancement suggests that SA affects various physiological and biochemical processes, including photosynthesis, water processes, ion homeostasis, antioxidant capacity generates and a wide array of metabolic responses in plants (Hayat and Ahmad, 2007). Also, SA modulates several physiological responses such as maintain Indole-3-acetic acid (IAA, 3-IAA) and gibberellin levels, inhibit ethylene biosynthesis and prevent auxin oxidation in plants (Pierpoint, 1994), which could be one reason for the SA-enhanced vegetative growth of peppermint in our study.

Essential oils

Peppermint essential oil (EO) is a rich source of monoterpenes such as menthol and menthone used in pharmaceutical, food, cosmetic and cleaning industries (Kamatou *et al*., 2013) and its commercial value depends on the essential oil contents. In this study, essential oil content was affected with both water stress and SA application (Table 2). Essential oil content increased from 1.146% (control plants) to 1.192% (mild stress treated plant) and decreased to 0.782% in moderate stress treated plant (Table 3). Exogenous application of SA alleviated this adverse effect especially in moderate stress condition and also SA application enhanced the EO yield in mild stress condition. In control irrigation conditions, 2 mM SA treatment increased total essential oil content to 1.256% while application of SA at higher concentrations caused a reduction in total essential oil content. Under water deficit stress conditions, SA significantly increased the total essential oil content when compared to the untreated plants. Particularly, 2 and 2.5 mM of SA increased essential oil content more than 2-fold compared to the untreated plants in the presence of moderate water deficit stress.

Responses of plant to environmental stress such as drought stress in terms of volatile emissions are species specific and depend on the duration and intensity of the stress period (Jord´anm *et al*., 2003). EO yield increased under mild water stress in this study. In medicinal and aromatic plants, the effect of

Table 2 - Effect of foliar spray of SA on biochemical properties of peppermint plants grown at different level of FC

Irrigation treatment	SA (mM)	Membrane permeability (s)	Lipid peroxidation (nM g^{-1})	Proline (mol g^{-1})
Control	0	37.11 def	5.81 d	0.58 ab
	1	27.43 efg	6.35 d	0.42 bc
	2	28.32 efg	5.38 d	0.28 c
	2.5	21.43 fg	6.05 d	0.66 ab
FC (75%)	0	41.02 cd	14.48 a	0.67 ab
	1	53.74 c	12.01 a	0.59 ab
	2	50.26 cd	6.09 d	0.4 bc
	2.5	25.49 fg	7.11 cd	0.62 ab
FC (50%)	0	87.04 a	11.71 ab	0.83 a
	1	41.52 cde	11.29 ab	0.79 a
	2	74.28 b	8.66 c	0.59 ab
	2.5	49.91 cd	7.03 cd	0.76 a

* Differences between means indicated by the same letters are not statistically significant (p≤0.05).

Table 3 - Effect of foliar spray of SA on phenolic and essential oil contents of peppermint plants grown at different level of FC

Irrigation treatment	SA (mM)	Total phenolic content (mg g^{-1})	Flavonoid content (mg GAE/g)	Total essential oil content (%)
Control	0	8.37 e	17.7g	1.146 d
	1	16.51 d	22.2e	1.153 d
	2	14.28 de	38.7d	1.256 c
	2.5	14.32 de	34.81e	1.092 e
FC (75%)	0	28.08 ab	37.2d	1.191 d
	1	22.71 bc	42.3c	1.201 d
	2	18.08 cd	47.4b	1.418 b
	2.5	17. 94 cd	48.2b	1.482 b
FC (50%)	0	29.39 ab	40.7c	0.782 f
	1	31.99 a	55.3a	1.010 d
	2	18.28 cd	53.2a	1.597 a
	2.5	33.65 a	55.7a	1.575 a

* Differences between means indicated by the same letters are not statistically significant (p≤0.05).

water deficit stress on essential oil yield is variable and depends on plant species. For example, drought stress had a negative effect on the essential oil yield of *M. piperit*a (Khorasaninejad *et al*., 2011). By contrast, water stress had a positive effect on *Salvia officinalis* and showed a higher concentration of monoterpenes such as cineole, camphor and α/ß-thujone (Nowak *et al*., 2010). Increasing in essential oil yield under stress condition may be related to induction changes in morphological or physiological traits such as high trichome density and smaller leaf size that prevent excessive water loss and allow them to survive in arid or semi-arid environmental conditions (Nobel, 1999). Foliar spray with Triazole (A triazole refers to any of the heterocyclic compounds with molecular formula $C_2H_3N_3$, having a five-membered ring of two carbon atoms and three nitrogen atoms) decreased the leaf area in *M. pulegium* (Hassanpour *et al*., 2012) and increased trichome density in *Bougainvillea spectabilis* (Mansouri and Kurup, 2009). The results suggest that the stimulation of essential oil production under drought stress could be due to low allocation of carbon to the growth and high terpene concentrations under stress conditions. Plant growth regulators (PGRs) favorably affect the yield and quantity of essential oil in lemon grass, rose grass, peppermint, spearmint, and sage (Shukla and Farooqi, 1990; Khan *et al*., 2014 b, 2015). Among different concentration of SA tested on *M. piperita* in none stress condition, 2 mM of SA showed the most effective in improving the essential oil content. The positive effect of SA on essential oil content and essential oil yield may be ascribed to the improvement in overall plant growth and metabolism by SA. It seemed that SA, considered to be a signaling molecule, might be involved in the signal transduction system, leading to the balance and improved quantity of the secondary metabolites, i.e., essential oil (Hayat and Ahmad, 2007). Thus, SA-mediated enhanced plant growth, photosynthesis, and the overall plant metabolism might account for oil accumulation in the present study. These results corroborate with the findings on *Artemisia annua*, *Salvia macrosiphon*, *Cumium cyminum*, *Ocimum basilicum*, and *Majorana hortensis* (Gharib, 2007; Aftab *et al*., 2010; Rowshan *et al*., 2010; Rahimi *et al*., 2013). Moderate stress significantly decreased essential oil content in current study (Table 3). Similarly, Khorasaninejad *et al*. (2011) reported significant decreases in total essential oil content in water stressed peppermint plants. These results suggest that a high level of water stress can suppresses the essential oil biosynthesis pathway in peppermint.

Reduction in photosynthesis rate and/or any changes in metabolic pathway could be caused some disorders of oil biosynthesis resulted in reduction in oil content (Srivastava *et al*., 1998). In control irrigation condition, foliar application of SA at 2 mM concentration resulted to increase in essential oil content of plants compared to the control plants while higher concentrations of SA decreased the oil content. Another interesting result is that SA foliar applications increased the essential oil content in water stressed plants. Under water deficit stressed conditions, essential oil contents of peppermint plants treated with SA were higher in comparison with the group exposed to water stress alone. The highest oil content was found in plants treated with SA at 2.5 mM concentration grown under moderate water stress. In this condition, oil content was more than 2 fold greater than that of plants under combination of moderate water stress and 0 mM of SA. According to the commercial importance of peppermint, our results represented that peppermint plants can be grown in mild or in moderate stress in the presence of SA applications.

Membrane permeability, lipid peroxidation and proline content

Both water deficit stresses resulted to a significant increase in the levels of membrane permeability, a sign of injury to the cells (Table 2). The maximum increase in membrane permeability was obtained in plants subjected to 50% FC and SA treatments reduced significantly this increase was in under water stress. The maximum reduction in membrane permeability was observed in plants treated with 2.5 mM SA. Our results indicated that water stress increased the membrane permeability levels of peppermint plants and they were alleviated significantly by foliar applications of SA.

Water stress significantly increased the lipid peroxidation compared to the control irrigation plants (Table 2). The maximum lipid peroxidation content was recorded in plants grown under high level of water deficit stress. However, SA treatments, especially at 2 mM concentration, significantly overcame the toxicity generated by water stress alone and almost leveled the values with those of control. However, SA did not affect the lipid peroxidation level in control irrigation plants.

The proline content increased by increasing the water stress but foliar spraying of SA at 2 mM concentration upon water stressed plants reduced the

proline content.

Ion leakage is known as a index for evaluating the cell wall damage caused from stress. Membrane permeability increased in line with the elevating levels of water deficit stress in this study. Also, there is a close relationship between producing oxidative damage under water stress condition of cell membrane permeability. However SA significantly decreased the membrane permeability in plants under water deficit stress (Table 4). This result can approve the capability of SA spraying treatment in negative effect of drought stress. Water deficit stress resulted to the displacement of membrane proteins, cellular compartmentalization disruption, loss of membrane selectivity, integrity and a loss of activity of enzymes (Mahajan and Tuteja, 2005).

Malondialdehyde is one of the main known indicator or reliable biomarker for lipid peroxidation, which is part of the oxidative damage at cell level. Malondialdehyde increase the cell permeability by deterioration of membrane integrity. Our results showed that there is a significant relationship between lipid peroxidation and water deficit stress (Table 4). Under water deficit stress conditions the amount of lipid peroxidation increased in peppermint plants, moreover, it was determined that MDA level increased as a result of deterioration of the cell structure. Karray-Bouraouia *et al*. (2009) and Karlıdag *et al*. (2011) reported a positive correlation between stress condition and lipid peroxidation. Water deficit stress caused considerable membrane injuries leading to membrane lipid peroxidation. Foliar spray of SA did not show any significant changes in lipid peroxidation content in control irrigation condition while it significantly decreased lipid peroxidation content in water stress-treated plants. The results showed that SA had an important role in membrane integrity and maintenance of membrane structure via preventing damages caused by water deficit stress. Low membrane lipid peroxidation in drought-exposed and SA (0.5 mM)-supplemented *T. aestivum* reported by Kang *et al*., (2012).

Table 4 - Effect of foliar spray of SA on antioxidant enzyme activities of peppermint plants grown at different level of FC

Irrigation treatment	SA (mM)	SOD (unit mg protein^{-1})	CAT (mol min^{-1} mg protein^{-1})	APX (mol min^{-1} mg protein^{-1})
Control	0	25.17 cd	6.44 cde	311.99 c
	1	17.47 ef	3.11 e	153.71 d
	2	12.31 f	6.63 cde	189.48 d
	2.5	21.19 de	4.15 de	279.11 c
FC (75%)	0	30.88 bc	15.92b	392.95 b
	1	21.17 de	8.52 c	158.21 d
	2	23.46 d	7.43 cd	264.01 c
	2.5	21.13 de	5.41 cde	314.64 c
FC (50%)	0	41.79 a	21.33 a	501.28 a
	1	19.94 de	16.21 b	183.23 d
	2	31.95 b	9.15 c	331. 53 c
	2.5	47.61 a	9.58 c	483.12 a

* Differences between means indicated by the same letters are not statistically significant (p≤0.05).

Amino acids play an important role in plant development and metabolism. Proline, an amino acid, plays main roles in plants exposed to stressful conditions (like water deficit stress, low temperature, salinity, heavy metal exposure and UV radiations, etc) such as osmoprotective, protein compatible hydrotrope, scavenging free radicals and buffering cellular redox potential, energy supply, stabilize the function and structure of protein and DNA, alleviating cytoplasmic acidosis and maintaining appropriate NADP+/NADPH ratios compatible with metabolism, metal chelator, antioxidant properties and a signaling molecule (Naidu *et al*., 1991; Bassi and Sharma, 1993; Schat *et al*., 1997; Hare *et al*., 1998; Rhodes *et al.*, 2002; Kavi Kishor *et al.,* 2005; Munns, 2005; Sharma and Dietz 2006; Ashraf and Foolad, 2007; Chookhampaeng, 2011). So, overproduction of proline is a common physiological response of plants against stressful environment. In this study, proline contents were increased with increasing water deficit stress (Table 4). So, more proline accumulation has been associated with increasing the stress tolerance of plant. In general, results showed that drought stress affected the proline biosynthesis in the leaves. The foliar spray of SA on stressed plants cause in reduction of proline content but these declines were not statistically significant in some concentration. It could be due to positive effects of SA in maintaining the membrane maintaining cell turgor or osmotic balance as an osmotic regulator (Wang and Zeng, 1993) or in stabilizing cell membranes, preventing electrolyte leakage and bringing concentrations of ROS within normal ranges.

Phenolic and flavonoids contents

Total phenolic and total flavonoid contents were increased under water deficit stress significantly as compared to control irrigation (100% FC) (Table 3). Minimum phenolic content was measured in control plants while its level increased with increasing the

water deficit stress intensity and SA application. Foliar application of SA (2 mM) under control irrigation increased total phenolic content less than two times compared to controls. Moreover, not only water stress but also SA applications enhanced total phenolic contents compared to controls. The plants under moderate stress were sprayed with a solution of 2.5 mM SA showed the highest phenolic contents. Phenolic compounds as stress markers accumulate under stressful environment. It is believed that soluble phenolics compounds act as scavengers of ROS and membrane stabilizer during abiotic stress (Moyer *et al.,* 2002; Taiz and Zeiger, 2006). The increase in total phenolic contents in our study is similar to that observed by El-Danasoury*et al.* (2010) in a study with *Mentha spicata* and Queslati *et al.* (2010) with *Mentha pulegium* under salinity stress. Also, foliar SA application resulted to increase in total phenolic contents in control plants. Foliar SA application at 2 mM was the most effective dose under stress free conditions for peppermint plants. In water stressed plants, foliar spry of SA generally have positive effects on phenolic accumulations. Especially at moderate stress and 2 and 2.5 mM concentrations of SA showed the highest amount of phenolics. Pepper-mint treated with SA or drought stress showed an increase in total phenolic and flavonoid content in our study. This effect was previously reported in ginger (*Zingiber officinale*) plants, where the total phenolic content increased by approximately 20% after treatment with 10^{-3} M SA (Ghasemzadeh and Jaafar, 2012). Also, Figueroa-Pérez *et al*. (2014) previously reported in peppermint plants, where the flavonoids, increased by approximately 93%, 100% and 56% treated with 0.5, 1.0 and 2.0 mM SA, respectively, when compared with the control. Flavonoids were increased in water deficit stress condition and SA treated plants in our study. Flavonoids are the main bioactive secondary metabolites in plants and they can serve as scavengers of ROS by locating and neutralizing radicals before they damage the cells and are thus important for plants under adverse environmental conditions (such as wounding, drought, metal toxicity, and nutrient deprivation) (Løvdal *et al.,* 2010; Agati *et al.,* 2012). Plants often respond to environmental stresses with an increase in the endogenous SA level (Janda *et al*., 2014). SA is a promising compound for the reduction of stress sensitivity in the practical agriculture (Horváth *et al.,* 2007).

ROS enzyme activity

The activities of SOD, CAT and APX enzymes were investigated in the leaves of peppermint plants. SOD, CAT and APX enzymes activities significantly increased in parallel to the water stress in the present study. Activity of SOD was found at 25.17 unit/mg protein, 30.88 unit mg protein^{-1} and 41.79 unit mg^{-1} protein in plants under control, mild and moderate stress, respectively (Table 4). In general, foliar application SA on stressed and non-stressed plants alleviated the activity of SOD. However, 2.5 mM of SA increased the SOD activity in plants under control and moderate stress condition. Also, CAT activity increased in the plants under water deficit stress (Table 4). In control irrigation plants, foliar spray of SA treatments did not show a significant change in CAT activity whereas they substantially decreased the CAT activity in mild and moderate stress. APX activity almost showed the similar trend with SOD activity and its activity increased with water deficit stress, gradually (Table 4). SA application at 2.5 mM concentration had no significant effect in terms of the reducing the APX activity in control and moderate stress treatments. The maximum APX activities were found in under moderate stress without SA application or with 2 mM SA while the highest reductions in APX activity content were recorded in plants treated with 1 mM SA in both mild and moderate water stress treatments. Developing enzymatic and non-enzymatic antioxidant defense mechanisms against reactive oxygen species is the main strategy of plants in stressful condition (Noctor *et al.,* 2002; Gong *et al.,* 2005; Cheeseman, 2007). In this study, ROS enzyme activities enhanced by increasing the intensity of water stress and APX enzyme activity increased with increasing stress conditions and the highest increases occurred in plants under moderate stress condition (Table 4).

4. Conclusions

According to the obtained results, it may be concluded that prolong water stress especially under moderate water deficit stress had negative effect on peppermint. Under water deficit stress, peppermint plants showed a significant decline in growth measurement in term of fresh and dry weights of aerial parts and leaf dry weight while increased antioxidant enzyme activities, the membrane permeability and lipid peroxidation. In addition, water stress enhanced total phenolic and flavonoids contents while significantly decreased the total essential oil content in moderate stress condition (mild stress increased the total essential oil content but, it was not significant).

However, the findings of the present study showed that foliar spray of SA increased grown parameters in non-stress plants while there were no significant effects of SA on water stressed plant. Severity of water stress has a great impact on the physiological and biochemical process of plants. SA applications in this study reduced the severity of both water stress condition and decreased the level of grown parameters reduction rate. SA significantly reduced the water stress-induced negative effects by improving the antioxidant enzyme activities and decreasing the lipid peroxidation and membrane permeability. In terms of the secondary metabolites, SA treatment increased the total phenolic contents in control plants while, especially in plants exposed to water stress, total essential oil content increased. SA contributed to the plant growth and development without any toxic effects and 2 mM of SA significantly increased the essential oil content in non-stressed plants. Also, it was determined that 2 mM of SA for control irrigation and 2 and 2.5 mM of SA for both water stress condition were the optimum concentrations in terms of dry leaf weight, total phenolic contents and essential oil content in peppermint plants.

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