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Alleviating the Adverse Effects of Salt Stress in Rosemary by Salicylic Acid Treatment.

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ABSTRACT

The current study was conducted to investigate the influence of salinity stress, salicylic acids (SA) treatments and their combinations on growth, yield , volatile oil content and some physiological and biochemical characters of (*Rosmarinus officinalis* L.) plant. Salinity levels were 0, 25, 50 and 100 mM NaCl and SA concentrations were 0, 0.2 and 0.4 mM. Salinity treatments significantly decreased fresh and dry weights of herb, relative water content (RWC) compared with the control. The volatile oil percentage was increased with increasing salinity; however the volatile oil yield was not significantly affected. The chlorophyll content was reduced unlike membrane permeability, malondialdehyde (MDA) and proline content which increased as a result of applying salinity. The percentages of N, P, K, and Mg were reduced with increasing salinity concentrations. Meanwhile, Na and Cl were gradually increased with increasing salinity levels. The foliar application of SA alleviated the abovementioned negative effects of salinity on growth, herb and volatile oil yield and the physiological and biochemical characters investigated. The contents of RNA and DNA as well as RNA/DNA ratio were significantly decreased due to salinity application. However, SA treatment increased those parameters relative to the control. The increment of RNA and DNA as well as proline accumulation as a result of SA treatment are suggested to involve as a part of the defense against salinity in rosemary plant.
Keywords: salinity, rosemary, SA, proline, nutrient content, volatile oil, RNA/DNA.

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INTRODUCTION

Soil salinity is one of the most common problems in arid and semiarid regions that negatively affect crop production. Salinity is one of the main stressors limiting plant development and crop productivity worldwide, and could be exacerbated by global climate change. Salinity affects plants at different levels morphological, physiological, and molecular and decreases their growth and development. Furthermore, salinity is the major constraints affecting physiological processes, and their effects may have severe consequences for plant growth and survival in semiarid regions. Crops of arid and semi-arid areas are frequently exposed to high soil salinity [1]. High salinity in the soil results in the accumulation of high levels of Na^+ and Cl^- in plant cells via roots through the symplastic pathway and causes an imbalance in cellular ionic flux inside cells [2]. This ionic flux causes oxidative stress, which influences photosynthesis rate, lipid bilayer functioning, and cellular metabolism. The negative impact on these processes lead to reduced plant growth and crop yield [3]. In addition, Soil salinity induces water stress, nutritional imbalance, specific ion toxicity, hormonal imbalance and generation of reactive oxygen species which may cause membrane destabilization.

Plants respond to stress by activating defense-signaling pathways, producing phytohormones, and adjusting their metabolism [4]. It is well established that salinity inhibits growth and reduces yield in many crop plants [5-7]. The growth parameters, RWC and photosynthetic pigments of rosemary plants were progressively decreased as salt stress increased [5-10]. The treatment of NaCl resulted in MDA accumulation in rosemary leaves relative to the control [9]. Since MDA content of rosemary plants was increased under saline condition, it is regarded as a marker for evaluation of lipid peroxidation that increases with environmental stress [8]. A positive correlation between salt stress and MDA level has been reported Fayez and Bazaid [7], Zou et al. [11] and therefore salinity treatment induced a significant increase in electrolyte leakage and increased the membrane permeability [5,6].

For alleviation of adverse effects of salinity stress, several strategies have been adopted and efforts are made to explore mechanisms for salinity tolerance. Salicylic acid (SA) is a phenolic-type endogenous regulator, which regulates varieties of plants physiological processes [12] and thus alleviates the deleterious effect of various stresses [13]. SA is also considered as a plant growth regulator, which plays an important role in regulating a number of plant physiological processes including photosynthesis and improves the plant growth under salt stress [12]. The exogenous application of SA has been reported to induce tolerance to salt stress [14]. SA alleviated the adverse effect of high salinity by decreasing K^+ leakage from root tissues and by enhancing the H^+-ATPase activity [15], which provides a driving force for Na^+/H^+ exchanger at the plasma membrane and leads to reduced Na accumulation in the cytosol [16]. SA foliar application significantly increased the vegetative growth and herb yield of rosemary plants compared with the control and the values obtained by SA treated plants under stress were significantly higher than those of stressed plants only [17]. Moreover, the volatile oil components of rosemary plants were significantly improved due to SA application relative to the control [18].

Herbs play an important role in maintaining human health and volatile oils have been of great interest due to their antioxidant capacities. Moreover, volatile oils have been considered the sources of natural products and used for many medical products. Rosemary (*Rosmarinus officinalis* L.), belongs to Lamiaceae family is a plant known worldwide as a culinary spice and a natural preservative due to its high antioxidant and antimicrobial activities. Rosemary has long been considered an important plant for its volatile oil used in perfumes and traditional and modern medicine [17]. With the main component of 1,8-cineole (35.8 %) volatile oil of rosemary exhibits some medicinal purposes such as anti-inflammatory, antiseptic, antispasmodic and anti-diabetic [19] . There is limited published research on rosemary growing criteria under salinity conditions despite its popularity and its several uses. Therefore, an attempt was made in the present investigation to study the effects of salinity concentrations, SA levels and their interaction on the herb growth and volatile oil content of rosemary plant. In addition, to provide information for understanding the different mechanisms by which SA can protect the rosemary plants against salt stress. The effects of salinity and SA on some biochemical, physiological and molecular changes on rosemary plant were also investigated.

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MATERIALS AND METHODS

Plant material

Pot experiment was conducted at the greenhouse of Biology Department, Faculty of Science, Taif University, Saudi Arabia during 2014 and 2015 seasons. Stem cuttings of rosemary (*Rosmarinus officinalis*, L.) were sown in the nursery and two months later, homogenous seedlings were transplanted into (30 x 20 cm) pots contained sandy soil. The physical analysis of soil was (Sand, 83.45%; Silt, 6.70%; Clay, 9.85%) and the chemical analysis was (Organic Matter, 0.12% ; pH, 8.17; E.C, 2.14 dSm^{-1} ; $CaCO_3$, 0.91% ; Total N^+,0.16%; Total PO_4^{-3}, 0.041%; Total K^+,0.047% ; Ca^{+2},42.18 ($meqL^{-1}$);Total SO_4^{-2}, 49.32 ($meqL^{-1}$); HCO_3, 2.10 ($meqL^{-1}$);Cl^-,0.59 ($meqL^{-1}$) according to Hassan et al. [17].

Salinity and SA treatments

Plants subjected to saline irrigation water after 14 days from transplanting. To prepare irrigation water with different salinity levels, NaCl salt was used. Salinity treatments used in this experiment were 0, 25, 50 and 100 mM . The salinity levels were obtained by addition of appropriate amount of NaCl to water and were adjusted by a portable EC meter instrument. Plants were subjected to saline irrigation water every 7 days using 0.5 L irrigation water per pot and pots were flushed out with saline water every two weeks to ensure homogeneity of salinity and to prevent the induction of salt build up. Irrigation started with 25 mM saline water and was increased by 25 mM every other irrigation time until reaching the exact salinity level to prevent shock to plants. Water content of the pots was maintained at 80 % field capacity with distilled water till the end of the experiment. Control plants were irrigated using 0.5 L tap water at the same period of salinity treatments.

Salicylic acid (SA; 2-hydroxybenzoic acid) was initially dissolved in 100 mL dimethyl sulfoxide and 0, 0.2 and 0.4 mM SA (pH 6.5) were prepared with distilled water containing 0.02 % Tween 20. The treatments applied as foliar spray and were started one week after salinity treatment. Spraying with SA was applied weekly in the early morning till the end of the experiment. Control plants were sprayed with tap water containing 0.02 % Tween 20 only. The salinity and SA treatments arranged in factorial design contained 12 treatments (4 x 3) with three replicates each. The experiment was repeated twice and data was combined.

Fresh and dry weight determination

Sample of fresh weight of herb was measured and then oven-dried at 70°C for 48 h till constant weight to determine the dry weight. It has been expressed as (g).

Relative water content (RWC %)

RWC was determined and calculated according to Weatherley [20] by the following relationship: $(W_{fresh}- W_{dry})/(W_{turgid}- W_{dry})$ x 100, where W_{fresh} is the sample fresh weight, W_{turgid} is the sample turgid weight after saturating with distilled water for 24 h at 4°C, and W_{dry} is the oven-dry (70°C for 48 h) weight of the sample.

Volatile oil %

The volatile oil percentages in rosemary leaves obtained from each replicate of every treatment were determined by a water distillation method described in British Pharmacopea [21], using the following equation: Volatile oil percentage = oil volume in the graduated tube / fresh weight of sample x 100. Then, the oil yield /plant was calculated.

Chlorophyll content

Samples of fresh leaves were taken for chlorophyll determination. Extraction in acetone was repeated until all pigments were extracted. Chlorophyll content was determined in samples according to Sadasivam and

Manickam [22]. The absorbance of extracts was determined by a spectrophotometer (type Pharmacia, LKB-Novaspec II). The chlorophyll content was calculated as mg g^{-1} fresh weight.

Membrane permeability

Membrane permeability of the excised leaves was measured by the method of Yan et al. [23]. Fresh part from the middle of leaves was weighed into a glass beaker containing reverse osmosis water. The beakers were immersed at 30 ± 1˚C for 3h, and then the conductivity of the solution was measured with a conductivity meter. The conductivity was measured again after boiling the samples for 2 min when the solution was cooled to room temperature. The percentage of electrolyte leakage was calculated using the equation, EC % = (C1/C2) X 100, since C1 and C2 are the electrolyte conductivities measured before and after boiling, respectively.

Malondialdehyde determination

MDA content was determined as described by Hodges et al. [24]. Fresh samples of leaves (0.2 g) were homogenized with 2 mL of 0.1 % trichloroacetic acid (TCA) and centrifuged at 14,000 rpm for 15 min. Two mL of the obtained supernatant were mixed with 3 ml 0.5 % TBA in 5 % TCA and incubated in hot water (95 °C) for 30 min. Then, it was immediately cooled on ice to stop the reaction and centrifuged at 5000 rpm for 15 min. The supernatant was spectrophotometrically detected at 450, 532 and 600 nm. The concentration of MDA was estimated by using the formula: MDA content = 6.45 × (A532 - A600) - 0.56 × A450, where A450, A532 and A600 are the absorbance at 450, 532 and 600 nm, respectively and were expressed as µmol mL^{-1}.

Proline content (µmol g^{-1} FW)

The free proline content was determined as described by Bates et al. [25]. Frozen leaf tissue (0.5 g) was homogenized with 10 mL of 3% sulfosalicylic acid at 4°C. Then, the obtained extract was filtered with Whatman No. 2. Mixture of 2 mL of filtrate, 2 mL of acid- ninhydrin, and 2 mL of glacial acetic acid were mixed in a test tube and incubated at 100°C for 1h. The reaction was terminated on ice, and the reaction mixture was then extracted with 4 mL of toluene. The chromophore-containing toluene was separated from the hydrated phase. The absorbance at 520 nm was spectrophotometrically determined with toluene as the blank. The proline concentration was calculated based on a standard curve and was expressed as µmol g^{-1} FW.

Nutrient elements

Nitrogen, phosphorus, potassium, magnesium, sodium and chloride were determined as described in A.O.A.C. [26].

Nucleic acids extraction and determination

Genomic DNA was isolated from leaf tissue using the Plant Genomic DNA extraction kit Promega according to the manufacturer's procedure. Total RNA was isolated from leaf tissues using Trizol method according to the manufacturer's procedure. DNA and RNA were quantitatively determined using (Genesys 10 UV) at 260 nm. The purity of DNA and RNA was calculated as OD260 / OD280 ratio.

Statistical analysis

The obtained data were subjected to statistical analysis using MSTAT program, USA. The analysis of variance (ANOVA) was performed to compare means. Data was combined for two experiments (n=6). Means were separated using LSD test at a significance level of 0.05.

RESULTS

Herb fresh and dry weights

Increasing salinity level from 0 to 100 mM resulted in a gradual decrease in plant fresh and dry weight and the differences were significant compared with the control. Meanwhile, both SA applications significantly increased the fresh and dry weight per plant relative to untreated plants and

the treatment of 0.4 mM was more effective in this regard than 0.2 mM (Table 1). Moreover, the combination between salinity and SA levels minimized the reduction of fresh and dry weight occurred due to salinity. This promotion effect of SA in fresh and dry weight was observed under any salinity level.

Table 1. Effect of salinity levels, salicylic acid (SA) treatments and their combination on fresh & dry weights (g) and RWC% of rosemary plant

Salinity (mM)	SA (mM)	Fresh weight (g)	Dry weight (g)	(RWC) %
0		55.67 ± 1.71	10.81 ± 0.08	87.56 ± 0.77
25		53.78 ± 1.4	10.28 ± 0.15	86.67 ± 0.58
50		50.78 ± 1.53	9.87 ± 0.15	83.67 ± 0.77
100		45.22 ± 0.89	8.96 ± 0.31	79.33 ± 1.03
LSD at P ≤ 0.05 %		**1.36**	**0.13**	**0.54**
	0	46.50 ± 5.28	9.24 ± 0.41	81.75 ± 3.26
	0.2	51.58 ± 4.96	10.07 ± 0.46	84.75 ± 1.87
	0.4	56.00 ± 4.35	10.63 ± 0.47	86.42 ± 1.98
LSD at P ≤ 0.05 %		**0.99**	**0.20**	**0.60**
	0	50.67 ± 2.08	10.39 ± 0.03	86.67 ± 1.1
0	**0.2**	56.67 ± 1.5	10.77 ± 0.1	87.33 ± 0.5
	0.4	59.67 ± 1.5	11.26 ± 0.1	88.67 ± 0.5
	0	49.33 ± 1.5	9.40 ± 0.15	85.67 ± 0.6
25	**0.2**	53.67 ± 1.5	10.40 ± 0.14	86.67 ± 0.5
	0.4	58.33 ± 1.1	11.04 ± 0.14	87.67 ± 0.5
	0	45.67 ± 1.5	9.07 ± 0.11	80.33 ± 1.1
50	**0.2**	50.33 ± 1.5	10.05 ± 0.11	84.33 ± 0.5
	0.4	56.33 ± 1.5	10.49 ± 0.20	86.33 ± 0.5
	0	40.33 ± 0.5	8.09 ± 0.3	74.33 ± 1.5
100	**0.2**	45.67 ± 1.5	9.04 ± 0.4	80.67 ± 0.5
	0.4	49.67 ± 0.5	9.74 ± 0.1	83.00 ± 1
LSD at P ≤ 0.05 %		**1.9**	**0.40**	**1.44**

Relative water content (%)

RWC was gradually decreased with increasing salinity concentrations, A sharp decrease was observed especially with the highest salinity level (100 mM) was applied. On the other hand, applying SA whether at (0.2 or 0.4 mM) resulted in a significant increase in RWC relative to the control (Table 1). However, the negative effect of salinity on RWC was alleviated when SA was used under any salinity level. The alleviation effect of SA at 0.4 mM was clearer than 0.2 mM .

Volatile oil percentage

A gradual increase in volatile oil percentage was observed with increasing salinity level. The highest and significant percentage was obtained by applying salinity at 100 mM relative to the control (Table 2). A promotion effect on volatile oil was also recorded when SA was used. The highest SA dose (0.4 mM) gave the highest volatile oil percentage (1.04%) as compared with control (0.91%). When

salinity was combined with SA, the volatile oil percentage was increased as well. The highest volatile oil percentage (1.32%) was recorded when plants sprayed with SA at 0.2 mM under 100 mM NaCl.

Table 2. Effect of salinity levels, salicylic acid (SA) treatments and their combination on Volatile oil percentage, volatile oil yield, Chlorophyll content of rosemary plant

Salinity (mM)	SA (mM)	Volatile oil % (%)	Volatile oil yield (mL/plant)	Chlorophyll (mg g^{-1} FW)
0		0.89 ± 0.02	0.50 ± 0.02	1.22 ± 0.02
25		0.95 ± 0.02	0.51 ± 0.02	1.18 ± 0.01
50		1.00 ± 0.02	0.51 ± 0.02	1.13 ± 0.03
100		1.15 ± 0.16	0.52 ± 0.06	0.89 ± 0.02
LSD at P ≤ 0.05 %		**0.14**	**0.06**	**0.04**
	0	0.91 ± 0.05	0.32 ± 0.04	1.01 ± 0.07
	0.2	1.04 ± 0.15	0.39 ± 0.09	1.89 ± 0.06
	0.4	1.04 ± 0.06	0.44 ± 0.07	2.16 ± 0.05
LSD at P ≤ 0.05 %		**0.10**	**0.05**	**0.01**
	0	0.84 ± 0.02	0.43 ± 0.01	1.16 ± 0.02
0	**0.2**	0.87 ± 0.01	0.49 ± 0.007	1.22 ± 0.02
	0.4	0.96 ± 0.02	0.57 ± 0.02	1.28 ± 0.005
	0	0.88 ± 0.01	0.43 ± 0.008	1.12 ± 0.01
25	**0.2**	0.95 ± 0.01	0.51 ± 0.01	1.18 ± 0.01
	0.4	1.01 ± 0.02	0.59 ± 0.02	1.24 ± 0.01
	0	0.94 ± 0.01	0.43 ± 0.01	0.99 ± 0.03
50	**0.2**	1.01 ± 0.02	0.51 ± 0.02	1.05 ± 0.02
	0.4	1.05 ± 0.01	0.59 ± 0.02	1.13 ± 0.02
	0	0.99 ± 0.02	0.4 ± 0.008	0.76 ± 0.02
100	**0.2**	1.32 ± 0.41	0.6 ± 0.1	0.91 ± 0.02
	0.4	1.13 ± 0.02	0.56 ± 0.01	0.99 ± 0.02
LSD at P ≤ 0.05 %		**0.21**	**0.09**	**0.01**

Volatile oil yield

Data presented in Table (2) indicate that the volatile oil yield/plant was slightly increased as a result of increasing salinity level from (0 to 100 Mm) and there were no significant difference between any salinity treatment and control in this respect. However, SA treatment resulted in a significant increase in the volatile oil yield/plant compared with the control. When plants grown under different salinity treatments and interacted with SA application, an improvement of volatile oil yield were observed compared with SA non-treated plants.

Chlorophyll content

All salinity treatments significantly reduced total chlorophyll content in rosemary leaves compared with the control and this reduction was gradually with increasing salinity level from 25 to 100 mM (Table 2). On the other hand, SA treatment showed significant positive role on chlorophyll content since it was increased as a result of applying any SA concentrations. Concerning the interaction between salinity and SA treatments, indicate that applying SA treatment maintained the total chlorophyll content on plants treated with any salinity level. Moreover the adverse effects of salinity were ameliorated due to any SA dose.

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Membrane permeability

The obtained results show that membrane permeability was significantly increased with increasing salinity levels relative to the control and reached its maximum value by applying the highest salinity level (100 mM). However, SA treatments resulted in a significant and gradual reduction of the membrane permeability compared with untreated plants (Table 3). Moreover, the interacted treatment of SA and NaCl reduced the membrane permeability occurred due to salinity. Therefore, SA treatment alleviated the negative effect of salinity on membrane permeability.

Table 3. Effect of salinity levels, salicylic acid (SA) treatments and their combination on Membrane permeability (%), Malondialdehyde content (MDA), Proline content (µmol g^{-1} FW) of rosemary plant

Salinity (mM)	SA (mM)	Membrane permeability (%)	Malondialdehyde content (µmol mL-1)	Proline content (µmol g-1 FW)
0		8.39 ± 0.13	0.52 ± 0.01	1.61 ± 0.03
25		9.74 ± 0.09	0.66 ± 0.02	1.93 ± 0.04
50		11.77 ± 0.09	0.77 ± 0.02	2.06 ± 0.02
100		13.34 ± 0.1	0.94 ± 0.03	2.13 ± 0.02
LSD at P ≤ 0.05 %		**0.19**	**0.03**	**0.03**
	0	12.61 ± 0.36	0.87 ± 0.07	1.87 ± 0.15
	0.2	10.67 ± 0.41	0.69 ± 0.03	1.94 ± 0.05
	0.4	9.15 ± 0.22	0.61 ± 0.04	1.98 ± 0.08
LSD at P ≤ 0.05 %		**0.07**	**0.01**	**0.03**
	0	9.66 ± 0.1	0.54 ± 0.01	1.57 ± 0.06
0	**0.2**	8.31 ± 0. 1	0.52 ± 0.05	1.63 ± 0.01
	0.4	7.19 ± 0.09	0.50 ± 0.05	1.64 ± 0.01
	0	11.4 ± 0.01	0.75 ± 0.02	1.87 ± 0.05
25	**0.2**	9.70 ± 0.06	0.64 ± 0.01	1.94 ± 0.02
	0.4	8.12 ± 0.04	0.57 ± 0.01	1.99 ± 0.03
	0	13.71 ± 0.05	0.96 ± 0.02	1.99 ± 0.03
50	**0.2**	11.45 ± 0.15	0.72 ± 0.05	2.07 ± 0.01
	0.4	10.14 ± 0.06	0.63 ± 0.02	2.12 ± 0.02
	0	15.65 ± 0.08	1.22 ± 0.04	2.06 ± 0.03
100	**0.2**	13.21 ± 0.14	0.86 ± 0.02	2.13 ± 0.02
	0.4	11.15 ± 0.08	0.74 ± 0.02	2.19 ± 0.02
LSD at P ≤ 0.05		**0.14**	**0.01**	**0.05**

Proline content (µmol g^{-1} FW)

The results about the effects of salinity and SA levels on proline content were presented in Table (3). All salinity treatments significantly improved the proline content in rosemary leaves compared with the control. The proline content was gradually increased with increasing salinity level. In the same time, both SA levels resulted in a significant increase in proline content compared with untreated plants. Moreover, the interacted treatment of NaCl and SA increased the proline content in rosemary leaves compared with the individual application with salinity or SA treatment.

Malondialdehyde content (MDA)

Data presented in Table (3) show that MDA content in rosemary leaves were significantly increased with increasing salinity level relative to the control. On the other hand, a significant reduction of MDA content in leaves was observed by foliar application with SA compared with the control. Concerning the interaction between salinity and SA treatments, the results indicate that both SA treatments decreased the accumulation of MDA which occurred due to salinity and 0.4 mM SA was superior than 0.2 mM in this concern under any salinity level.

RNA, DNA and RNA/DNA ratio

The results show that the contents of RNA, DNA and RNA/DNA ratio were significantly decreased as a result of applying any salinity level compared with the control in Table (4). On the other hand, both of SA levels significantly increased RNA, DNA and RNA/DNA ratio in rosemary leaves relative to the control. Foliar spraying with SA at 0.2 or 0.4 mM increased RNA/DNA ratio in rosemary leaves under any salinity level. There is no any significant difference between both levels of SA in this respect. The positive effects of SA on RNA/DNA ratio was more pronounced when the highest salinity level (100 mM NaCl) was applied.

Table 4. Effect of salinity levels, salicylic acid (SA) treatments and their combination on RNA and DNA contents as well as RNA/DNA ratio of rosemary plant

Salinity (mM)	SA (mM)	RNA content (mgg^{-1} DW)	DNA content (mgg^{-1} DW)	RNA/DNA ratio
0		1.71	0.18	9.50
25		0.99	0.14	7.05
50		0.68	0.10	6.55
100		0.36	0.07	5.40
LSD at P ≤ 0.05 %		**0.04**	**0.02**	**0.36**
	0	0.68	0.10	6.80
	0.2	0.94	0.12	7.83
	0.4	1.18	0.14	8.43
LSD at P ≤ 0.05 %		**0.03**	**0.01**	**0.56**
	0	1.08	1.14	7.71
0	**0.2**	1.76	1.18	9.78
	0.4	2.29	0.22	10.41
	0	0.87	0.13	6.69
25	**0.2**	0.97	0.14	6.93
	0.4	1.12	0.15	7.47
	0	0.55	0.09	6.11
50	**0.2**	0.65	0.10	6.50
	0.4	0.83	0.12	6.92
	0	0.22	0.05	4.40
100	**0.2**	0.37	0.07	5.29
	0.4	0.49	0.08	6.13
LSD at P ≤ 0.05 %		**0.05**	**0.03**	**0.63**

Nutrient elements

Data presented in Tables (5 and 6) indicate that salinity treatments were affected the nutrient elements in rosemary leaves. The percentages of N, P, K and Mg were significantly decreased due to salinity treatment compared with the control. Meanwhile, salinity treatment significantly increased Na and Cl contents in rosemary leaves relative to untreated plants. On the other hand, SA treatment significantly increased the N,

P, K and Mg percentages relative to the control. Moreover, SA treatment markedly decreased Na and Cl contents in rosemary leaves compared with the control and the reduction was gradually with increasing SA level. Concerning the interaction between salinity and SA treatments, it could be observed that SA treatment ameliorated the adverse effects of salinity on nutrient contents in rosemary leaves. Both SA treatments improved the percentages of N, P, K and Mg under any salinity level compared with SA-untreated plants. In addition, the contents of Na and Cl were significantly reduced as a result of SA treatment in salt treated plants and the accumulation of Na and Cl occurred by salinity was prevented due to SA application and also reduced Na:K ratio compared with the control under 0.4 mM SA treatment.

Table 5. Effect of salinity levels, salicylic acid (SA) treatments and their combination on N, P and K percentages of rosemary plant

Salinity (mM)	SA (mM)	N (%)	P (%)	K (%)
0		1.91 ± 0.02	0.42 ± 0.01	2.15 ± 0.02
25		1.83 ± 0.01	0.40 ± 0.01	1.93 ± 0.01
50		1.75 ± 0.02	0.36 ± 0.01	1.90 ± 0.02
100		1.69 ± 0.01	0.34 ± 0.01	1.80 ± 0.02
LSD at P ≤ 0.05 %		**0.04**	**0.01**	**0.01**
	0	1.72 ± 0.06	0.35 ± 0.04	1.90 ± 0.07
	0.2	1.82 ± 0.05	0.38 ± 0.03	1.95 ± 0.04
	0.4	1.85 ± 0.04	0.41 ± 0.03	1.98 ± 0.04
LSD at P ≤ 0.05 %		**0.01**	**0.01**	**0.01**
	0	1.86 ± 0.03	0.39 ± 0.01	2.11 ± 0.02
0	**0.2**	1.92 ± 0.02	0.42 ± 0.01	2.16 ± 0.01
	0.4	1.97 ± 0.01	0.45 ± 0.01	2.19 ± 0.05
	0	1.77 ± 0.01	0.36 ± 0.01	1.90 ± 0.01
25	**0.2**	1.84 ± 0.01	0.41 ± 0.01	1.94 ± 0.01
	0.4	1.89 ± 0.01	0.43 ± 0.005	1.96 ± 0.01
	0	1.67 ± 0.02	0.33 ± 0.01	1.84 ± 0.02
50	**0.2**	1.77 ± 0.02	0.37 ± 0.01	1.9 ± 0.01
	0.4	1.81 ± 0.01	0.39 ± 0.005	1.94 ± 0.02
	0	1.59 ± 0.01	0.31 ± 0.01	1.76 ± 0.03
100	**0.2**	1.76 ± 0.01	0.34 ± 0.01	1.81 ± 0.01
	0.4	1.71 ± 0.01	0.37 ± 0.01	1.84 ± 0.05
LSD at P ≤ 0.05 %		**0.01**	**0.01**	**0.02**

Table 6. Effect of salinity levels, salicylic acid (SA) treatments and their combination on Mg, Na, Cl contents and Na/K ratio of rosemary plant

Salinity (mM)	SA (mM)	Mg (mgg^{-1} FW)	Na (mgg^{-1} FW)	Cl (mgg^{-1} FW)	Na/K ratio
0		0.73 ± 0.01	2.85 ± 0.07	4.13 ± 0.06	1.3
25		0.71 ± 0.01	3.11 ± 0.05	5.33 ± 0.09	1.6
50		0.65 ± 0.01	3.62 ± 0.06	6.27 ± 0.11	1.9
100		1.62 ± 0.01	4.46 ± 0.09	7.72 ± 0.14	2.4
LSD at P ≤ 0.05 %		**0.01**	**0.08**	**0.16**	**0.01**

	0	0.64 ± 0.04	4.79 ± 0.28	7.84 ± 0.37	2.5
	0.2	0.69 ± 0.03	3.02 ± 0.21	5.15 ± 0.30	1.5
	0.4	0.71 ± 0.02	2.72 ± 0.11	4.60 ± 0.17	1.3
LSD at P ≤ 0.05 %		**0.008**	**0.61**	**0.07**	**0.01**
	0	0.71 ± 0.01	3.09 ± 0.1	4.25 ± 0.07	1.47
0	**0.2**	0.73 ± 0.01	2.88 ± 0.09	4.12 ± 0.07	1.33
	0.4	0.75 ± 0.01	2.58 ± 0.02	4.03 ± 0.02	1.18
	0	0.68 ± 0.01	3.65 ± 0.06	6.99 ± 0.1	1.92
25	**0.2**	0.71 ± 0.01	3.00 ± 0.04	4.84 ± 0.07	1.55
	0.4	0.74 ± 0.005	2.67 ± 0.02	4.17 ± 0.04	1.36
	0	0.61 ± 0.01	4.96 ± 0.08	9.08 ± 0.1	2.70
50	**0.2**	0.66 ± 0.01	3.08 ± 0.05	5.28 ± 0.1	1.62
	0.4	0.68 ± 0.005	2.83 ± 0.06	4.47 ± 0.08	1.46
	0	0.57 ± 0.01	7.46 ± 0.1	11.05 ± 0.1	4.23
100	**0.2**	0.64 ± 0.01	3.13 ± 0.09	6.38 ± 0.1	1.73
	0.4	0.66 ± 0.01	2.78 ± 0.02	5.74 ± 0.1	1.52
LSD at P ≤ 0.05 %		**0.01**	**0.12**	**0.15**	**0.01**

DISCUSSION

In this study, the RWC, herb fresh weight and herb dry weight of rosemary plant was negatively affected due to salinity treatments. The RWC, herb fresh weight and herb dry weight were significantly and gradually decreased with increasing salinity levels compared with the control. Reducing RWC leaves as our data indicated may be also a possible explanation for growth reduction of rosemary because it considers as an important parameter for water statues. As a result of a reduction in RWC under salt stress, a loss of turgor was occurred and resulted in limited water availability for the cell extension process [27]**.** These results support the previous results obtained by Ali et al. [5] on rose and Yu et al. [10] on mint plants.

On the other hand, SA treatment alleviated the deleterious effects of salinity on growth of rosemary plant. SA has been shown as an important signal molecule for modulating plant responses to environmental stress. In addition to facilitating the growth of plant, SA has been shown to play a role in mitigating the deleterious effects of some environmental stresses [28]**.** The results of this study have shown that SA treatment increased RWC as well as fresh and dry weights of herb. These results are in accordance with the Coronado et al. [29] who reported that foliar spray of SA significantly increased the growth of shoots in soybean. Further, SA treated maize plants showed higher dry mass as compared to those of untreated seedlings grown under salt stress [30]. Misra and Saxena [31] have reported that the increase in dry matter of salt stressed plants in response to SA may be ascribed to the induction of protective role of membranes that increase the tolerance of plant to damage. Increasing RWC may be attributed to the fact that foliar SA application can increase the leaf diffusive resistance and lower transpiration rates [32] or the role of SA in accumulation of compatible osmolytes in plants subjected to stress [33]. The inhibition on RWC in the leaves of NaCl treated seedlings was dramatically alleviated by the exogenous application of SA [33-35].

The results of this study show also that although salinity treatments increased the volatile oil percentage of rosemary, there were no significant differences concerning volatile oil yield/plant (Table 2). It has been suggested that under stress a higher density of oil glands due to the reduction in leaf area results in an elevated amount of volatile oil accumulation [36]. Antioxidant producer crops like rosemary may increase antioxidant phenolic compound production, and may lead to an economic advantage over regular water irrigation [37] and plants use these antioxidants to protect themselves against drought damage [38]. Otherwise, increasing volatile oil percentage by salinity may be also due to the increment in total soluble sugars as our data indicated since volatile oils are formed as secondary metabolites. On the other hand, higher herb yield obtained from control plants or lower salinity level may be a reason for obtaining higher volatile oil yield/plant. Similar trend on different aromatic plants has been documented [6]. Meanwhile, SA treatment increased the volatile oil percentage and yield of rosemary plant in current study. Similarly, Gharib [39] foliar

spray of both basil and marjoram plants with SA increased essential oil percentage and yield per plant. This increment might be due to the increase in vegetative growth, changes in leaf oil gland population, carbohydrates content and the beneficial effect of SA on metabolism and enzymes activities responsible for mono or sesqueterpene-biosynthesis [39].

In current study, the chlorophyll content of rosemary leaves was reduced as a result of salinity treatments. This reduction could be explained through one or more mechanisms from the following: a reduction in the uptake of minerals i.e. Mg needed for chlorophyll biosynthesis [40], membrane deterioration [41], the salt-induced water stress reduction of chloroplast stoma volume and regeneration of reactive oxygen species which play an important role in the inhibition of photosynthesis seen in salt-stressed plants [42], damaging to the photosynthetic apparatus [43] or the suppression of specific enzymes that are responsible for the synthesis of photosynthetic pigments [44]. The results obtained in this study support the first two reasons because we observed a significant decrease in Mg and increase in membrane permeability under salt stress. Reduction of photosynthetic pigments under stress could be also related to degradation of chloroplast structure and photosynthetic apparatus, chlorophyll photo oxidation, destruction of chlorophyll substrate, inhibition of chlorophyll biosynthesis, and the increase of chlorophyllase activity [33]**.** SA treatment however, positively affected the total chlorophyll content and ameliorated the adverse effects of salinity. In this regard, Singh and Usha [45] found that chlorophyll content increased significantly with SA application under stress as compared to the stressed plants without SA. The application of SA improved barley plant growth by promoting protective reactions involving the photosynthetic pigments and maintaining membrane integrity [46]. Strawberry plants treated with SA exhibited greater growth, as did higher chlorophyll concentrations under salt stress [32]. Exogenously applied SA increased the photosynthetic rate in different crops, *Nigella sativa* [33] and bean [34].

In this experiment, the salinity treatment led to a significant increase in membrane permeability compared with the control (Table 3). These results may be due to the negative effects of salinity on Ca^{+2} level since Ca^{+2} is required to improve membrane stability [47]. Also, Serrano et al [48] found that when salinity results from the excess of NaCl, which is by far the most common type of salt stress, the increased intercellular concentration of Na^{+} and Cl^{-} is deleterious to cellular systems. Increasing membrane permeability as a result of salinity treatment has been previously reported [5, 6, 49]. On the other hand, SA treatment maintained the membrane stability and hence ameliorated the negative effects of salinity. It has been reported that SA treatment reduced the amount of ion leakage (measured as electrolytes) in salt stressed rosemary seedlings indicating that SA treatment has facilitated the maintenance of membrane functions under stress conditions [50]. Exogenous application of salicylic acid enhanced the photosynthetic rate and also maintained the stability of membranes, thereby improved the growth of salinity stressed barley plants [46]. Further, the lipid peroxidation and membrane permeability were decreased by SA in maize under salinity stress, leading to the enhancement of plant growth [30]**.** The protective role of SA in membrane integrity and regulation of ion uptake has also been reported [30].

In view of our results, salinity treatment increased lipid peroxidation and hence increased membrane permeability (Table 3). It is important to mention that reduced lipid peroxidation and retained membrane stability have been demonstrated to be inversely proportional with salt stress. These results are in agreement of Kiarostami et al. [8] and Tounekti et al [9] on *Rosmarinus officinalis* L. plants who reported that the treatment of NaCl resulted in MDA accumulation in leaves relative to the control. Hence, MDA is regarded as a marker for evaluation of lipid peroxidation that increases with environmental stress. In this regard, Fayez and Bazaid [7] reported that there was a positive correlation between salt stress and MDA level in barley plants. The MDA content of leaves was significantly increased with increasing salt doses. Similar results have been previously reported [11, 51, 52]. Unlike salinity, SA treatment reduced MDA content and decreased its level indicates reduced lipid peroxidation. Reduced lipid peroxidation participates to maintained membrane stability in response to SA treatment. Such effect of SA as lipid peroxidation reduction and maintained cell stability was previously reported by [35, 53, 54]**.**

The results of current study also show that the proline content in rosemary leaves was increased as a result of salinity treatment. It has been reported that proline may play a role in stress adaptation within the cell [55]**.** Proline plays a protective function against salinity stress in plants [56, 57]. It acts as a compatible osmolyte, enzyme protectant, free radical scavenger, cell redox balancer, cytosolic pH buffer and stabilizer for subcellular structures to bring about salinity tolerance [56, 57]. It has been reported that proline acts as an

osmoprotectant and plays vital role in balancing osmotic stress. It protects sub-cellular structures, enzymes and increases cellular osmolarity, probably by scavenging ROS that provides the turgor necessary for cell expansion under stress treatments [49]. Otherwise, Jaleel et al [58] stated that one of the mechanisms to contribute salt tolerance is the compatible solutes accumulation of such as proline in the cytoplasm which provides an environment compatible with the macromolecular structure and function and helps to adapt the salinity injury. Such proline accumulation as a result of salt stress is well documented [51, 52].

A substantial increase in proline levels with both treatments (SA and NaCl), might be attributed to the strategies adapted by plants to cope up with stress conditions and this accumulation of proline under stress conditions has been correlated with stress tolerance [59]. The interaction treatment of SA and NaCl had an additive effect on increasing proline content and antioxidant enzymes and hence the stress generated by NaCl was alleviated [60]. It has been reported that, the higher accumulation of proline under stress conditions was attributed to enhanced activities of proline biosynthesis enzymes, ornithine aminotransferase and pyrroline-5-carboxylate reductase, as well as due to inhibition of proline degradation enzymes, proline oxidase and proline dehydrogenase [56]. These results are in accordance with Liu et al. [35] and Hassan and Ali [54] who reported that foliar application of SA increased proline content under stress.

Applying salinity treatments decreased N, P, K, Ca and Mg contents however Na and Cl were increased. Decreasing N under salinity treatment has been previously reported [61]. Moreover, reduction of P uptake in saline soils was attributed to precipitation of $H_2PO_4$with Ca^{2+} ions in soil and of K and Ca to a competition with Na [62]. The reduction of K percentage could be explained through the competition exists between Na^+ and K^+ leading to a reduced level of internal K^+ at high external NaCl concentration [63]. Increasing Na and Cl absorption under salinity in this study is agreeing with Turan et al. [64]. Moreover, the accumulation of NaCl disturbed the homeostasis not only Na^+ and Cl^- but also of essential cations such as K^+ and Ca^{2+} Roussos et al. [65] and hence a decrease in K^+ and Ca^{2+} in rose leaves was observed. In a recent study of Caia et al [66] they concluded that salinity treatment enhances the accumulation of leaf Na^+ and Cl^- ions, thereby reducing plant growth rate and hence minimizing the ion uptake by the roots and ion accumulation in the shoots are important mechanisms of salt tolerance. SA treatment also stimulated the uptake of N, P, K and Mg by rosemary plants even under salt stress. Otherwise, Na and Cl were significantly reduced due to SA application. These results are in agreement of Grattan and Grieve [67] who found that SA application stimulated N, P, K and Mg uptake but inhibited Na accumulation. They added that this effect of SA on mineral uptake could ameliorate the deleterious effects of salinity on growth and yield. Therefore, alteration of mineral uptake from SA applications may be one mechanism for the alleviation of salt stress [32].

In this experiment, increasing salinity level resulted in a significant reduction in both RNA and DNA and hence a reduction in RNA/DNA ratio was observed. In this regard, Bulow [68] reported that RNA/DNA ratio is believed to provide estimates of growth because protein synthesis is intensive during active growth and cell enlargement. RNA is directly involved in protein synthesis and therefore increases in RNA content are observed during periods of rapid growth, whereas DNA content is usually stable making the RNA/DNA ratio an indicator of protein synthesis capacity per cell. The RNA/DNA ratio is thus a frequently measured indicator of growth rate. The obtained results concerning RNA/DNA ratio are comparable to those of vegetative growth of rosemary plants. In addition, Humphrey [69] reported that RNA/DNA ratio is a measure of protein synthesis that has been used as a biochemical biomarker of growth reflecting a general response to environmental stress. Therefore, increasing salinity level decreased RNA/DNA ratio and consequently decreased the vegetative growth. These results support the findings of Reef et al. [70] who reported that RNA/DNA ratio may be a good indicator of growth rates and can reliably predict interspecific differences in growth rates. The correlation between vegetative growth and RNA/DNA ratio under salinity has been previously reported [71]. Their major function is to maintain the redox balance in vivo and protect plants from oxidative damage under abiotic stress [72]. On the other hand, the increase in DNA and RNA in rosemary plants in response to SA treatment could be attributed to the enhancement of nucleic acid biosynthesis and/or inhibition of their degradation [73].

As a conclusion, from the results of this study it could be concluded that the foliar application of SA alleviated the negative effects of salinity on RWC, herb fresh weight and herb dry weight and the physiological and biochemical characters investigated. SA treatment increased the content of nucleic acids (RNA and DNA) and proline as well as prevented ion homeostasis which may consider possible mechanisms for salinity tolerance in rosemary plant.

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