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Exogenous Salicylic Acid Ameliorates the Adverse Effects of Salt Stress on Antioxidant System in *Rosmarinus officinalis* L

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SALT stress is a main factor limiting plant growth and productivity. Salicylic acid (SA) has been shown to alleviate the adverse effects of different environmental stresses on plants. To investigate the protective role of salicylic acid (SA) in alleviating salt stress on *Rosmarinus officinalis* L. (rosemary) plant, a pot experiment was conducted to measure the growth parameters such as plant height and branch number. An antioxidant defense system, represented by catalase (CAT), superoxide dismutase (SOD) and peroxidase (POX), was evaluated at both biochemical and transcriptional levels. NaCl was applied at 0, 25, 50 and 100mM and 0, 0.2 and 0.4mM of SA were used. The exposure of rosemary plant to salt conditions resulted in significant reduction of plant height, branches number and increases in the three measured antioxidant enzyme activities, compared to control. The foliar application of SA effectively increased growth rates and enhanced the activities of CAT, SOD and POX enzymes. The PCR (RT-PCR) analysis revealed that the relative transcript levels of SOD, CAT, and POX genes were changed and up-regulated compared to the control due to NaCl stress and SA treatments, reached the highest expression level by applying 25mM NaCl and 0 SA treatments. The results implied that SA regulated the transcript levels of the antioxidant genes, resulting in the increased contents of antioxidant enzymes and enhanced salt tolerance.

Keywords: Salinity, Rosemary, Antioxidant enzymes, Gene expression.

Introduction

Salt stress is one of the fundamental limiting factors in agricultural crop production throughout the world. It causes water deficit, ion toxicity, and nutrient deficiency, leading to growth, yield and development reduction, and even to plant death (Gezi et al., 2013). Additionally, it has a negative impact on photosynthesis, water management and enzymatic activity (Salachna et al., 2017). It has been reported that salinity reduces plant productivity by reducing plant growth during the phase of osmotic stress and subsequently by inducing leaf senescence during the phase of toxicity after excessive salt accumulation in the transpiring leaves (Munns, 2002). Growth retardation is the most typical morphological symptom of saline injury in plants due to inhibition of cell expansion. Razmjoo et al. (2008) found that, increased salinity caused reduction in the number of branches per plant, flowers per plant, peduncle length and head diameter of chamomile (*Matricaria chamomile*). Also, medium and high salt concentrations have been reported to cause significant reduction in shoot lengths of maize plants (Forieri et al., 2016).

Moreover, salinity stress conditions enhance plants to produce cytotoxic activated oxygen that can seriously disrupt normal metabolism, through

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oxidative damage of lipids, proteins, and nucleic acids (Abbaspour, 2012 and Abdul Qados, 2015), resulting in the formation of reactive oxygen species(ROS) (Sudhir & Murthy, 2004; Nazar et al., 2011; He et al., 2014 and Li et al., 2014). To mitigate the harmful effects of these ROS, plants have improved an effective scavenging system composed of non-enzymatic antioxidants (carotenoids, ascorbate, and tocopherol) and enzymatic antioxidants, such as catalase (CAT) and peroxidase (POD) (Li et al., 2014). Enhancement of SOD, CAT, and POX activities has been reported in two cultivars of *Poa pratensis* L. grown under salinity stress (Puyang et al., 2015).

As an important signaling molecule, salicylic acid (SA) impacts various physiological and biochemical functions in plants and has different effects on the tolerance to biotic and abiotic stresses (Li et al., 2014). The role of SA in plant tolerance to abiotic stresses such as heavy metal, heat, UV-B, ozone, and osmotic stress has been reported (El-Tayeb, 2005 and Wang et al., 2010). In mung bean, exogenous SA application alleviated the harmful effects of salt stress through the improvement of plant photosynthesis, growth and also enhancing antioxidant system. Also, SA has proved to have an important contribution to growth stimulation of wheat seedlings by an enhancement of cell division and extension of root cells (Shakirova et al., 2003). In other plant species such as squash (*Cucurbita pepo* L.), growth parameters and vegetative characters of stressed plants were maximized due to SA application (Abd El-Mageed et al., 2016).

However, the improvement of salt tolerance by exogenous SA depends on genotype and the concentration of SA used. For example, in a salt-tolerant wheat genotype, exogenous SA counteracted the salt stress-induced growth inhibition, whereas no improvement occurred in a salt-sensitive cultivar (Arfan et al., 2007).

Adaptation to salt stress besides the application of exogenous substances such as salicylic acid requires alterations in gene expression (Abdul Qados., 2015). The role of SA against stress is relevant in its regulation of antioxidant enzymes and compounds containing active oxygen species in plant (Stevens et al., 2006 and Hosseini et al., 2015). Various studies have shown that the cytotoxicity effects induced by salt stress can be increased by the exogenous application of SA (Simaei et al., 2012 and Abdul Qados., 2015). Enzymatic antioxidant systems (e.g. SOD, CAT and POX) are engaged in defense against ROS induced byabiotic stress (Mittler, 2002; Carillo et al., 2008 and Gezi et al., 2013). The activities of antioxidant enzymes including SOD, POX and CAT were up-regulated by NaCl stress and the activities of these enzymes were further enhanced by SA treatment of *Nitraria tangutorum* Bobr (Liu et al., 2016).

Gene expression studies have become very important in the identification of genes that perform directly in signal transduction, stress protection, and gene expression regulation as noticed in *Arabidopsis* (Seki et al., 2002). Increased expression of antioxidant enzyme systems in transgenic plants might award protection from ROS generated as a result of salt stress in several plants. On the other hand, the pre-treatment of SA could effectively preserve plant seedlings from oxidative damage of abiotic stress such as chilling and salinity through promoting antioxidant enzymes activities and related gene expression (Chen et al., 2011 and Li et al., 2013).

Rosemary (*Rosmarinus officinalis* L.) is one of the most effective spices among the Lamiaceae, commercially used as natural source of antioxidants (Yanishlieva et al., 2006; Okoh et al., 2011 and Gharib et al., 2014) being an alternative to synthetic antioxidants which have carcinogenic potential and negative health influences (Botsoglou et al., 2002). The major constituents of *R. officinalis* oil was found to be 1,8-cineol, camphor, α-pinene and borneol (Minaiyan et al., 2011 and Gharib & Teixeira daSilva, 2012) were found to decrease in response to exposure to different salinity levels (Langroudi et al., 2013). As different environmental stresses such as salinity may change the efficiency of oil composition and antioxidants level in rosemary, there was a persistent need to use inexpensive, non-polluting, and non-hazardous way to overcome stress effects on plant (Gharib et al., 2014). There are limited published researches on growth criteria of rosemary under salinity stress conditions despite its popularity and its several uses. Rosemary is comparatively tolerant to salt stress, partly because of its ability to regulate stomata aperture and activate osmotic adjustment. However, nothing is known about how this species tolerate salt stress in terms of mechanisms

of photo-protection and antioxidant protection (Tounekti et al., 2011).

The objectives of this work were to study the effect of salinity stress on growth of rosemary plants, examine whether the harmful effects of salinity stress can be recompense by the exogenous application of SA, by analyzing growth criteria such as plant height and branches numbers, some antioxidant enzyme activities at both biochemical and transcriptional levels. CAT, SOD, and POX were selected as the target genes encoding antioxidant enzymes in this study. The information obtained is essential for propagation and cultivation of *R. officinalis* species under salt stress condition.

Materials and Methods

Pot experiment was conducted at the greenhouse of Biology Department, Faculty of Science, Taif University, Saudi Arabia during 2014 and 2015 seasons. The aim of this study was to investigate the effects of salt stress, salicylic acid (SA) and their interaction on the growth, biochemical, physiological and molecular parameters of *Rosmarinus officinalis* L. plant.

Plant material

Stem cuttings of rosemary (*Rosmarinus officinalis* L.) were sown in the nursery and two months later, homogenous seedlings were transplanted into (30 x 20cm) pots contained sandy soil. The physical constituents of soil were (Sand, 83.45%; Silt, 6.70%; Clay, 9.85%). Organic Matter was 0.12% with 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 $SO4^{-2}$, 49.32 ($meqL^{-1}$); HCO_3, 2.10 ($meqL^{-1}$); Cl^-, 0.59 ($meqL^{-1}$) (Hassan et al., 2013).

Experimental design and treatments

Two weeks after transplanting, a completely randomized design with 3 replications per treatment and 5 plants per replication was adopted. Twelve treatments with different NaCl concentrations (0, 25, 50, 100mM) and different SA concentrations (0, 0.2, 0.4mM) were implemented as shown in Table 1.

The salinity levels were obtained by addition of appropriate amount of NaCl to water and adjusted by a portable EC meter instrument. Plants were subjected to saline irrigation water every 7 days using 0.5L irrigation water per pot. Pots were flushed out with saline water (NaCl) every two weeks to ensure homogeneity of salinity and to prevent the induction of salt build up. To avoid osmotic shock, the NaCl solution was gradually added to the soil every other irrigation time to achieve the final concentrations. Water content of the pots was maintained at 80% field capacity with distilled water till the end of the experiment.

Salicylic acid (SA; 2-hydroxybenzoic acid) was initially dissolved in 100ml dimethyl sulfoxide. The SA concentrations (0, 0.2 and 0.4mM) were attained using double-distilled water (DDW). The treatments were applied as foliar spray one week after salinity treatment. Spraying with SA was applied weekly in the early morning till the end of the experiment. Control plants were irrigated using 0.5L distilled water. The same environmental conditions were maintained throughout the experiment.

Growth parameters analysis

Plant height and branch number/plant of rosemary herb were estimated. The length of main stem from soil surface to the plant apex was recorded to obtain plant height (cm). Number of branches on the main stem was counted to obtain branches number.

TABLE 1. Salinity levels, SA treatments and their combination.

No.	NaCl (mM)	SA (mM)
T1	0	0 (control)
T2		0.2
T3		0.4
T4	25	0
T5		0.2
T6		0.4
T7	50	0
T8		0.2
T9		0.4
T10	100	0
T11		0.2
T12		0.4

Antioxidant enzyme activity

One gram of leaf tissue was homogenized using a chilled pestle and mortar with 5ml of 0.05M potassium phosphate buffer (pH 7.8) containing 1 MKCl, 0.5% polyvinyl pyrrolidone, 0.1mM EDTA and 2mM dithiothreitol. The homogenate was filtered through four layers of cheesecloth. The extract was centrifuged at 20000g at 4°C for 15 min. The resulting supernatant was used as an enzyme extract to determine superoxide dismutase (SOD), catalase (CAT) and peroxidase (POX) activities.

SOD (Ec 1. 15. 1.1) activity was assayed by measuring its ability to inhibit the photochemical reduction of nitrobluetetrazolium (NBT). SOD activity was expressed as SOD unitsmin^{-1} mg^{-1} protein. One unit of SOD was considered to be the amount of enzyme required to inhibit NBT reduction by 50% as described by Giannopolitis & Ries (1977) by measuring the absorbance at 560nm by a spectrophotometer (type GBC, UV/VIS 916).

CAT (Ec 1. 11. 1.6) activity was spectrophotometrically estimated by method of Clairbone (1985), following the disappearance of H_2O_2 at 240nm. The level of enzyme activity was expressed as µmol min^{-1} mg^{-1} protein.

POX (Ec 1. 11. 1.7) activity was tested according to Shanon et al. (1966). Sodium acetate buffer (0.1M) and 0.5% guaiacol were added to the enzyme extract. The reaction was started with 0.1% H_2O_2. The rate of change in absorbance was spectrophotometrically measured at 470nm and the level of enzyme activity was expressed as µmol min^{-1} mg^{-1} protein.

Gene expression analysis

RNA isolation

Total RNA was extracted from frozen tissue using Trizol reagent (Life Technologies, Ambion) following the protocol of MacRae (2007). Tissue was ground and homogenized in TRIzol solution (100mg of tissue per 1ml of TRIzol), extracted and precipitated as described by the manufacturer. To quantify and assess the degree of purity, a 2.5µl aliquot of RNA samples were diluted with 1 ml of water. Absorbance was measured in a spectrophotometer at 260 and 280nm. The concentration of RNA was calculated by the formula: OD260 × dilution factor (1/100) ×40/1000 µg/µl RNA. To assess whether extracted RNA is degraded and to confirm the quantification, an aliquot of 1-2.5µl RNA samples and 10µl loading buffer were loaded on 1% agarose gel and visualized by UV transilluminator (Biometra UV star 15).

Primer designand PCR optimization

Specific primers using known cDNA sequences were supplied by Macrogen Inc. (Korea) for amplification of antioxidant genes (Table 2).Total RNA was reverse transcribed to generate c-DNA using the Access RT-PCR System (Promega) and a PXE 0.5 thermocycler (Thermo Scientific) following the manufacturer's instructions. The densitometry data for band intensities in different sets of experiments was generated by analyzing the gel images on the Gel Pro analysis program (Version 32, USA). Actin transcript level was used as housekeeping standard gene.

Statistical analysis

All experimental data were statistically analyzed using the IBM/SPSS version 22 software.The analysis of variance (ANOVA) was performed to compare means using the Duncan Multiple Range Test. Different letters indicate statistical difference at $P<0.05$. Figures were plotted by Excel software 2010. Each treatment was analyzed in three replications.

TABLE 2. Primers of genes coding for antioxidant enzymes used in semi-quantitative RT-PCR analysis.

Gene	Forward primer 5′– 3′	Reverse primer 5′– 3′
Cu/Zn SOD	5-ATGGTKAAGGCTGTGGCAGTTC-3	5-ACCTTTCCCAAGATCATCAGGAGGATC-3
POD	5-CTCAGAGGYTTMATCGCTGAGAAG-3	5-CAGCAAACCCAAGCTCRGARAGC-3
CAT	5-TTCAAGCAGGCTGGAGAGAG-3	5-CCAGACACTGCGGATTTCAT-3
Actin	5-CCTGGCATCCACGAGACAA-3	5-ATCAGCAATGCCTGGGAACA-3

Results

Effect of salinity, SA and their interaction on growth characters

Data analysis of the growth parameters indicated that the salt stress caused a significant inhibition in plant growth, revealed by a pronounced reduction in plant height and number of branches (Table 3). A Dose response relationship is clearly indicated by a gradual significant reduction in both plant height and number of branches through increasing salinity level, compared to the control. The lowest values in this concern were obtained by the highest salinity level (100mM). However, in case of SA applications, a significant enhancement in both plant height and branches number was clearly exhibited in a dose–dependent manner, relative to the control, as the highest SA dose recorded higher values of growth parameters. Moreover, in case of the treatments including interaction between salinity and SA, a pronounced mitigating effect of SA on plant height and branches number at all salinity levels, with the highest values recorded at 0.4mM SA (Table 3).

Effect of salinity, SA and their interaction on antioxidant enzyme activity

The activities of SOD, CAT, and POX in rosemary plants were significantly affected by the salt stress and SA treatments. With the increasing extent of salt stress, enhancement of SOD, CAT, and POX activities was substantially occurred. This increase in the activity of enzymes was salinity dose-dependent as the highest activities in CAT, SOD and POX were recorded by the treatment of 100mM NaCl. CAT, SOD and POX activities were subsequently increased by 47.17%, 66% and 25.3% respectively, relative to control plants. Both SA levels caused a significant increase in CAT, SOD and POX enzyme activities in rosemary leaves, compared with untreated plants, in a dose–dependent manner. The increases in the activities of CAT, SOD, and POX were approximately 7.55%, 10%, and 9.4% under 0.2mM SA conditions and 15.09%, 20%, and 15.33% under 0.4mM SA conditions, respectively. In case of salinity-SA combination, the activities of CAT, SOD and POX enzymes were higher, compared to those obtained by SA treatment alone (Fig. 1). Under the treatment of 100mM NaCl combined with 0.4mM SA, the activities of CAT, SOD and POX were elevated by 75.47%, 144% and 84.8%, respectively relative to the control. Elevation of SOD activity was more pronounced compared to the other enzymes.

TABLE 3. Effect of salinity levels, salicylic acid (SA) treatments and their combination on plant height and number of branches of rosemary plant.

Treatment		Plant height (cm)	Number of branches/plant
Salinity (mM)	SA (mM)		
0	0	27.67±0.5 ^{d}	6.67 ± 0.5 ^{cde}
	0	25.00 ±0.1 ^{e}	6.33± 0.5 ^{de}
25	0.2	27.67 ± 0.5 ^{d}	7.0± 0.1 ^{cd}
	0.4	31.67 ± 1.53 ^{ab}	8.33± 0.5 ^{ab}
	0	21.33 ± 1.15 ^{f}	5.67± 0.5^{e}
50	0.2	24.67 ± 1.5 ^{e}	6.33± 0.5 ^{de}
	0.4	29.67 ± 1.5^{c}	7.67± 0.5 ^{abc}
	0	19.00 ± 0.1^{g}	4.33± 0.5 ^{f}
100	0.2	21.67 ± 1.5^{f}	5.67± 0.5^{e}
	0.4	26.00 ± 0.2 ^{de}	6.33± 0.5^{de}

-The values refer to means ± S.D.

-(n = 3) For a given measurement.

-Means followed by the same letter within a row are not significantly different at $P < 0.05$ level by one-way ANOVA.

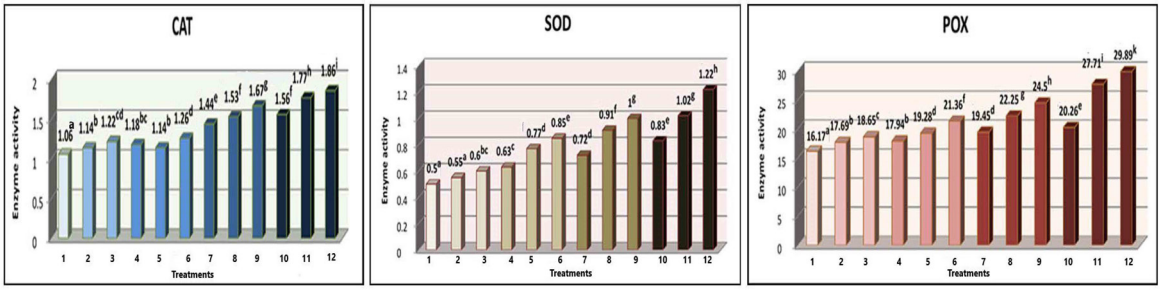


Fig. 1. Effect of salinity levels, salicylic acid (SA) treatments on :CAT, SOD, POX activity (µmol min^{-1} mg^{-1} protein) of *R. officinalis* leaves. 1: (control), 2: (0 Salinity- 0.2mM SA), 3: (0 Salinity- 0.4mM SA), 4: (25mM Salinity- 0 SA), 5: (25mM Salinity- 0.2mM SA), 6: (25mM Salinity- 0.4mM SA), 7: (50mM Salinity- 0 SA), 8: (50mM Salinity- 0.2mM SA), 9: (50mM Salinity- 0.4mM SA), 10: (100mM Salinity- 0 SA), 11: (100mM Salinity- 0.2mM SA), 12: (100mM Salinity- 0.4mM SA). Mean values with the same letter are not significantly different at P < 0.05 level by one-way ANOVA.

Effect of salinity, SA and their interaction on gene expression of antioxidant enzymes

The expression patterns of SOD, CAT, and POX genes were generated in rosemary genotype by performing sq RT-PCR procedure to investigate the effect of salinity, SA and their interaction at the transcriptional level (Fig. 2). A dramatic change in the relative transcript levels of those three genes was observed in response to both NaCl stress and SA application. Generally, in all treatments, CAT, SOD and POX genes were upregulated compared to the control. At 100mM NaCl- 0.4mM SA interaction, CAT, SOD and POX gene expression was increased by 29.8%, 51.3% and 32.14% respectively. It is noticeable that SOD was the most induced gene relative to the other two analyzed genes. Surprisingly, the highest recorded transcript level of all genes was caused by salinity concentration of 25mM. On the other hand, 0.4mM SA revealed more effect on gene induction than 0.2mM at all salinity levels (Fig.2).

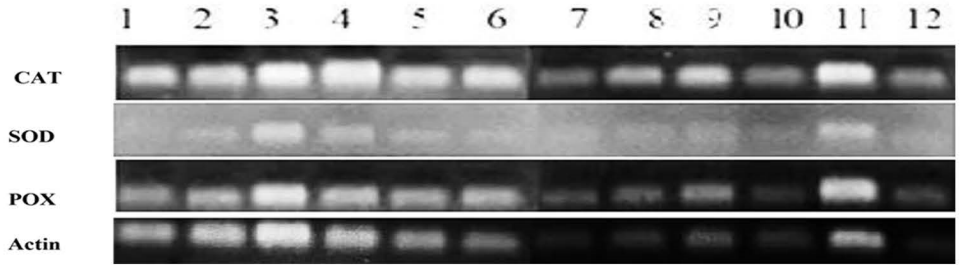
Discussion

In this study, the vegetative growth of rosemary plant was negatively affected due to salinity treatments. The plant height and branches number were significantly decreased with increasing salinity levels, compared to the control. The reduction in vegetative growth could be attributed to inhibition in both cell division and cell enlargement (Yasseen et al., 1987). Furthermore, salt stress has been found to restrict the uptake of water and some mineral nutrients causing retardation of plant growth and development (Cai et al., 2014). This delay of growth has been considered as a sort of plant adaptation to salt stress (Mulholland et al., 2003 and Qaderi et al., 2006). Other researchers (Abdel Latef, 2010; Hassan & Ali, 2014; Lee et al., 2014 and Zou et al., 2015) ascribed this retardation to either direct toxic effects of saline ions (Na^+ and Cl^-) or indirect effects that may cause soil/plant osmotic imbalance.

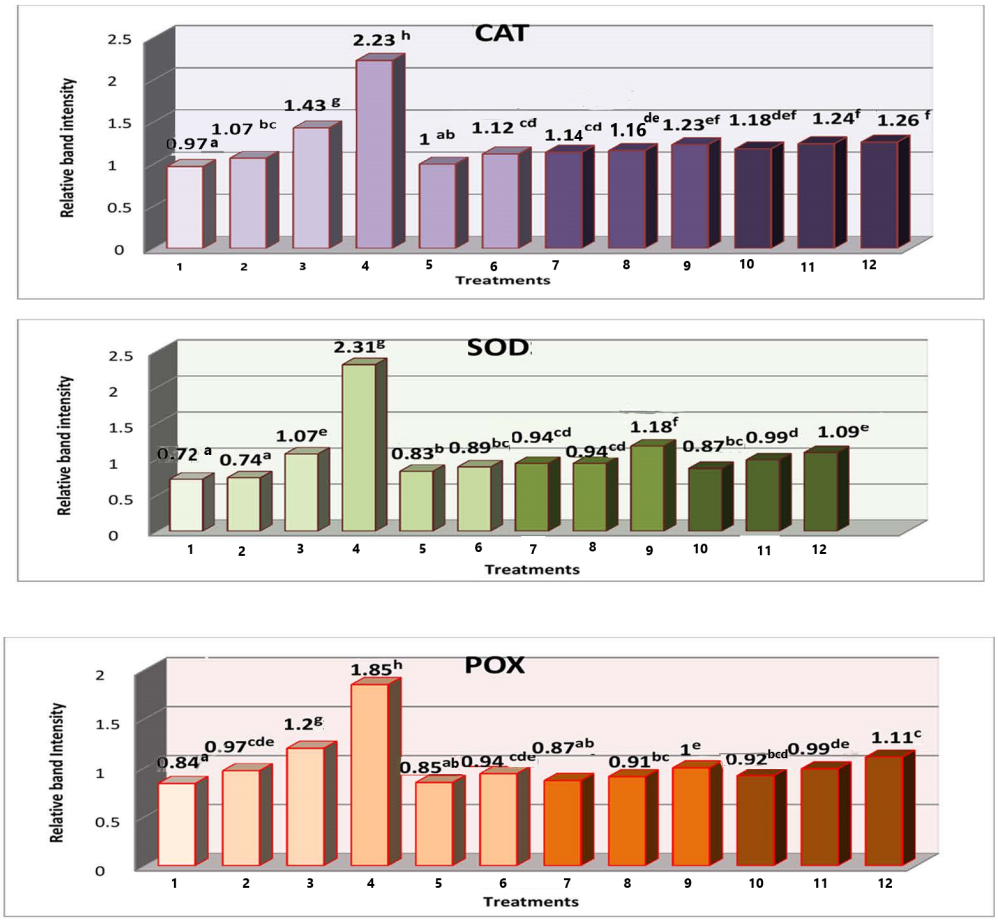
Concerning the SA role, results of this study proved that exogenous application of SA alleviated the deleterious effects of salinity on growth of rosemary plant. SA has been suggested as an important signal molecule for modulating plant responses to environmental stress, as well as facilitating the growth of plant, it has been shown to play a role in mitigating the deleterious effects of some environmental stresses (Hela et al., 2009). Coronado et al. (1998) reported that foliar spray of SA significantly increased the growth of shoots in soybean. These findings are in accordance with the results of this study, which revealed stimulation in growth of rosemary plants upon application of SA, through increasing of plant height and branches number.

It has been also found that salt stress can induce oxidative stress (Gill & Tuteja., 2010 and Malik et al., 2011), as a result of reactive oxygen species (ROS) generation. They are harmful to plant growth because of their detrimental effects on the subcellular components and metabolism of the plant, causing the oxidative destruction of cells, deterioration of membrane lipids, and subsequently led to leakage of solutes from membranes (Mishra & Choudhuri, 1999). Results of biochemical analysis showed a significant increase in the antioxidant enzyme activities

(CAT, SOD and POX) after salinity treatment. An increase in the activities of the antioxidative enzymes under salt stress could be considered as an indicator of an increased production of ROS and a construction of a protective mechanism to reduce oxidative damage triggered by stress experienced by plants.



(A)



(B)

Fig. 2. Expression pattern of genes antioxidants (CAT, SOD, POX) in leaves of *R. officinalis* in response to salinity and SA treatments: 1 (0 Salinity- 0 SA), 2: (0 Salinity- 0.2mM SA), 3: (0 Salinity- 0.4mM SA), 4: (25mM Salinity- 0 SA), 5: (25mM Salinity- 0.2mM SA), 6: (25mM Salinity- 0.4mM SA), 7: (50mM Salinity- 0 SA), 8: (50mM Salinity- 0.2mM SA), 9: (50mM Salinity- 0.4mM SA), 10: (100mM Salinity- 0 SA), 11: (100mM Salinity- 0.2mM SA), 12: (100mM Salinity- 0.4mM SA). A: Transcripts accumulation; B: antioxidant genes/ Actin expression ratio. Mean values with the same letter are not significantly different at P < 0.05 level by one-way ANOVA

The combined action of CAT and SOD converts thetoxic superoxide radical (O_2) and hydrogen peroxide (H_2O_2) into water (H_2O) and molecular oxygen (O_2), consequently preventing the cellular damage under unfavorable conditions like water stress (Noctor et al., 2000 and Ahmad & Haddad, 2011). Meantime, hydrogen peroxide which is also toxic, has to be scavenged by CAT or POX to water and oxygen (Sairam et al., 2005). Hence, the mechanisms that contribute in reduction of ROS and increase the activity of antioxidant enzyme system as well, have crucial roles in imparting tolerance in plants under environmental stress conditions (Abd El-baky et al., 2003). The increase in antioxidant enzyme activity under salt stress has been reported by other several authors (Hassan & Ali, 2014; Zou et al., 2015 and Puyang et al., 2015). Also, high POX activity has proved to correlate with the reduction of plant growth and this increment is considered to have an important role in defense mechanism against salt stress.

In the present study, rosemary plants treated with SA revealed higher activity of antioxidant enzymes. It has been reported that, SA enhances salt tolerance of rosemary plants by reducing ionic and osmotic stress and inducing antioxidant machinery (Fetouh, 2016). Exogenous application of SA can organize the activities of intracellular antioxidant enzymes and accelerate plant tolerance to different environmental stresses (Senaratna et al., 2000; Sakhabutdinova et al., 2004 and Sahu & Sabat, 2011). Likewise, enhancement of the activities of CAT, POX and SOD upon exogenous SA spraying on salinity-stressed plants has been previously reported (Yusuf et al., 2008; Hassan & Ali, 2014; Iqbal et al., 2014; Wang et al., 2015 and Liu et al., 2016). SA has been found to protect photosynthetic machinery from salt-induced oxidative stress hence, alleviated adverse effects of salt stress on photosynthesis and growth. Therefore, it has been concluded that these enzymes may have an important role in protecting the photosynthetic apparatus by scavenging active oxygen species arising during stress.

To investigate salt tolerance in rosemary at molecular level, sq RT-PCR was performed in this study to evaluate changes in the transcript levels of genes encoding the three antioxidant enzymes (CAT, SOD and POX). Results of gene expression analysis demonstrated a significant increase in the transcript levels of these three genes under saline stress and SA treatment as well. Since plants can activate antioxidative defense systems to protect themselves from oxidative stress induced by abiotic stresses such as salinity, analyses of the transcript levels of antioxidant defense genes may reflect the contributions of their products to salt stress response in plants (Lu et al., 2007; Carvalho et al., 2013 and Zhang et al., 2014). Transcriptional analysis has the advantage of quantifying the changes in transcript levels of different genes. Therefore, this could lay the foundation for the identification of genes involved in the regulation of metabolism and provide valuable insights into the molecular mechanisms of many biosynthetic pathways (Ohdan et al., 2005 and Li et al., 2013).

The present research is one of few studies (Zhang et al., 2016 and Herrera-Vasquez et al., 2015), which has investigated the effect of SA at the transcriptional levels of CAT, SOD and POX genes in response to salinity stress and proved the increase in expression of these genes occurred during exposure to NaCl treatments. Gene expression statistical analysis of this study demonstrated that salinity stress induced drastic changes in the expression of all antioxidative enzymes encoding genes. In contrary to biochemical data, there were dramatic increases in the activities of SOD, CAT, and POX under 25mM salt stress conditions, compared to 50 and 100mM salt stress conditions (Fig. 2 B), suggesting that increase in salinity level may led to the decrease in the ability of rosemary plants to resist salt stress. This finding is concurrent with a previous study on *Torreya grandis* plants (Li et al., 2014). On the other hand, inconsistent expression profile of SOD, CAT and POX genes with the corresponding enzyme activity under 25mM salt stress conditions was also recorded. In accordance with other researchers (Gill &Tuteja, 2010 and Harb et al., 2015), this finding could be attributed to the presence of isoforms of the antioxidant enzymes. At the biochemical level, the activities of all isoforms were assayed, whereas at the transcriptional level, the expression of only one isoform was examined (Furlan et al., 2013). In this situation, the complex regulation mechanisms of gene expression cannot be directly correlated with enzyme activity.

Upon the application of exogenous SA, the plants showed higher expression of SOD, CAT, and POX than those of the non-SA treated plants grown under 50 and 100mM salt stress conditions (Fig. 2). In this regard, SA has been mentioned

as a potential intermediate in signal transduction pathways involved in defense mechanism that represented by alterations in gene expression (Durner et al., 1997; Poór & Tari, 2012 and Csiszár et al., 2014). Application of exogenous SA could alter the activity of antioxidant enzyme system through upregulation of their genes and, led to ameliorating the effect of salinity stress in rosemary plants. This finding could be explained on the basis that addition of SA to $NaCl^-$ stressed rosemary plants might alter the activity of mRNA via enhancement of its transcripts level of CAT, SOD, and POX genes, and this could improve the effect of salinity.

With regard to the comparative expression profiling of the studied genes, higher level of SOD transcripts was recorded compared to the other two genes in response to both salinity and salicylic acid treatments. This result is in accordance with the biochemical data which revealed higher percentage of increment in enzyme activity of SOD in all NaCl and SA treatments, compared to CAT and POX enzymes. In salinity- stressed plants, SOD plays as a major scavenger which catalyzes the dismutation of superoxide (a main cause of membrane damage) to hydrogen peroxide and oxygen (Racchi., 2013). Therefore, SOD is considered as one of the most effective and functional antioxidant enzymes in limiting oxidative damage (Asada, 1999 and DaCosta & Huang, 2007). The increment in the expression level of SOD gene yet could also be as a result of increased stability of transcribed mRNAs (Soydam et al., 2013 and Alharby et al., 2016). Furthermore, studies on the alleviative influence of various compounds in stressed plants, confirmed the increase in SOD expression as one of the most important mechanisms to improve the effects of saline stress (Zhu & Scandalios, 1994 and Sakamoto et al., 1995). Similarly, SA application on rosemary saline treated plants caused significant increase in SOD expression, suggesting that SA might serve as a signal to induce expression of the studied Cu /Zn SOD gene which may have acertain role in protection against salt stress.

Since the level of H_2O_2 increases by increasing the activities of SOD, consequently, CAT activity increases. Therefore, SOD cannot be considered solely responsible for membrane protection against peroxidation because it converts O^2 to H_2O_2, which is also a ROS. Other enzymes, such as CAT and POX, should then scavenge this ROS. Induction of CATgene expression under other abiotic stress conditions has been reported in other plant species (Meyer et al., 2011). Zhang et al. (2014) found that upregulation of transcript levels in CAT was consistent with the increase in CAT enzyme activity, suggesting that CAT might play a key role in scavenging excessive ROS in salinity and salicylic acid treated L. *sinense.* Furthermore, Sofo et al. (2015) reported that increased CAT activity appears linked to lower oxidative damage, and gene expression regulation, was detected in plants with higher CAT activity, considering the protective function of this enzyme. Another explanation for the expression of CAT gene under salt stress was suggested by Bueno et al. (1998) who recorded the senescence of cells and the activation of biochemical pathways associated with it as one of the most important outcomes of abiotic stress. On the other hand, increase in POX activity has been reported as one of the main defense mechanisms in response to a number of stress conditions, including salt stress (Asada, 1992 and Bhatt et al., 2013). Interestingly, the overexpression of POX led to reducing the extent of lipid peroxidation (Guan et al., 2015 and Sofo et al., 2015), removing H_2O_2 and minimizing photo-oxidative damage (Xu et al., 2011; Poór et al., 2011 and Csiszár et al., 2014).

Conclusion

Results of this study showed that salt stress inhibited the vegetative growth in *R. officinalis* associated with a significant increase in both antioxidant enzyme activities and their genes expression. SA efficiently ameliorated the negative effects of salt stress over the growth and defense mechanism by enhancing the plants antioxidant enzyme activities and upregulating the transcript levels of the genes encoding antioxidant enzymes, confirming its ability to alleviate the membrane oxidative damage by salinity. It seems that, SA increases the salt tolerance via neutralizing or scavenging of ROS, especially in saline environment. However, the role of exogenous SA to resist salt stress depends on genotype and the concentration of SA used. In consequence, the questions of whether the effectiveness of SA in the alleviation of salt stress is dependent on the plant species must be further elucidated by investigating different species and cultivars of salt stressed *Rosmarinus*. The results of this study could also provide a useful guide for the conservation and large-scale plantation of *R. officinalis*.

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حمض السالسيليك الخارجي يخفف من الآثار الضارة لإجهاد الملوحة على نظام مضاد للأكسدة في نبات أكليل الجبل

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يعتبر الإجهاد الملحي من أهم العوامل المحددة لنمو النبات وإنتاجيته ومن المعروف أن حامض السالسيليك من الهرمونات التى لها تأثير يخفف من الإضرار التى قد يتعرض لها النبات من العوامل البيئية المسببة للإجهاد. اجريت التجربة لدراسة التأثير الوقائي لحامض السالسيليك لنبات أكليل الجبل تحت ظروف الإجهاد الملحي بشكل رئيسي على أنشطه الإنزيمات المضادة للأكسدة ممثله في الكاتاليز (CAT)، السوبر أوكسيد ديسميوتيز (SOD) والبيروكسيديز (POX) وذلك على المستويين البيوكيميائى والنسخى (التعبير الجيني). صممت التجربة بعاملين هما تراكيز السالسيليك (0، 0,2 و 0,4 ملى مول) وكلوريد الصوديوم كمصدر للملوحة (0 ،25 ، 50 و 100 ميلى مول)، كما تم رصد تأثير المعاملات على بعض عوامل النمو مثل طول النبات وعدد الأفرع. وقد اظهرت النتائج فروقا معنويه في النمو وذلك بإختزال في طول النبات وعدد الأفرع في النباتات التى تعرضت لإجهاد الملوحة مقارنه بالعينة القياسية، أما على المستوى البيوكيميائى فقد أدت معاملات الملوحة إلى زياده مستويات الإنزيمات قيد الدراسة، وفى النباتات التى عوملت بحامض السالسيليك فقد لوحظ زياده في كلا من معدلات النمو والنشاط الإنزيمى في تلك النباتات. ومن ناحيه أخرى، فانه على مستوى النسخ الجيني للجينات المشفرة لتلك الإنزيمات فقد اظهرت نتائج تفاعل النسخ العكسي تغييرا في معدلات النسخ مقارنه بالعينات القياسية حيث وصلت أعلى مستويات التعبير الجيني في معامله كلوريد الصوديوم 25 ملى مول. كما أثبتت النتائج الدور المنظم لحامض السالسيليك للتعبير الجيني والذى يؤدى بدوره إلى زياده الأنشطة الأنزيمية مما له اكبر الأثر على زياده مقاومه النبات لإجهاد الملوحة.