

Research Article

Effect of exogenous melatonin on growth, electrolyte leakage and antioxidant enzyme activity in rosemary under salinity stress

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(Received:21/01/2020-Accepted:17/03/2020)

Abstract

Melatonin is a new plant hormone that plays an important role in stress tolerance. For investigation the effect of exogenous application of melatonin on salt tolerance in rosemary (*Rosmarinus officinalis* L.), a factorial experiment was conducted in a completely randomized design with three replications. The first factor was melatonin (50, 100 μM) and the second factor was salinity stress (6, 9, 12 ds m^{-1}). Water was used for control treatment. In the treated plants with concentrations of 6, 9 and 12 dsm^{-1} NaCl, stem height decreased compared to the control. Melatonin treatment (100 μM) reduced the effects of NaCl stress. 100 μM melatonin increased leaf growth compared to the control. The highest activity of SOD and POD was recorded in pretreatment plants with 50 and 100 μM melatonin. Melatonin prevented the reduction of chlorophyll content due to salt stress. The lowest ion leakage was related to 100 μM melatonin in control, 6 and 9 dsm^{-1} NaCl. Also, the highest reduction of electrolyte leakage (with 28.5% reduction) was related to 100 μM MT at 12 dsm^{-1} NaCl. According to the results, 100 μM melatonin was more effective in reducing the effects of salinity stress.

Key words: Antioxidants, Electrolyte leakage, Oxidative damage, plant development, Salt tolerance

Introduction

Environmental stresses can delay plant growth, inhibit seed germination, delay growth, increase aging and even lead to plant death. One of the most important environmental stresses is salinity that severely restricts agricultural production and causes ion-specific toxicity, disrupting nutritional balance and reactive oxygen species (Zhu, 2001; Abbasi *et al.*, 2016; Guo *et al.*, 2018). Exposure of plants to salt stress causes excessive production of reactive oxygen species (ROS), which ultimately results in membrane damage (Shalata *et al.*, 2001; Hasanuzzaman *et al.*, 2018).

In plants, strategies to protect against salt stress include controlling toxic ions uptake by roots, controlling the movement of ions from roots to shoots, altering photosynthetic pathways, altering the activity of antioxidant enzymes, and regulation of hormone levels (Zhao *et al.*, 2010; Liu *et al.*, 2018). Various studies have shown that melatonin (N-acetyl-5-methoxy tryptamine) plays an important role in the resistance of plants to salinity (Li *et al.*, 2012; Li *et al.*, 2019; Zhan, 2019). Melatonin is a multi-regulatory molecule that is probably present in most animals and plants (Zhao *et al.*, 2019). Melatonin was discovered in plants in 1995 (Hattori *et al.*, 1995). It is a biological hormone that plays an important role in stress tolerance (Li *et al.*, 2019). Melatonin plays an important role in improving antioxidant systems and eliminating free radicals under salt stress. As a result, it improves photosynthesis,

hormone activation, polyamine metabolism, and ionic homeostasis. Melatonin regulates gene expression responses to salt stress (Zhan, 2019).

Extensive studies have shown the essential roles that melatonin plays in enhancing salt tolerance in various plant species. Melatonin treatment reduces H_2O_2 and O_2^- concentrations by activating antioxidant enzymes. This performance has been proven in many plants, such as radish, rapeseed, cucumber, kiwi fruit, corn, bermuda grass, malus, okra, rice, wheat and watermelon (Besma and Denden, 2012; Li *et al.*, 2012, 2016; Chen *et al.*, 2015). In one study, melatonin treatment increased salt tolerance of rice seedlings by reducing the amount of chlorophyll degradation (Liang *et al.*, 2015). Jiang *et al.* (2017) reported that, Chlorophyll a, chlorophyll b, and total chlorophyll content in melatonin-treated radish seedlings were increased under salt stress, also 100 μM melatonin has been suggested as the best treatment. In general, melatonin treatment improves photosynthetic efficiency by reducing chlorophyll degradation and stomata closure under salt stress (Li *et al.*, 2012; Zhou *et al.*, 2016). In a study on watermelon, the effects of melatonin (50, 150 and 500 μM) on leaf photosynthesis and redox homeostasis under salt stress (300 mM) were investigated. Salt stress inhibited photosynthesis and increased ROS accumulation and membrane damage in watermelon seedlings. However, melatonin treatment prevented photosynthetic rate reduction and oxidative stress (Li *et al.*, 2017).

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Rosemary (*Rosmarinus officinalis* L.) is an attractive and drought tolerant plant, and is used as an ornamental plant in gardens and for landscaping (Arteche *et al.*, 1992). It is easy to grow and resistant to pests. In soils with high electrical conductivity, the growth of rosemary bushes is restricted and leaf yellowing symptoms are observed in plants (Tounekti *et al.*, 2008). According to the studies, there is no evidence about the effect of melatonin application on the improvement of rosemary growth in saline conditions. Therefore, the present study aimed to investigate the effects of exogenous melatonin treatment on some morphological and physiological parameters of rosemary plant under salinity stress condition.

Materials and Methods

Plant materials and treatments: In order to investigate the effect of exogenous melatonin on salt tolerance ability of rosemary, a factorial experiment was conducted in a completely randomized design with three replications. The first factor was melatonin (MT) (50-100 μM) and the salinity stress (6, 9 and 12 dsm^{-1}) (Tounekti *et al.*, 2008; Taieb *et al.*, 2011) was considered as the second factor, also in both factors water was used as control treatment. Two-year-old of rosemary plant (*R. officinalis*) with uniform sizes was grown in a greenhouse in plastic pots (15 cm diameter) containing sand and garden soil (3:1 v/v). Soil properties are presented in Table 1. Plants were kept in a greenhouse with average night and day temperature 17 and 22°C, respectively and relative humidity of 75% under natural light.

All plants (except control treatment) were sprayed with different concentrations of melatonin after 60 days from planting date. A second spraying was applied after one week. The soil surface was covered in each spraying. One week after the second foliar application, salinity was applied. NaCl with purity of 99.9% was used for salinity stress. To prevent osmotic shock, salt concentration was gradually increased until the desired concentration was reached. For control treatment, plants were irrigated with tap water.

Plant growth: Stem length, leaf length and new shoot growth were recorded after 60 days of stress. Plants were removed completely from the pot after 60 days of salinity stress. Stem height and fresh weight of shoot and root were recorded. Dry weight of shoots was determined after drying of the samples at 80°C. The roots were washed using tap water and dried at 80°C to obtain their dry weight.

Chlorophyll content: At the end of the experiment, chlorophyll was extracted from fresh leaves with 80% acetone and the contents of Chlorophyll a, b and total chlorophyll contents were determined spectrophotometry, according to the method of Arnon (1949). The last extended leaves of the main branch of each plant were used for all biochemical traits.

Determination of electrolyte leakage (EL): At the end of the experiment, 0.2 g of fresh leaf was washed

with deionized water. The leaves were placed in closed tubes containing 5 ml of deionized water and incubated at 10°C for 24 hrs. Subsequently, the initial electrical conductivity of the solution (EC1) was determined using conductor. Then, the samples were immersed in a water bath at 95°C for 20 mins., cooled to 25°C and their EC2 was measured. Electrolyte leakage was calculated from $\text{EL} = (\text{EC1} / \text{EC2}) \times 100\%$ (Deshmukh *et al.*, 1991).

Antioxidant enzyme activity assay: Three weeks after salinity stress, antioxidant enzyme activities were assayed in leaves by using spectrophotometric methods. Protein contents were determined following the method of Bradford and Williams (1976).

Peroxidase (POD) (EC 1.11.1.7) activity was assayed according to the method of Aebi (1983). Phosphate buffer (0.1 M, pH 7.0) containing 15% (w/w) PVPP, 2 mM EDTA and 0.5% (v/v) Triton X-100. The homogenate was centrifuged at 10,000 rpm for 20 mins. and the supernatant was assayed for POD. Peroxidase activity was determined following oxidation of *o*-Dianisidine in the presence of H_2O_2 at 470 nm.

Superoxide dismutase activity (SOD) (EC 1.15.1.1) was assayed according to the method of Stewart and Bewley (1980). The reaction mixture was prepared by mixing 0.1 mM nitroblue tetrazolium, 0.1 mM EDTA, and 50 μM xanthine and xanthine oxidase in 50 mM potassium phosphate buffer, pH 7.8. One unit of SOD is defined as the amount of enzyme that inhibits the control rate by 50% (0.025 units of absorbance at 550 nm min^{-1}).

For catalase (CAT) (EC 1.11.1.6) assay, the reaction mixture contained 25 mM phosphate buffer (pH 7.0), 10 mM H_2O_2 , and the enzyme extract (Cakmak and Marschner, 1992).

Statistical analysis: Data were statistically analyzed using SAS software (Version 9.1). Mean comparisons were performed using the least significant difference (LSD) at the level of $P < 0.05$.

Results

The interaction of salinity and MT on all traits was significant, however the main effects of salinity on the shoot fresh weight was not significant (Table 1 and 2)

Plant height: In the treated plants with concentrations of 6, 9 and 12 dsm^{-1} NaCl, stem height decreased compared to the control by 6.97%, 13.29% and 33.37%, respectively. MT treatment reduced the effects of salinity. Also, MT treatment (100 μM) reduced the effects of NaCl stress (6, 9 and 12 dsm^{-1} NaCl) with 14.00%, 15.38% and 40.11%, respectively (Figure 1A).

Leaf length: As shown in Figure 1B, 100 μM melatonin pretreatment increased leaf growths compared to the control in non-stressed plants. When plants were exposed to salt stress, leaf length decreased. Leaf length was higher in rosemary's treated with MT

Table 1. Properties of the soil used in this study as media

Mg ⁺	Ca ⁺	K ⁺	Na ⁺	Cl ⁻	Field capacity %	EC dS m ⁻¹
cmol/kg						
6.34	15.11	1.22	8.8	6.3	19.12	1.33

Table 2. Mean squares obtained from variance analysis of measured traits in rosemary under different levels of melatonin and salinity

SOV	df	Plant height	Leaf length	New shoot height	Chlorophyll content	Shoot fresh weight	Shoot dry weight	root fresh weight	Electrolyte leakage
Salt stress (A)	3	524.5**	3.28**	70.54**	2.87**	0.81 ^{ns}	922**	25.0**	203.7**
Melatonin (B)	2	434.4**	2.87**	83.52**	3.15**	58.42**	458**	177**	126.1**
A×B	6	30.1**	0.86**	13.31**	1.98*	3.97**	44.9**	26.9**	10.51*
Error	24	1.0	0.42	3.26	1.30	7.46	3.49	1.11	3.51
CV (%)		1.4	21.02	14.19	13.49	7.81	7.21	11.5	7.73

ns, * and **Non-significant and significant at $P \leq 0.05$ and $P \leq 0.01$ respectively

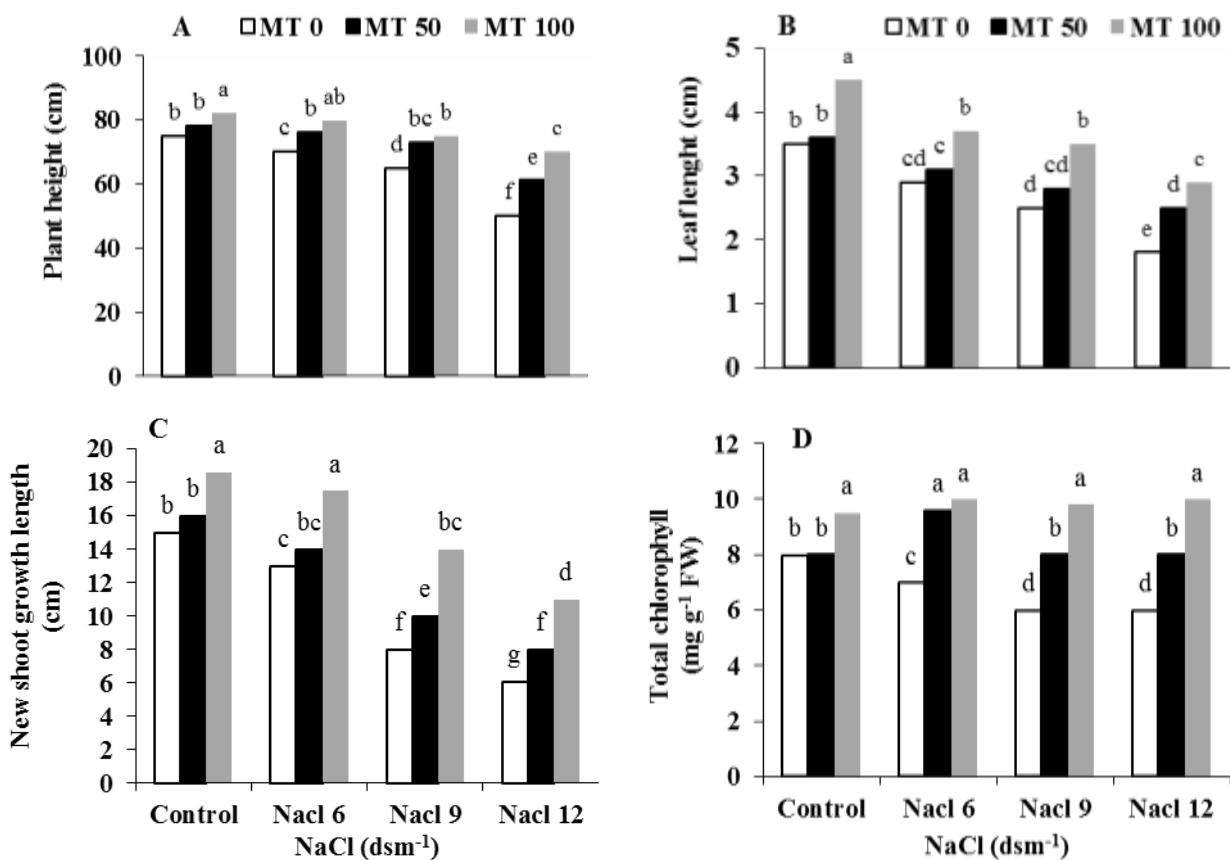


Figure 1. Effects of exogenous melatonin (0, 50 and 100 μM) on plant height, leaf length, new shoot length and leaf chlorophyll in rosemary under salt (6, 9 and 12 dsm⁻¹) stress. The columns with the same letters are not significantly different at the least significant difference (LSD) the level of $P < 0.05$.

before salt stress. The 100 μM melatonin performed better than the 50 μM (Figure 1B).

New shoot growth height: Plants treated with only 100 μM MT had significantly higher shoot growth than the control (0 MT). Plants treated with NaCl at concentrations of 6, 9 and 12 dsm⁻¹ showed a significant decrease of 13.33%, 46.66% and 60% in growth compared to the untreated plants, respectively. While melatonin reduced the negative effect of salinity (Figure 1C).

Chlorophyll content: The results showed that NaCl

treatment reduced chlorophyll contents in rosemary leaf. However, melatonin prevented the reduction of chlorophyll content due to salt stress. The concentration of 100 μM was the most effective in reducing salt stress. Compared with 6, 9 and 12 dsm⁻¹ NaCl, the chlorophyll contents increased by 40.1 %, 63.1 % and 66.6 %, respectively, under 100 μM MT + 6, 9 and 12 dsm⁻¹ NaCl (Figure 1D).

Fresh and dry weight of shoots and roots: NaCl treatment decreased fresh and dry weight of shoots and roots in rosemary. However, pretreatment with 50 or

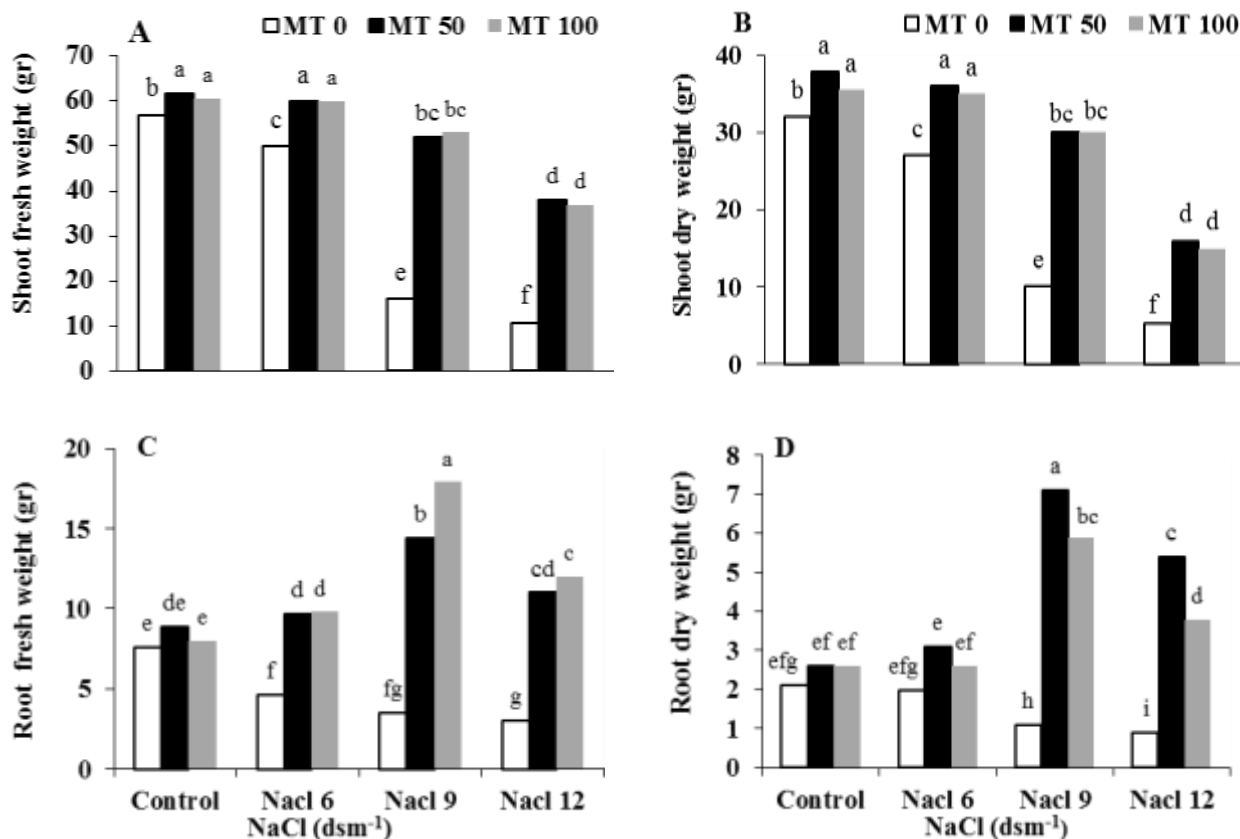


Figure 2. Effects of exogenous melatonin (0, 50 and 100 μM) on fresh and dry weight of shoots and roots in rosemary under salt (6, 9 and 12 dsm^{-1}) stress. The columns with the same letters are not significantly different at the least significant difference (LSD) the level of $P < 0.05$.

100 μM of melatonin obviously alleviated salt stress. The lowest shoot fresh weight was belonged to 12 dsm^{-1} in 0 melatonin (Figure 2A). The effect of salt stress and melatonin treatment on shoot dry weight significant. Melatonin 50 and 100 μM (without salt stress) increased shoot dry weight compared to the control. With increasing salt concentration, shoot dry weight decreased. (Figure 2B). Salinity stress decreased root growth. The highest growth reduction was at 12 dsm^{-1} without melatonin. Melatonin reduced the negative effect of salinity. The highest fresh and dry weight of root belonged to 50 and 100 μM melatonin in 9 dsm^{-1} salinity (Figure 2C and D).

Enzyme activity: The results showed that melatonin treatment significantly increased the activity of three antioxidant enzymes under salinity stress. The highest activity of SOD and POD was recorded in pretreatment plants with melatonin 50 and 100 μM under salt stress (6, 9 and 12 dsm^{-1}). The activity of CAT enzymes was maximal at 50 and 100 μM melatonin + 6 and 9 dsm^{-1} NaCl. Compared with 9 dsm^{-1} NaCl stress alone, the activities of, SOD, CAT and POD increased by 28.5%, 16.2% and 65.1%, respectively, under 100 μM MT+9 dsm^{-1} NaCl (Figure 3A, B and C).

Electrolyte leakage: The results showed that the mean electrolyte leakage was significantly increased at 6, 9 and 12 dsm^{-1} compared to the control treatment. The highest ion leakage was observed at the highest salt

concentration. Melatonin treatment reduced the negative effect of salinity. The lowest ion leakage was related to 100 μM melatonin in control, 6 and 9 dsm^{-1} NaCl. Also, the highest reduction of electrolyte leakage (with 28.5% reduction) was related to 100 μM MT at 12 dsm^{-1} NaCl stress treatment (Figure 3D).

Discussion

Plants have different strategies to different environmental stresses. Various studies suggest melatonin as a novel plant growth regulator and has been implicated in different biotic and abiotic stress responses (Yin *et al.*, 2013; Li *et al.*, 2016; Zeng *et al.*, 2018). In the present study, the positive protective role of melatonin in rosemary against salt stress was investigated. According to the results, vegetative growth of rosemary was inhibited by salt stress, but exogenous melatonin treatment prevented growth inhibition under salt stress.

Salinity decreases growth by reducing leaf water potential and altering various metabolic activities such as alteration in solute accumulation, ion imbalance and inhibition of enzymatic activity (Munns *et al.*, 2006). The results showed that melatonin treatment was effective in increasing shoot growth. In rosemary plants under salinity stress, melatonin-treated plants had higher growth than the untreated plants. Melatonin is both a stimulant and an inhibitor of growth. The stimulant and

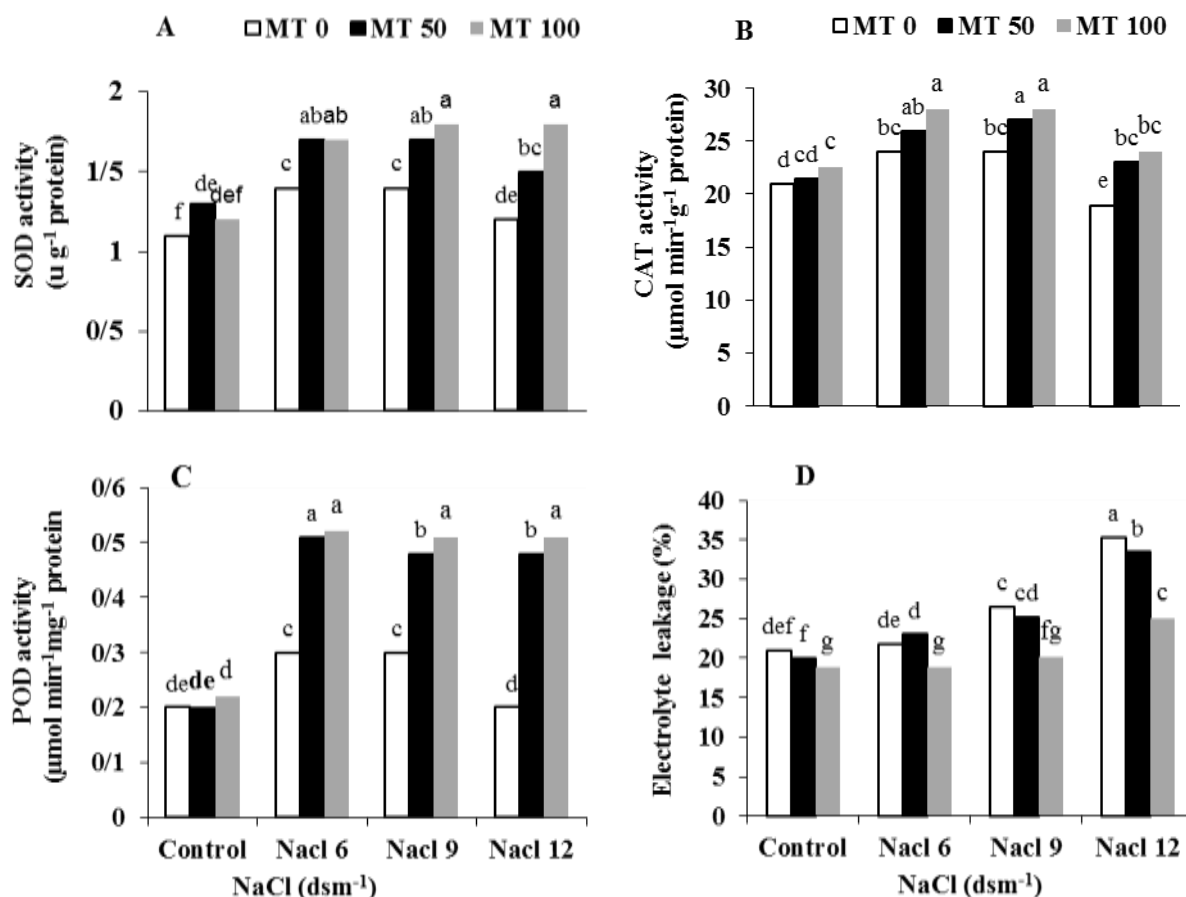


Figure 3. Effects of exogenous melatonin (0, 50 and 100 µM) on the activities of CAT, SOD, POD and electrolyte leakage in rosemary under salt (6, 9 and 12 dsm⁻¹) stress. The columns with the same letters are not significantly at the least significant difference (LSD) the level of P < 0.05.

inhibitory effect of melatonin depends on its concentration (Sarropoulou *et al.*, 2012). In a study conducted on *Prunus avium* × *Prunus cerasus*, melatonin was the forefront in tackling drought and salinity, and other antioxidants act as post-melatonin support (Sarropoulou *et al.*, 2012).

Auxin, as a rooting hormone, plays a key role in root formation. In plants, MT is very similar to IAA, since both compounds are indole and share a common biosynthetic pathway (Liang *et al.*, 2017). Various studies have shown that one of the roles of melatonin in plants is the growth promoting activity and induction of rhizogenesis, so they act in a similar manner as auxin (Tan *et al.*, 2016). In the present study, melatonin treatment increased root fresh and dry weight. In the study of Liang *et al.* (2017), treatment with melatonin significantly increased the formation and development of lateral roots in rice plant.

Through evolution, plants have gained systems, such as activating a set of antioxidants to eliminate excess ROS that are harmful to the plant cells. Melatonin is a well-documented antioxidant and plays an important role in reducing environmental stress by scavenging RNS (reactive nitrogen species) or ROS directly or indirectly in plants (Bonfont-Rousselot *et al.*, 2011; Li *et al.*, 2016). Much evidence has shown that

melatonin is not able to directly remove O₂⁻ or H₂O₂ (Bonfont-Rousselot *et al.*, 2011; Li *et al.*, 2016). Thus, melatonin induces antioxidant systems, such as antioxidant enzymes and non-enzymatic antioxidants for eliminating ROS. In the current study, results showed that melatonin treatment significantly increased the activity of three antioxidant enzymes (CAT, SOD and POD) under salinity stress. The highest activity of SOD and POD was recorded in pretreatment plants with melatonin 50 and 100 µM under salt stress. In plant cells, O₂⁻ is rapidly converted to H₂O₂ by the SOD enzyme, while H₂O₂ is degradable by CAT (Noctor and Foyer, 1998).

Li *et al.* (2012, 2017) have reported that exogenous treatment of melatonin induced the activities of some antioxidant enzymes including SOD and CAT under salt stress, which were in line with our findings. In this study, different concentrations of MT effectively lessened the decrease of chlorophyll contents caused by salinity stress. Also, the contents of chlorophyll were higher in melatonin-treated plants (without salinity stress), suggesting that MT may promote chloroplast gene expression and protein turnover to facilitate chlorophyll accumulation (Yin *et al.*, 2013; Suo *et al.*, 2015). In a study on tomato, treatment of 2 mM melatonin increased the leaf chlorophyll content under

salinity stress (Yin *et al.*, 2019). Chlorophyll a, chlorophyll b, and total chlorophyll content of radish seedlings increased under salt stress as a result of melatonin-treated. In this experiment, a concentration of 100 μM was reported as the best treatment (Jiang *et al.*, 2017). Studies show excessive accumulation of ROS promotes chlorophyll degradation and reduces photosynthetic function. MT reduces H_2O_2 and O_2^- concentrations by activating antioxidant enzymes. Therefore, it can prevent the degradation of chlorophyll (Woo *et al.*, 2004; Allakhverdiev *et al.*, 2008).

Results of Li *et al.* (2019) showed that melatonin pretreatment in tea (*Camellia sinensis*) increased resistance to salinity by increasing the efficiency of photosynthetic system. In a study on wheat (*Triticum aestivum* L.) seedlings, melatonin maintained photosynthesis during salt stress (Ke *et al.*, 2018).

In the present study melatonin reduced electrolyte leakage in rosemary under salinity stress. In maize seedlings, 1 μM melatonin significantly reduced the electrolyte leakage induced by salt stress (Jiang *et al.*, 2016). Li *et al.* (2017) reported that salt stress-induced accumulation of H_2O_2 and O_2^- is consistent with

increased electrical conductivity, and excess ROS may be responsible for salt-induced membrane damage. They stated that exogenous melatonin reduced ROS accumulation due to salt stress, thereby reducing EC.

Conclusion

Exogenous melatonin treatment induced salt tolerance of rosemary plants, which can be manifested as follows: melatonin improves plant height, leaf length, new shoot growth height, chlorophyll content, enhances the activity of antioxidant enzymes, protects chlorophyll pigment, and reduces electrolyte leakage. Therefore, it is recommended to use melatonin pretreatment, especially at a concentration of 100 μM , for increasing resistance to salinity in rosemary.

Acknowledgement

The authors acknowledge the Sari Agricultural Sciences and Natural Resources University (Sari, Iran) for supporting this research.

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