



# Melatonin improves salinity stress tolerance of *Phaseolus vulgaris* L. cv. Pak by changing antioxidant enzymes and photosynthetic parameters

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## Abstract

Plants face many stresses in their natural environment. Different types of phytohormones help the plant to adapt to stressful conditions. Melatonin is a newly known substance in the plant kingdom, that is effective in relieving stress for plants. In this study, the effect of melatonin on *Phaseolus vulgaris* L. cv. Pak that was exposed to salinity stress at concentrations of 100 and 200 mM NaCl was investigated. Treatment of stressed plants and control group (0 mM NaCl) with concentrations of 100, 200  $\mu$ M melatonin improved effects of salinity on the dry weight of shoot, the root and the photosynthetic pigments, net photosynthesis rate, leaf stomatal conductance, transpiration rate,  $K^+$ ,  $Na^+$ , and  $Ca^{2+}$  content, and the ratio of  $K^+/Na^+$ . Melatonin application increased proline and sugar content while decreasing malondialdehyde and  $H_2O_2$  content that were increased by salinity stress. Also, melatonin increased the activity of antioxidant enzymes of catalase, peroxidase, ascorbate peroxidase, polyphenol oxidase, and superoxide dismutase. Overall, lower concentrations of melatonin had significant ameliorating effects on salinity stress-induced damage to the bean plant. While, the use of 400  $\mu$ M melatonin could somewhat exacerbate the effects of salinity in *P. vulgaris* L. cv. Pak.

**Keywords** Catalase · Proline accumulation · Salinity · Stomatal conductance · Transpiration

## Introduction

Salinity is a soil condition characterized by a high concentration of soluble salts such as NaCl, which is the most abundant and soluble salt in the soil (Munns and Tester 2008). Salinity stress has an unfavorable impact on agricultural products worldwide, with a greater impact in the arid and semi-arid areas (PasandiPour et al. 2013). According to the Food and Agriculture Organization of the United Nations (FAO) above 6%, more than 800 million hectares of land throughout the world are affected by salinity problems (Munns and Tester 2008). Salinity causes ionic imbalance both outside and inside the plant. First, soil salinity reduces soil water potential leading to a decrease in water level in

plants and consequently resulting in a lower rate of cell division and plant growth. Then, the high density of the  $Cl^-$  and  $Na^+$  ions inside the cells and decreased  $K^+/Na^+$  ratio impair plant enzymes and membranes function, induce cell dehydration, stomata closure, and minimize  $CO_2$  level inside the photosynthetic cell. As well, the decrease in  $Mg^{2+}$  and toxicity caused by the increase of  $Cl^-$  in leaf cells decreases the quantity of chlorophylls, resulting in reduced photosynthesis (Parihar et al. 2015; Rady and Mohamed 2015; Sytar et al. 2017; Liu et al. 2018). For plants to grow in saline soil, they need to adjust osmotically to maintain a positive turgor pressure (Flowers et al. 2015). The plant strives to adapt to osmotic stress and ion toxicity due to salinity by producing osmolytes such as proline and soluble sugars and intracellular compartmentation of ions (Li et al. 2017).

Another consequence of salinity is oxidative stress. As a result of oxidative stress, reactive oxygen species (ROS) damage proteins and nucleic acids, oxidize carbohydrates, negatively affect the  $K^+$  and  $Ca^{2+}$  cation channels, and eventually damage pigments and membranes. Lipid peroxidation disrupts membrane integrity and metabolism balance. The most sustained ROS produced in saline stress is  $H_2O_2$ , which plants must balance its production and decomposition

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to avoid its effects. The use of enzymatic and non-enzymatic antioxidant systems to neutralize ROS is one of the strategies that plants take in the stress conditions (Zhang et al. 2014b; Demidchik 2015; Rady and Mohamed 2015).

Plants differ greatly in their tolerance of salinity, as reflected in their different growth responses (Munns and Tester 2008). Previously, researchers have applied a variety of chemical compounds to raise the extent of resistance of plants to salinity stress conditions (Khadri et al. 2007). In recent years, Melatonin (MEL) has been at the core of attention. MEL is a tryptophan-derived indoleamine-type hormone that is first found in the bovine pineal gland and then in many vertebrate and invertebrate animals (Arnao and Hernández-Ruiz 2014). In vertebrates, several physiological roles like regulation of circadian rhythms, photoperiodism, acting as an antioxidant compound and impacting on sleep, reproduction, and the immune system as an immune buffer or as an anti-inflammatory compound in the presence of exacerbated immune responses (Carrillo-Vico et al. 2013), have been attributed to MEL (Li et al. 2017; Afreen et al. 2006). Its discovery in higher plants dates as back as 1995. Plants' chloroplasts are where the biosynthesis of MEL occurs. The MEL content of plant cells is much higher than that of animal cells (Afreen et al. 2006; Zheng et al. 2017). What makes the study of MEL important in plants is the multiple roles it plays in plant growth and defense against environmental factors. There have been reports of the role of MEL in improving plant responses to stresses such as drought (Ye et al. 2016; Li et al. 2019a, b), salinity (Huang et al. 2017; Jiang et al. 2016a, b; Liu et al. 2018; Zhang et al. 2014a), cold stress (Yang et al. 2018; Zhang et al. 2014a), attack of pathogens and protection against herbicide (Park et al. 2013), and ion toxicity (Posmyk et al. 2008; Yadu et al. 2018).

A report of increased levels of endogenous MEL in UV-B-treated *Glycyrrhiza uralensis* indicates its effective role in protecting against UV-induced oxidative damage (Afreen et al. 2006). The use of external MEL in Melon with the support of chlorophyll stability improved photosynthetic efficiency and light absorption by increasing the activity of superoxide dismutase (SOD), peroxidase (POX), and catalase (CAT) enzymes mitigated the effects of cold stress (Zhang et al. 2017). MEL improved salinity in watermelon, apple, and cucumber by improving photosynthesis and ion homeostasis (Gao et al. 2019). Pre-treatment with MEL increases the photosynthetic capacity and development of the root system. Increased expression of one of the genes in the biosynthetic pathway of MEL (*MzASMT9*) in Arabidopsis increased its tolerance to salt than wild-type plants (Zheng et al. 2017). A similar result was observed for transgenic *Panicum virgatum* L. with more MEL biosynthetic ability in salt tolerance. Proline content in this plant was higher than in the control group (Huang et al. 2017). The

application of MEL in the oats exposed to salinity increased chlorophyll content and leaf area and increased fresh and dry weight of the plant, while the activity of SOD, POX, CAT, and APX enzymes (Gao et al. 2019). Liang et al. (2015) showed that using external MEL will promote tolerance of rice to salinity by direct/indirect inhibition of H<sub>2</sub>O<sub>2</sub> accumulation (Liang et al. 2015).

Common bean (*Phaseolus vulgaris* L.), with high protein and other nutrients such as fiber, vitamins, and nutrient ions, has high food intake in the developing countries and is considered as one of the most important crops in areas such as the Middle East, Latin America, and Africa (Talaat et al. 2015; Rady and Mohamed 2015; López-Barrios et al. 2016; Hnatuszko-Konka et al. 2014). This plant shows low tolerance to salinity and is known as a glycophyte plant. A decrease in growth and weight loss of plants due to salinity has been reported in *P. vulgaris* L. (Syta et al. 2017; Brugnoli and Lauteri 1991; Rady and Mohamed 2015). In this study, we attempt to understand the effect of MEL treatment on *P. vulgaris* L. cv. Pak plant under salinity stress conditions. We hypothesize that the use of MEL improved bean's tolerance to salt stress by an increase in antioxidant enzyme activity and ion homeostasis.

## Materials and methods

### Plant material and growth conditions

A total of 0.058 g of MEL (Sigma-Aldrich) were dissolved absolutely with 25 mmol/L concentration and it was then stored at the temperature of  $-20^{\circ}\text{C}$ . Twenty-five mmol/L of MEL was further diluted to 400, 200, and 100  $\mu\text{M}$ . To soak the sterilized seeds of *P. vulgaris* distilled water or MEL solutions with a respective concentration of 100, 200, and 400  $\mu\text{M}$  were used for 12 h. As the seedlings sprouted, they were transplanted into perlite-containing, 10 cm pots which were placed in a greenhouse and exposed to the light of 16/8 h interval along with other controlled conditions (heat  $25 \pm 5^{\circ}\text{C}$ , approximate humidity of  $60 \pm 10\%$ , and 1200–1400 light flux). A half-strength (pH range of 6.8–7) solution of Hoagland was used to irrigate them. In the four-leaf stage, 21 days after planting, melatonin spray was used with a concentration of none, 100, 200, and 400  $\mu\text{M}$  (40 mL). Then, the pots were divided into three groups as each group was exposed to either 0, 100, or 200 mM NaCl. Concentrations of 100 and 200 mM salt were supplied by adding NaCl by weight to the half-strength Hoagland nutrient solution. Salinity stress was applied to the plants by irrigating them with a half-strength solution of Hoagland containing NaCl, and 12 plants received each treatment 48 h after salinity stress, plants were harvested, and were either frozen at  $-80^{\circ}\text{C}$  using liquid nitrogen or dried for measurements. A

minimum of three biological replicates per condition were used.

### Measurement of photosynthetic characteristics

Evaluations for net photosynthesis rate ( $P_N$ ), leaf stomatal conductance ( $C$ ), and transpiration rate ( $E$ ) were taken from not separated young, healthy, and well-grown leaves of the main stem. To comply with the standard conditions for all treatments, the conditions of the leaf chamber were adjusted according to the culture room conditions. Measurements were taken using a portable photosynthesis system (CI-340, CID Bio-Science). Measurement was carried out on the second and third leaves of bean plants under similar developmental stages and environmental conditions for all plants.

### Photosynthetic pigments' measurement

For measuring chlorophyll (Chl) and carotenoid (Car) contents, Arnon method was used: 0.5 g of leaf sample in 20 mL of 80% acetone was ground. Extracts were centrifuged (Model SIGMA 2-16KL) at 6000 rpm for 10 min, and the absorbance of the supernatant was measured at 663, 645, and 470 nm with a spectrophotometer (Model Epoch, BioTek company, England) for Chl *a*, Chl *b*, and Car, respectively. The total chlorophyll (Total Chl), Chl *a* and Chl *b*, and Car contents were calculated using the following equations (Arnon 1967):

$$\text{Chl } a = 19.5(A_{663}) - 0.86(A_{645})V/100W$$

$$\text{Chl } b = 19.3(A_{645}) - 3.6(A_{663})V/100W$$

$$\text{Car} = (1000A_{470} - 1.8 \text{ Chl } a - 85.02 \text{ Chl } b)/198$$

$$\text{Total Chl} = \text{Chl } a + \text{Chl } b.$$

### Evaluation of proline and sugars

The Bates et al.'s method was used to measure proline content. The leaf sample (0.2 g) was homogenized with aqueous sulfosalicylic acid (3%). Ninhydrin, acetic acid finally, toluene were added to the supernatant. The colored product of the reaction was read at 520 nm using toluene as the blank (Bates et al. 1973). For Sugar measurement, approximately 0.2 g of the leaves was extracted with distilled water based on the Somogyi method (1952). The mixture of extract and  $\text{CuSO}_4$  was heated for 20 min. After cooling, the phosphomolybdic acid solution was added to the mixture and the absorbance of the product of the reaction was measured at 600 nm (Somogyi 1952).

### Measurement of $\text{H}_2\text{O}_2$ content and evaluation of malondialdehyde

To determine the relationship between melatonin use and reduction of  $\text{H}_2\text{O}_2$ , its concentration in stressed plants and the control group was measured in the presence of melatonin test concentrations based on the Sergiev et al.'s method. A sample of leaf tissues (0.5 g) was homogenized with 0.1% (w/v) trichloroacetic acid (TCA). After centrifuging at  $12,000\times g$  for 15 min, the supernatant was mixed with 10 mM potassium phosphate buffer (pH 7) and 1 M potassium iodide (KI). The sample absorbance was read at 390 nm (Velikova et al. 2000). MDA content, as a product of unsaturated fatty acid peroxidation, was determined by the thiobarbituric acid (TBA) reaction as described by Heath and Packer. The leaf material (0.2 g) was homogenized in 0.1% (w/v) TCA. The absorbance of MDA was read at 532 nm (Heath and Packer 1968).

### Antioxidant enzyme activity measurement

For total protein extraction, 0.1 g of leaf tissue was ground in a chilled mortar with 1% (w/v) polyvinylpyrrolidone. Extract homogenized with 10 mL of 50 mM potassium phosphate buffer (pH 7.8) containing 1 mM EDTA- $\text{Na}_2$ , and then centrifuged at  $12,000\times g$  for 20 min. The supernatant was used to measure total protein and antioxidant enzyme activity. Leaf soluble protein was measured by the Bradford method using bovine serum albumin as a standard (Bradford 1976). To measure the activity of CAT (CAT; EC 1.11.1.6) according to the method of Kato and Shimizu, the initial rate of disappearance of  $\text{H}_2\text{O}_2$  was measured. The CAT reaction solution (1 mL) contained 20  $\mu\text{L}$  of enzyme extract. The absorbance of the sample was measured every 5 s for 1 min at 240 nm (Kato and Shimizu 1987). Peroxidase (POX; EC 1.11.1.7) activity was measured using the Mac-Adam et al. method. The reaction solution consisted of 0.1 M potassium phosphate buffer, 3%  $\text{H}_2\text{O}_2$  solution, and pure guaiacol. The activity of POX was evaluated as a result of guaiacol oxidation by increasing the adsorption at 436 nm at 30-s intervals (MacAdam et al. 1992). Ascorbate peroxidase (APX; EC 1.11.1.11) was determined according to Nakano and Asada method. The reaction solution (1 mL) consisted of 50 mM potassium phosphate buffer (pH 7), 0.5 mM ascorbate, 0.25 mM  $\text{H}_2\text{O}_2$ , and 100  $\mu\text{L}$  enzyme extract. The absorption of the reaction solution was read at 290 nm (Nakano and Asada 1981). Polyphenol oxidase (PPO; EC 1.14.18.1) activity was examined using the method of Raymond et al. with slight modifications, and the reaction mixture (1 mL) contained 0.2 M phosphate buffer (pH 7.6), 20 mM pyrogallol, and 200  $\mu\text{L}$  enzyme extract. The increase of absorbance was recorded at 430 nm and the temperature of the reaction mixture was 25 °C (Raymond et al. 1993). All of

these enzyme activities were expressed as units per mg protein. To measure the activity of superoxide dismutase enzyme (SOD; EC 1.15.1.1) according to Giannopolitis and Ries method. The reaction mixture included 50 mM sodium phosphate buffer (pH 7.5), 13 mM L-methionine, 75  $\mu$ M NBT, 0.1 mM EDTA, 2  $\mu$ M riboflavin, and 10  $\mu$ L enzyme extract used. The reaction solution was exposed to the white fluorescent light for 15 min. Then, the reduction in NBT was measured by reading the absorbance at 560 nm (Giannopolitis and Ries 1977).

### Measurement of $\text{Na}^+$ , $\text{K}^+$ , and $\text{Ca}^{2+}$

$\text{Na}^+$ ,  $\text{K}^+$ , and  $\text{Ca}^{2+}$  contents were assayed based on the Buendia et al.'s method. The samples of shoot and root of bean plants ash (0.1 g) were mixed with 50 mL of 6 M HCl, then the added acid was evaporated by the heater, and 30 mL of 0.1 M  $\text{HNO}_3$  was added and remained for 2 h. The residual solution volume (about 1 mL) was reached 100 mL by deionized water and was filtered. The ion's content was measured using atomic absorption (Model AAS240FS, Agilent Company, USA) and was reported based on  $\text{mg g}^{-1}$  DW (Buendía-González et al. 2010).

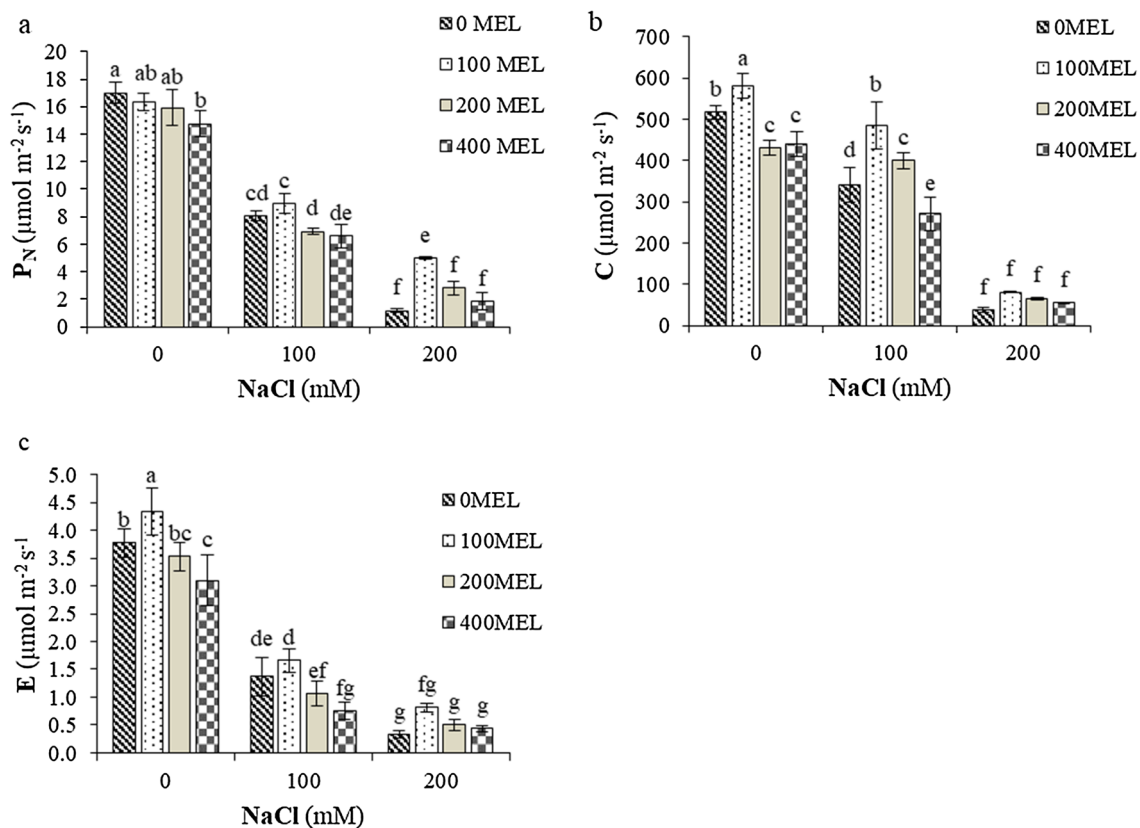
### Statistical analysis

Data are reported as mean  $\pm$  standard error (SE) values of triplicate experiments and were analyzed using SPSS 20. General Linear Model analysis of variance (ANOVA) and Duncan's tests were performed to determine the significance of the difference among samples, with a significance level of 0.05. All data generated or analyzed during this study are included in this published article.

### Results

#### Photosynthesis, photosynthetic pigments, and plant growth

As salt levels rose,  $P_N$ ,  $C$ , and  $E$  decreased significantly in bean leaves. In unstressed plants, except for  $P_N$ , 100  $\mu$ M MEL had an improving effect on other factors. In salt stress conditions, compared to zero MEL, 100  $\mu$ M MEL affected better at any salinity level. At 200 mM NaCl, application of 100  $\mu$ M MEL, compared to zero MEL, increased  $P_N$ ,  $C$ , and  $E$  up to 334%, 118%, and 148%, respectively (Fig. 1).



**Fig. 1** Effect of MEL and salt stress on the photosynthetic characteristics of *P. vulgaris* L. cv. Pak. Net photosynthesis rate (a), leaf stomatal conductance (b), and transpiration rate (c). Columns with the same letter(s) are not significantly different at  $P \leq 0.05$

Different levels of salinity decreased leaf chlorophylls and carotenoid content significantly. Compared to other concentrations of MEL, its 400  $\mu\text{M}$  concentration had a small effect on these parameters at different levels of salt. Concentrations of 100 and 200  $\mu\text{M}$  MEL improved the effect of salinity on pigment content. The ratio of Chl *a/b* decreased as salinity increased. MEL application increased this ratio up to 58% (Fig. 2).

In this study, shoot dry weight (SDW) and root dry weight (RDW) were identified as a factor showing plant growth. Salinity induced a significant decrease in SDW and RDW, MEL at 100 and 200  $\mu\text{M}$  concentrations increased these factors. The highest amount of SDW and RDW was observed in salt-free conditions which were increased up to 25% and 7%, respectively, compared to controls (Fig. 3a, b).

### Sugar and proline contents

Salinity significantly increased the content of sugar and proline content in leaves of *P. vulgaris* cv. Pak. The highest levels were observed under the 200 mM NaCl condition and 100  $\mu\text{M}$  MEL treatment. At this salinity level, sugar and proline contents were increased by 54% and 52%, respectively, with MEL (Fig. 3c, d).

### The amount of hydrogen peroxide and malondialdehyde

Salinity significantly increased leaf  $\text{H}_2\text{O}_2$  and MDA content. The highest values of  $\text{H}_2\text{O}_2$  and MDA have been observed in 200 mM salt and 0  $\mu\text{M}$  MEL. At different levels of NaCl, the effect of MEL at 100  $\mu\text{M}$  concentration was better than others. In the best results, at 200 mM NaCl, 100  $\mu\text{M}$  MEL significantly reduced  $\text{H}_2\text{O}_2$  and MDA levels to 31% and 54%, respectively, and at 100 mM NaCl reduced MDA level to 60% compared to controls (Fig. 4).

### Antioxidant enzymes' activity

Increased activity of antioxidant enzymes is one of the plant's responses to oxidative stress induced by salinity. In this study, MEL increased the activity of SOD, CAT, POX, PPO, and APX. MEL at 400  $\mu\text{M}$  showed the least additive effect. At all levels of NaCl, SOD, and CAT activity significantly increased by 100  $\mu\text{M}$  MEL, compared to the control plants treated by 0  $\mu\text{M}$  MEL. The highest activity of APX was at 200 mM NaCl and 200  $\mu\text{M}$  MEL, with a 102% increase compared to the controls. The 100  $\mu\text{M}$  concentration of MEL significantly increased the activity of PPO at all salt levels. The positive effect of 200  $\mu\text{M}$  MEL on PPO activity at 100 mM NaCl was also significant, compared to the controls. The highest POX activity was measured in 100  $\mu\text{M}$  MEL and 200 mM NaCl, which was significantly

different from the control with an increase of 65%. Table 1 shows the effect of MEL and salinity interaction on the activity of these enzymes.

### Content of $\text{Na}^+$ , $\text{K}^+$ , and $\text{Ca}^{2+}$ ions

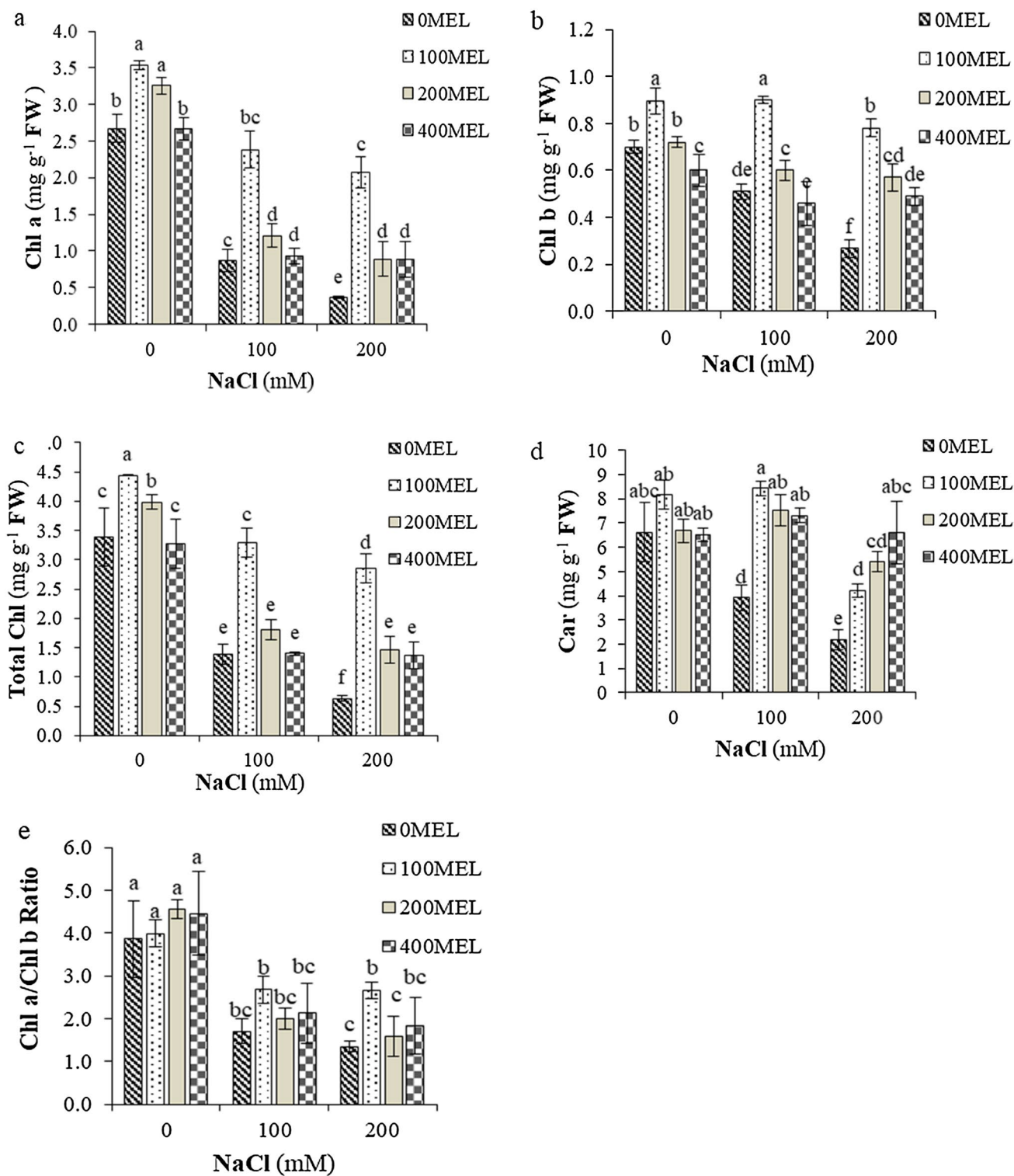
Increasing salinity level decreased root and shoot  $\text{K}^+$  and  $\text{Ca}^{2+}$  and increased  $\text{Na}^+$  in these organs. In roots, at 100 mM NaCl, and  $\text{K}^+$  did not decrease in the absence of MEL. Moreover, the  $\text{K}^+/\text{Na}^+$  ratio also decreased significantly in root and shoot. MEL decreased the salinity effect on  $\text{Na}^+$  root and shoot and increased  $\text{K}^+$  content of root and shoot. It also increased the  $\text{K}^+/\text{Na}^+$  ratio in these two fractions. In roots at 100 and shoots at 200 mM NaCl, MEL application increased  $\text{Ca}^{2+}$  levels by 16% and 69%, respectively. The increasing effect of MEL on  $\text{K}^+$  and  $\text{Ca}^{2+}$  content was observed at concentrations of 100 and 200  $\mu\text{M}$ , respectively, while 400  $\mu\text{M}$  concentration of MEL hurt ions content (Table 2).

## Discussion

### Melatonin improved photosynthesis and plant biomass

Salinity stress affects the water status of the tissue, the metabolic processes, and plant growth by limiting water absorption and ion toxicity (Rady and Mohamed 2015). Drought and salinity stress significantly decrease growth through decreasing chlorophyll content and photosynthesis rate (Zhang et al. 2014b). A decrease in photosynthesis occurs in salinity due to stomatal or non-stomatal reasons (Zhang et al. 2017). In the present study, salinity decreased  $P_N$ ,  $C$ , and  $E$  significantly. Although the improving effect of melatonin 100  $\mu\text{M}$  on these factors was significant, the highest amount was observed in control treatments of NaCl. This shows that melatonin cannot eliminate some of the damages that salinity has resulted in the plant's photosynthetic system.

There is a positive relationship between photosynthesis, stomatal conductance, intrinsic  $\text{CO}_2$ , and biomass production (Qu et al. 2017). When non-stomatal factors are involved, intracellular  $\text{CO}_2$  stays constant or decreases parallel to the decrease in stomatal conductance (Zhang et al. 2017). As the results of the present study show, MEL increased photosynthetic pigments levels in the control group and stressed plants. Chl *a/b* ratio analysis showed that 100  $\mu\text{M}$  MEL had a positive and significant role in raising this ratio under salinity conditions. In plants, the amount of Chl *a* is higher than that of Chl *b*, but with increasing salinity, Chl *b* decreases more than Chl *a* (Parihar et al. 2015). MEL stabilizes photosynthetic processes by preventing the breakdown of chlorophyll and proteins and regulating glucose and nitrogen metabolism (Siddiqui et al. 2019). Other

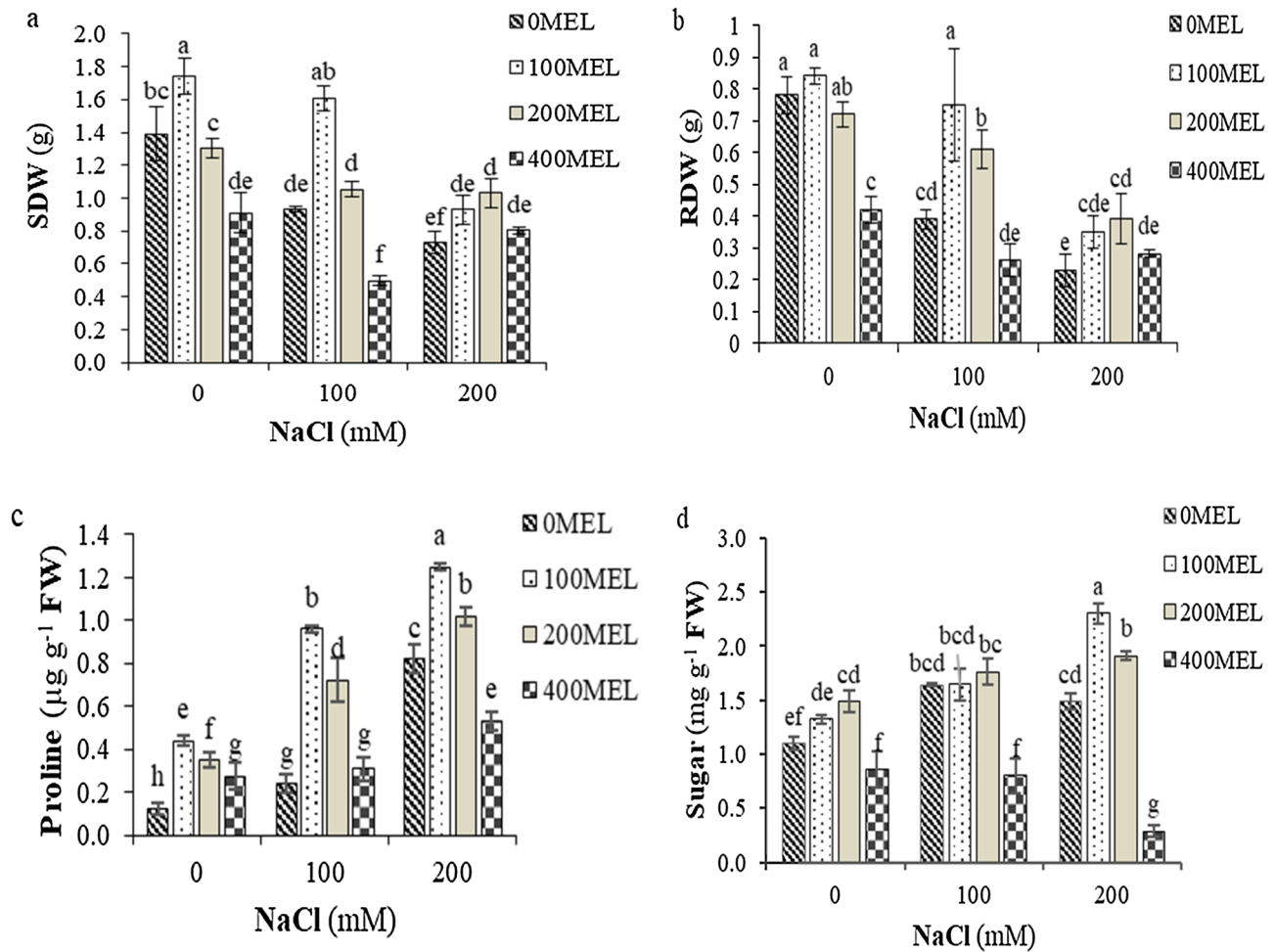


**Fig. 2** Effect of MEL and salt stress on the photosynthetic pigments of *P. vulgaris* L. cv. Pak. Chl a (a), Chl b (b), total Chl (c), Car (d), and Chl a/b ratio (e). Columns with the same letter(s) are not significantly different at  $P \leq 0.05$

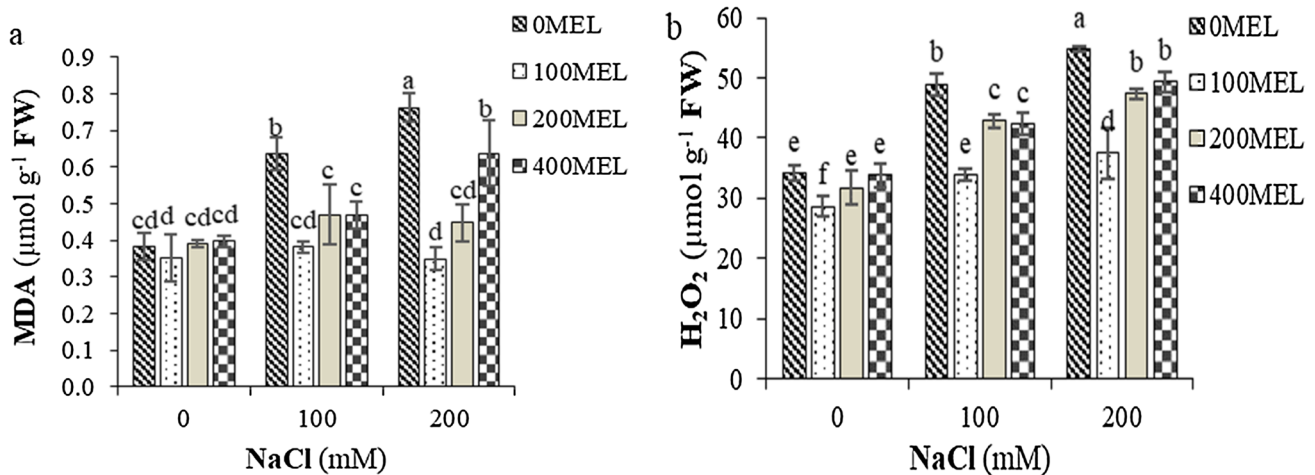
studies have also reported the beneficial effect of MEL on chlorophyll content, photosynthesis, stomatal conductance, and ion homeostasis under drought and salinity stress

conditions (Jiang et al. 2016b; Ye et al. 2016; Gao et al. 2019; Liang et al. 2015).

According to the results of this study, SDW and RDW reduction caused by salinity stress was compensated by



**Fig. 3** Effect of MEL and salt stress on the shoot dry weight (SDW) (a) and root dry weight (RDW) (b), proline (c), and sugar content (d) of *P. vulgaris* L. cv. Pak. Columns with the same letter(s) are not significantly different at  $P \leq 0.05$



**Fig. 4** Effect of MEL and salt stress on MDA (a) and  $\text{H}_2\text{O}_2$  (b) content of *P. vulgaris* L. cv. Pak. Columns with the same letter(s) are not significantly different at  $P \leq 0.05$

**Table 1** Effect of MEL and salt stress on the antioxidant enzymes activity of *P. vulgaris* L. cv. Pak

NaCl (mM)	Melatonin ( $\mu\text{M}$ )	SOD ( $\text{U mg}^{-1}$ FW)	PPO ( $\text{U mg}^{-1}$ protein)	APX ( $\text{U mg}^{-1}$ protein)	POX ( $\text{U mg}^{-1}$ protein)	CAT ( $\text{U mg}^{-1}$ protein)
0	0	1.13 $\pm$ 0.07fgh	7.59 $\pm$ 0.45de	162.5 $\pm$ 12.5d	8.58 $\pm$ 0.73cde	9.7 $\pm$ 1def
	100	1.51 $\pm$ 0.24bcd	14.83 $\pm$ 0.84b	165.67 $\pm$ 9.73d	10.22 $\pm$ 0.96bcde	12.45 $\pm$ 1.52cde
	200	1.03 $\pm$ 0.03h	6.84 $\pm$ 0.46de	165.77 $\pm$ 6.82d	9.93 $\pm$ 0.24bcde	8.2 $\pm$ 0.2gh
	400	1.05 $\pm$ 0.04gh	6.28 $\pm$ 0.48de	160.23 $\pm$ 16.07d	8.06 $\pm$ 0.62de	10.1 $\pm$ 0.82def
100	0	1.28 $\pm$ 0.08defg	7.15 $\pm$ 0.65de	176.17 $\pm$ 7.15d	9.67 $\pm$ 0.11cde	12.9 $\pm$ 1.1cd
	100	1.61 $\pm$ 0.08abc	13.33 $\pm$ 1.04bc	184.75 $\pm$ 12.75d	11.21 $\pm$ 0.86abcd	13.03 $\pm$ 1.7c
	200	1.23 $\pm$ 0.04efgh	10.29 $\pm$ 0.46cd	178.33 $\pm$ 8.35d	11.04 $\pm$ 0.71bcd	11.7 $\pm$ 0.36cdef
	400	1.29 $\pm$ 0.01def	8.03 $\pm$ 0.3de	233.33 $\pm$ 56.65c	7.52 $\pm$ 1.2e	10.43 $\pm$ 1.35cdefg
200	0	1.7 $\pm$ 0.2a	19 $\pm$ 1a	191.5 $\pm$ 0.5d	13.02 $\pm$ 0.56ab	16.97 $\pm$ 0.55b
	100	1.79 $\pm$ 0.06a	20.54 $\pm$ 1.46a	292.67 $\pm$ 35.34b	14.18 $\pm$ 0.87a	19.67 $\pm$ 0.15a
	200	1.54 $\pm$ 0.11b	15.14 $\pm$ 0.86b	328.5 $\pm$ 10.5a	11.68 $\pm$ 1.35abc	9.33 $\pm$ 0.15ef
	400	1.41 $\pm$ 0.13c	3.35 $\pm$ 0.77e	231.67 $\pm$ 28.35c	9.67 $\pm$ 0.67cde	6.17 $\pm$ 0.65h

POX (a), PPO (b), SOD (c), APX (d), and CAT (e)

Difference among data of each column followed by the same letter was not statistically significant ( $P < 0.05$ )

**Table 2** Effect of MEL and salt stress on the content of root and shoot ions of *P. vulgaris* L. cv. Pak

NaCl (mM)	Melatonin ( $\mu\text{M}$ )	$\text{K}^+$ content ( $\text{mg g}^{-1}$ DW)		$\text{Na}^+$ content ( $\text{mg g}^{-1}$ DW)	
		Root	Shoot	Root	Shoot
0	0	106.23 $\pm$ 0.6cd	141.72 $\pm$ 1.2f	25.51 $\pm$ 3.3e	6 $\pm$ 0.9e
	100	137.21 $\pm$ 6.7a	293.45 $\pm$ 9.3a	15.31 $\pm$ 3.6f	3 $\pm$ 0.7f
	200	127.04 $\pm$ 2.1b	273.89 $\pm$ 2.2b	17.44 $\pm$ 2f	4.1 $\pm$ 0.2f
	400	106.4 $\pm$ 2.5cd	107.12 $\pm$ 5.4g	18.2 $\pm$ 1.3f	9 $\pm$ 0.3d
100	0	106.34 $\pm$ 3cd	104.42 $\pm$ 6b	65.82 $\pm$ 1.8b	15.1 $\pm$ 0b.6
	100	111.57 $\pm$ 0.7c	260.98 $\pm$ 5.9c	45.23 $\pm$ 13.1d	9.1 $\pm$ 0.6d
	200	104.59 $\pm$ 3d	226.37 $\pm$ 4.7d	65.81 $\pm$ 3.6b	9 $\pm$ 0.2d
	400	62.02 $\pm$ 2.8f	101.39 $\pm$ 3g	93.83 $\pm$ 2.9a	12.3 $\pm$ 0.4c
200	0	94.69 $\pm$ 2.1e	82.26 $\pm$ 7.3h	96.51 $\pm$ 1.8a	26 $\pm$ 2.8a
	100	105.22 $\pm$ 0.3d	165.45 $\pm$ 2.2e	58.14 $\pm$ 5.1c	12.4 $\pm$ 1.4c
	200	97.68 $\pm$ 2.1e	147.74 $\pm$ 5.2f	71.9 $\pm$ 2.9b	17.2 $\pm$ 1.5b
	400	50.26 $\pm$ 1.6g	106.27 $\pm$ 8.6g	95.17 $\pm$ 2.8a	27.7 $\pm$ 2.6a
NaCl (mM)	Melatonin ( $\mu\text{M}$ )	$\text{K}^+/\text{Na}^+$		$\text{Ca}^{2+}$ content ( $\text{mg g}^{-1}$ DW)	
		Root	Shoot	Root	Shoot
0	0	4.2 $\pm$ 0.6d	24.1 $\pm$ 3.6cd	78.7 $\pm$ 2.3b	77.4 $\pm$ 3e
	100	9 $\pm$ 0.4a	103.04 $\pm$ 22.6a	74.3 $\pm$ 2.5bcd	95.2 $\pm$ 3.4c
	200	7.3 $\pm$ 0.5b	67.6 $\pm$ 0.2b	72.1 $\pm$ 1.1cde	90.3 $\pm$ 5d
	400	5.8 $\pm$ 0.9c	12 $\pm$ 0.5ef	49.5 $\pm$ 1g	72 $\pm$ 4.3f
100	0	1.7 $\pm$ 0.3f	7 $\pm$ 0.7fg	69.1 $\pm$ 1.8e	62.4 $\pm$ 3g
	100	2.5 $\pm$ 0.1e	28.8 $\pm$ 2.4bc	70.3 $\pm$ 1.3de	103.1 $\pm$ 2.7b
	200	1.6 $\pm$ 0.1f	25.1 $\pm$ 0.1c	85 $\pm$ 3.7a	92.4 $\pm$ 5.9cd
	400	0.7 $\pm$ 0.05h	8.2 $\pm$ 0.5efg	55.3 $\pm$ 8.4f	64.7 $\pm$ 2.8g
200	0	1 $\pm$ 0.04gh	3.2 $\pm$ 0.3g	46.9 $\pm$ 1.1g	44.9 $\pm$ 3.1h
	100	1.8 $\pm$ 0.1f	15.5 $\pm$ 1.8cd	77.1 $\pm$ 1bc	121 $\pm$ 2.4a
	200	1.4 $\pm$ 0.1fg	8.6 $\pm$ 0.4de	70.4 $\pm$ 1.5de	106.5 $\pm$ 5.7b
	400	0.5 $\pm$ 0.03h	3.8 $\pm$ 0.05fg	27.1 $\pm$ 1.2h	41.7 $\pm$ 1.5h

$\text{K}^+$  (a),  $\text{Na}^+$  (b),  $\text{K}^+/\text{Na}^+$  (c), and  $\text{Ca}^{2+}$  (d)

Difference among data of each column followed by the same letter was not statistically significant ( $P < 0.05$ )



MEL treatment. By downregulating enzymes involved in lipid degradation via beta-oxidation and slowing the degradation of chlorophyll proteins by changing those proteases, MEL improves chloroplast and chlorophyll stability. Also, the effect of MEL on photosynthesis and the activity of enzymes such as Rubisco can have a positive effect on early net production, which eventually leads to increased plant growth (Wang et al. 2014). Pre-treatment with MEL increases photosynthetic capacity and develops the root system (Zhang et al. 2013). In a study by Zhang et al. (2017) on *Cucumis melo* L., the use of external MEL at a concentration of 200  $\mu\text{M}$  as leaf spray significantly prevented the suppression of cold-induced growth and the plants treated by MEL showed higher growth and chlorophyll content than non-treated ones (Zhang et al. 2017).

### Melatonin enhanced sugar and proline content

Osmotic stress affects the metabolism of carbohydrates and osmolytes such as amino acids, and has a role in osmotic regulation by affecting carbohydrate metabolism (most of all proline) (Qian et al. 2015; Siddiqui et al. 2019). Similar to other studies (Palma et al. 2009), salinity in *P. vulgaris* cv. Pak significantly increased the concentration of soluble sugars and proline in the present study, and the additive effect of MEL on these factors under stress and non-stress conditions was depending on concentration. Since there is a positive relationship between the accumulation of proline osmolyte and plant tolerance to stress (Nanjo et al. 1999), it is possible to emphasize the effective role of MEL in plant tolerance to salinity based on the present study results.

Proline has positive effects on the enzymes and integrity of the cell membrane and plays an adaptive role as an osmotic mediator in plants under stress (Ashraf and Foolad 2007). Qian et al. (2015) showed that the use of MEL in *Arabidopsis* plants, exposed to bacterial invasion, increased the levels of sugars such as fructose, glucose, and glycerol. Among the 16 amino acids they measured, proline levels were significantly increased after treatment. Results of their study demonstrated the effective role of MEL in pathogen tolerance in *Arabidopsis* by affecting the metabolism of sugars. Huang et al. (2017) showed that transgenic *Panicum virgatum* L. with more MEL biosynthetic ability compared to the control group has increased proline content and more resistance to salt stress.

### Melatonin protected membranes by reducing $\text{H}_2\text{O}_2$ level

All stresses increase ROS levels and disrupt ROS homeostasis, causing damage to the membrane and enzymatic systems. Plant response to stress-induced damage usually begins in the early hours of the confronting to the stress

by applying a strong protein and non-protein antioxidant system. Damage to membranes is associated with increased levels of MDA due to environmental stresses such as salinity stress (Posmyk et al. 2008). MEL treatments improved the potential of the plant to tolerate oxidative stress, induced by salinity through increasing transcript levels and growing antioxidant enzyme activity. MEL significantly suppresses  $\text{H}_2\text{O}_2$  production and hydroxyl radicals (Zhang et al. 2013). In the present study in *P. vulgaris* L. cv. Pak, application of 100 and 200  $\mu\text{M}$  MEL significantly decreased  $\text{H}_2\text{O}_2$  and MDA in stress conditions, although higher concentrations of MEL increased these two factors which were observed in earlier studies (Posmyk et al. 2008; Zhang et al. 2013).

The results of Gao et al.'s (2019) research on oats showed that application of external MEL reduced the  $\text{H}_2\text{O}_2$  and MDA content under saline stress conditions (Gao et al. 2019). Similarly, the application of MEL reduced lipid peroxidation and MDA production in corn seedlings under drought stress (Ye et al. 2016). Li et al. (2017) showed that external MEL can reduce salt stress damage in rice, decrease  $\text{H}_2\text{O}_2$  levels, and increase the activity of SOD and CAT enzymes (Li et al. 2017). Similarly, the use of MEL in wheat and cucumber and melon seedlings increased plant resistance to cold stress (Zhao et al. 2017; Cao et al. 2018; Zhang et al. 2017), which was associated with a decrease in  $\text{H}_2\text{O}_2$  level compared to the control group (Cao et al. 2018) and with an increase in net photosynthesis (Zhao et al. 2017).

### Melatonin alleviated salt stress by increasing antioxidant enzyme activity

Oxidative stress is a complex chemical and physiological phenomenon that is caused by almost all biotic and abiotic stresses in plants. The produced ROS damages and deactivates all the important plant polymers, altering the activity of  $\text{K}^+$  and  $\text{Ca}^{2+}$  channels and catalyzing  $\text{Ca}^{2+}$ -dependent signaling events, causing  $\text{K}^+$  leakage, and cell death (Demidchik 2015). Among the antioxidant defense systems, SOD is the first line of defense against oxidative stress that reduces  $\text{O}_2^-$  to  $\text{H}_2\text{O}_2$ . CAT can then convert  $\text{H}_2\text{O}_2$  to  $\text{H}_2\text{O}$  in plant cells. APX can also convert  $\text{H}_2\text{O}_2$  to monodehydroascorbate (MDHA) using ascorbic acid (AsA) and neutralize its toxicity (Cao et al. 2018).

In addition to being directly ROS-scavenging (Park et al. 2013; Cao et al. 2018), MEL has an effective role in reducing oxidative damage by increasing the activity of antioxidant systems, including the SOD, POX, CAT, and APX enzymes. Increasing effects of MEL on alteration of antioxidant enzymes activity in plants exposed to salinity (Zhang et al. 2014a; Li et al. 2017), drought (Wang et al. 2013; Ye et al. 2016), oxidative (Afreen et al. 2006), and cold stress (Zhang et al. 2017) have been reported.

In the present study, MEL increased the activity of SOD, PPO, POX, CAT, and APX enzymes in *P. vulgaris* L. cv. Pak under salinity stress. The use of MEL at a concentration of 100  $\mu\text{M}$  increased the activity of these enzymes at the highest salinity level compared to the control group, which confirms the results of previous studies (Liang et al. 2015; Zhang et al. 2017; Gao et al. 2019). In a previous research MEL at the same concentration, improved fenugreek tolerance to drought stress by increasing CAT, POX, and PPO activity (Zamani et al. 2019). Jiang et al. (2016a, b) also reported an increase in the activity of the POX and APX enzymes by applying MEL in corn leaves under salinity stress (Jiang et al. 2016b).

### Melatonin alleviated salt stress by improving ion homeostasis

In the present study, MEL had an alleviative effect on increased  $\text{Na}^+$  and decreased amounts of root and shoot  $\text{K}^+$  and  $\text{Ca}^{2+}$  induced by salt stress. MEL also increased the  $\text{K}^+/\text{Na}^+$  ratio in the shoot and root of bean plants. Li et al. (2017) studied rice, showing that pretreatment with 75  $\mu\text{M}$  MEL reduced  $\text{Na}^+$  and  $\text{Cl}^-$  content and  $\text{Na}^+/\text{K}^+$  ratio in roots and leaves, but had no significant effect on  $\text{K}^+$  values (Li et al. 2017), whereas in the case of *P. vulgaris* L. cv. Pak, MEL increased  $\text{K}^+$  in the control group and the stressed plants. Research by Zhang et al. has shown that MEL increases the expression of genes for the  $\text{K}^+$  (*AKTI*) and sodium (*NHX1*) channels that are involved in the development of ion homeostasis (Zhang et al. 2014b). Increased  $\text{K}^+$  content in shoots after MEL application has a positive effect on stomatal conductance and increased  $\text{CO}_2$  uptake (Qu et al. 2017). The results of the present study showed that MEL (100  $\mu\text{M}$ ) improved stomatal conductance under NaCl 100 and 200 mM, which is consistent with increased amounts of potassium in the shoot.

### The effects of different MEL concentrations on alleviating salt-induced damages

Melatonin is involved in plant development and abiotic stress responses. However, the effect of MEL, like hormones such as auxin, is significantly related to its concentration. In this study, treatment with 100 or 200  $\mu\text{M}$  MEL alleviated the salt-induced inhibition of plant growth and biomass accumulation, and improved NaCl-induced damages by increasing antioxidant activity. Whereas in most cases, a concentration of 400  $\mu\text{M}$  MEL intensified salinity stress damages, which agrees with the results in the previous studies.

There are a very limited number of reports indicating the beneficial impact of high MEL concentrations on plant tolerance to stress conditions. Our results at 400  $\mu\text{M}$  concentration level demonstrate the inhibitory effects of MEL

concentration. The inhibitory effects of higher levels of MEL, such as auxin, have been reported in cases like root growth. In mustard, low MEL concentration (0.1 mM) stimulated root growth, whereas higher concentration (100 mM) had an inhibitory effect on growth. Low concentrations of MEL led to rooting and high concentration inhibited growth in the case of cherry under tissue culture (Zhang et al. 2014b). MEL in the high and low concentration ranges in different species and even close to one another have had varying effects (Arnao and Hernández-Ruiz 2009; Zhang et al. 2014b, 2017). For instance, in *Brassica oleracea* L., concentrations of 1 and 10  $\mu\text{M}$  MEL subsided the inhibitory effects of copper and 100  $\mu\text{M}$  MEL concentration exacerbated these effects (Posmyk et al. 2008), whereas MEL with the concentration levels of 200  $\mu\text{M}$  have had an ameliorative effect on cucumber seedlings, being subjected to the chilling stress (Zhao et al. 2017). MEL with a concentration level of 0.1  $\mu\text{M}$  improved corn growth under salinity stress conditions, while increasing net photosynthesis and antioxidant activity of its enzymes and improving the ion homeostasis (Jiang et al. 2016b).

### Conclusions

In the present study, NaCl stress inhibited plant growth and biomass accumulation in bean plants, which was associated with a decrease in photosynthesis and the content of photosynthetic pigments and a decrease in  $\text{K}^+$  in stressed plants. The results of atomic absorption and change in  $\text{K}^+$  values in shoots, as well as comparison of the results of changes in stomatal conductance and pigments content, indicate that possibly photosynthetic degradation, in this case, is related to non-stomatal factors such as chlorophyll degradation and damage to the membranes, which are also linked to stomatal causes.

In general, the relationship between different physiological parameters in *P. vulgaris* L. cv. Pak under salinity stress revealed that an increase in salinity level from 100 to 200 mM NaCl, in most cases, promoted significant changes, and treatment with MEL for both levels of salinity had alleviating effects. The effects are testified to be concentration-dependent to the extent that MEL concentrations of 100  $\mu\text{M}$  and, in some cases, 200  $\mu\text{M}$  had ameliorating effects, and in many cases, 400  $\mu\text{M}$  MEL concentration intensified the effects of salinity.

**Author contribution statement** F. Azizi took part in performing the experiments and preparation of the manuscript, H. Amiri designed experiments and revised the final version of the manuscript. A. Ismaili evaluated the statistical sections of the research and revised the final version of the manuscript.

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## Declarations

**Conflict of interest** The authors declare no financial or other competing conflicts of interest.

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