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Alleviation Mechanism of Melatonin in Chickpea (*Cicer arietinum* L.) under the Salt Stress Conditions

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Abstract: Melatonin (MT) is considered to be a hormone involved in increasing tolerance in plants under stress. The effect of different doses (0, 50, and 100 µM) of MT on the growth, biochemical and physiological properties of chickpea under salt stress was investigated. Salt stress significantly suppressed the growth, leaf relative water content (LRWC), chlorophyll reading value (CRV), chlorophyll a, chlorophyll b, and total chlorophyll. Salinity conditions also caused a decrease in macro- and micronutrients, while electrolyte leakage (EL), hydrogen peroxide (H_2O_2), malondialdehyde (MDA), and proline contents, catalase (CAT), peroxidase (POD), and superoxide dismutase (SOD) activities increased under salinity conditions. MT treatments increased plant fresh weight, plant dry weight, root fresh weight, root dry weight, plant height, stem diameter, LRWC, CRV, chlorophyll a, chlorophyll b, total chlorophyll, total carotene of chickpea seedlings under 75 and 150 mM NaCl compared to the non-MT treatment. Especially, 100 µM MT treatment under 75 and 150 mM salinity conditions reduced the H_2O_2 and MDA contents compared to the non-MT-treated plants. Moreover, exogenous MT increased the K^+/Na^+ and Ca^{+2}/Na^+ ratios under salt stress conditions. It could be concluded exogenous MT treatments alleviated the salt stress on chickpea by modulating physiological and biochemical properties. Especially 100 µM MT treatment can be suggested for decreasing the negative influence of salinity on chickpea seedlings.

Keywords: chickpea; growth; melatonin; physiology; salt stress

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1. Introduction

Plants often face many environmental constraints such as extreme temperatures, salinity, and drought stress [1]. Plant growth and productivity are negatively affected by these abiotic stresses. Salinity stress is one of the destructive environmental factors [2], which is an increasingly serious problem [3]. Approximately 7% of the world's land is affected by salt, which negatively affects both growth and development of plants [4]. Salt stress imposes major limitations on the growth, development, crop productivity, and quality [5] through impairing metabolic processes in plants. Secondary metabolite accumulation in stressed plants can be stimulated [6,7]. Excess salt ions have been recorded to change mineral uptake, disrupt nutrient balance, and minimize water absorption and reduce plant growth. As with many other stress factors, plants under salt stress close their stomata to reduce water loss, thus preventing the entry of CO_2 gas. In plants under stress, increased reactive oxygen species (ROS) damage cells, causing damage to cell components such as protein membrane lipids, nucleic acids, and chlorophyll [8].

Various technologies have been employed to improve the tolerance of plants to salt stress [9]. Plant growth regulators (PGRs) are promising substances in enhancing plant stress tolerance. Employment of PGRs against some stress conditions has been determined to enhance tolerance significantly in several crops.

Biostimulants are used to increase plant growth, plant nutrition, crop quality and yield; they are applied to plant leaves, in the soil, or as seed treatments. Furthermore, they can increase the resistance of plants to stress [1,10–12].

Melatonin (N-acetyl-5-methoxytryptamine, MT) was first identified in animals. It regulates circadian sleep rhythms [13] and the immune system [14] and also acts as an enhancer for the activities of antioxidant enzymes [15–17]. It has been determined that MT is found in almost all organs of various plant species. Chloroplasts and mitochondria are where the biosynthesis of MT occurs [18]. MT has been proven to show significant functions in response to abiotic stress in conditions (low temperature, high salinity, drought) as a growth regulator in crops [19,20]. The mitigating impacts of MT on abiotic stress are generally related to the increased activity of antioxidant enzymes [21]. Both exogenous administration of MT and endogenous MT can mitigate the negative impacts of abiotic stress conditions [19], regulating many physiological processes such as osmoregulation in plants [22]. MT also functions as an auxin-like regulator, acting upstream of the auxin pathway to alter the expression profiles of various auxin-related transcription factors [18,23].

Chickpea is one of the most widely produced edible legumes all over the world. Chickpea is generally grown in arid and semi-arid regions. Salinity, which creates a problem in both arid and semi-arid and irrigated areas, also affects chickpea cultivation. Although chickpea is less resistant to salt than many grain crops, it is the most resistant to salt among edible legumes [24]. Exogenous application of MT has been shown to enhance plant tolerance against some abiotic stress conditions by modulating the biosynthesis of endogenous MT and the activities of antioxidative enzymes [25–28]. However, this study is the first study to demonstrate effects of MT applications in chickpeas grown under saline conditions at the morphological, physiological, and biochemical levels. This study was conducted primarily to focus on the impacts of spraying seedlings with MT on enhancing salt stress tolerance of chickpea plants; secondly, to reveal the mechanism of MT effect on the growth of chickpea under salinity conditions.

2. Materials and Methods

2.1. Plant Material and Growth Conditions

Chickpea (*Cicer arietinum* L.) plants were grown in the greenhouse of Atatürk University, Erzurum, under ambient light, at 20–30 °C (the night–day temperature), and with a relative humidity of 50–60%. Chickpea (*Cicer arietinum* L.) seeds of uniform size were surface-sterilized using 5% NaOH and subsequently washed with double-distilled water. The seeds were sown into a mixture of soil, sand, and peat (2:2:1, v:v:v) in a controlled greenhouse.

The physical and chemical soil properties of the medium used in the study were determined: sandy loam texture (23.6% clay, 33.5% silt, and 42.9% sand); pH, 7.5; electrical conductivity, 0.23 $dS \cdot m^{-1}$; organic matter, 2.02%; NH_4–N, 1.44 $mg \cdot kg^{-1}$; NO_3-N, 0.85 $mg \cdot kg^{-1}$; P, 3.2 $mg \cdot kg^{-1}$; K, 2.60 $cmolc \cdot kg^{-1}$; Ca, 17.50 $cmolc \cdot kg^{-1}$; Mg, 2.40 $cmolc \cdot kg^{-1}$; Na, 0.24 $cmolc \cdot kg^{-1}$; Fe, 2.40 $mg \cdot kg^{-1}$; Mn, 1.05 $mg \cdot kg^{-1}$; Zn, 0.20 $mg \cdot kg^{-1}$; Cu, 0.60 $mg \cdot kg^{-1}$; B, 0.45 $mg \cdot kg^{-1}$; and $CaCO_3$, 2.8%.

2.2. MT Treatments

MT solutions of 0 µM, 50 µM, and 100 µM (pH, 6.0–6.5) were made with distilled water containing 0.02% Tween 20 (Sigma Chemicals, Gillingham, UK). The MT solutions (50 mL for each seedling) were applied as foliar application in the late afternoon hours with one-week intervals using a handheld sprayer. A control spray treatment consisting of 0.02% Tween 20 in deionized water was applied to the treatments not receiving MT. MT applications were initiated 7 days after seedling emergence. During spraying, care was taken to ensure that both surfaces of the leaves were completely wet.

2.3. Salinity Treatments

The first saline irrigations were made with 25 mM NaCl solutions in the day after the first MT application and gradually increased and fixed in the end after 150 mM doses.

Electrical conductivity (EC) of the soils was determined with a portable EC meter (HH_2 Moisture Meter, WET Sensor, Delta-T Devices, Cambridge, England). EC of the non-saline (S0), saline (75 mM), and saline (150 mM) soils as measured were 0.55 dS m^{-1}, 4.68 dS m^{-1}, and 7.92 dS m^{-1}, respectively, at harvesting. A soil moisture meter (WET Sensor) was used to calculate irrigation water amounts. The pots were irrigated to reach field capacity at each irrigation.

2.4. Growth Parameters

The study was terminated 45 days after seed sowing. Plant height, stem diameter, leaf area, plant fresh weight, plant dry weight, root fresh weight, and root dry weight were determined. The roots were carefully harvested from the pots and gently washed to remove the soil. Maximum attention was paid to avoid root loss. For dry weight measurements, the plant material was dried at 70 °C for 48 h. The leaf area was determined by using a leaf area meter (CI-202 Portable Laser Leaf Area Meter by CID Bio-Science, Ave Camas, WA, USA).

2.5. CRV and Chlorophyll Content

The CRV was detected using a SPAD-502 chlorophyll meter (SPAD-502, Konica Minolta Sensing, Inc., Osaka, Japan). The amount of chlorophyll was assessed using the method defined by Lichtenthaler and Buschmann [29].

2.6. Physiological Parameters

For the leaf relative water content (LRWC), the method described by Sahin et al. [30] was followed.

2.7. Antioxidant Enzyme Activity

Superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD) activities were determined based on the method described by Sahin et al. [30].

2.8. Proline Content

Proline content was determined according to the slightly modified method of Ekinci et al. [10].

2.9. H_2O_2 and MDA Content

The amount of H_2O_2 was quantified as described by Loreto and Velikova [31]. Thiobarbituric acid-reactive substances were determined by measuring the malondialdehyde (MDA) content, which is an output of lipid peroxidation [32].

2.10. Mineral Analysis

Mineral element analyses were performed to determine the K, Ca, and Na content. First, plant leaf samples were subjected to combustion in nitric acid–hydrogen peroxide (2:3) acid solutions in a microwave wet combustion unit resistant to 40 bar pressure (Speedwave MWS-2 Berghof Products + Instruments Harresstr.1. 72800 Enien, Germany). Three consecutive steps were completed: (1) 5 min at 145 °C and 75% microwave power, (2) 10 min at 180 °C and 90% microwave power (3), and 10 min at 100 °C and 40% microwave power [33]. Then, mineral element content was determined spectrophotometrically (Optima 2100 DV, ICP/OES; Perkin-Elmer, Shelton, CT, USA) [34].

2.11. Statistical Analysis

The data obtained were analyzed using the SPSS 18 statistical package program (IBM, New York, NY, USA). The data were subjected to two-way variance analysis (ANOVA), and differences of means were determined using Duncan's multiple comparison test [35].

3. Results and Discussion

The analysis of variance showed significant interactions of MT application and salinity stress for the plant growth characteristics of chickpea (Table 1). Salinity treatments significantly suppressed plant growth of bean seedlings. Compared with the non-salt-treated group, 150 mM NaCl caused a reduction in plant fresh weight by 64.0%, plant dry weight—by 62.2%, root fresh weight—by 43.2%, root dry weight—by 56.0%, plant height—by 22.8%, stem diameter—by 17.3% (Table 1 and Figure 1). In previous studies, it was reported that salt stress restricts plant growth by adversely influencing various aspects of physiology and biochemistry of plants [10,12,36,37]. Plants usually close or constrict their stomata to prevent water loss via respiration under salt stress, restricting nutrient uptake and CO_2 entrance to leaf cells. The decrease in the amount of CO_2 and water taken into the plant as well as irregularities in the intake of plant nutrients cause a reduction in photosynthesis, eventually resulting in the retardation of plant growth and death [12].

Table 1. Plant growth response of chickpea seedlings under salinity stress to MT applications.

NaCl (mM)	MT (µM)	Plant Fresh Weight (g plant^{-1})	Plant Dry Weight (g plant^{-1})	Root Fresh Weight (g plant^{-1})	Root Dry Weight (g plant^{-1})	Plant Height (cm)	Stem Diameter (mm)
0	0	6.58 ± 0.12 ^{b}	1.19 ± 0.05 ^{b}	6.49 ± 0.6 ^{b}	0.59 ± 0.03 ^{b}	26.8 ± 1.2 ^{b}	3.54 ± 0.05 ^{b,c}
	50	6.61 ± 0.14 ^{b}	1.36 ± 0.07 ^{a}	7.56 ± 0.3 ^{a}	0.65 ± 0.02 ^{a}	27.27 ± 1.3 ^{b}	3.91 ± 0.06 ^{a,b}
	100	7.48 ± 0.11 ^{a}	1.38 ± 0.05 ^{a}	7.28 ± 0.0.4 ^{a}	0.68 ± 0.02 ^{a}	31.19 ± 1.1 ^{a}	4.06 ± 0.03 ^{a}
75	0	2.89 ± 0.04 ^{e}	0.62 ± 0.02 ^{d}	3.78 ± 0.05 ^{f}	0.32 ± 0.05 ^{f}	19.84 ± 1.5 ^{e}	2.98 ± 0.02 ^{d}
	50	3.59 ± 0.07 ^{d}	0.73 ± 0.01 ^{c,d}	4.88 ± 0.07 ^{d}	0.42 ± 0.03 ^{c,d}	23.42 ± 1.3 ^{c,d}	3.41 ± 0.05 ^{c}
	100	3.84 ± 0.09 ^{cd}	0.87 ± 0.02 ^{c}	5.45 ± 0.03 ^{c}	0.47 ± 0.06 ^{c}	23.33 ± 1.6 ^{c,d}	3.74 ± 0.02 ^{a,c}
150	0	2.37 ± 0.3 ^{e}	0.45 ± 0.02 ^{e}	3.04 ± 0.02 ^{g}	0.26 ± 0.03 ^{g}	20.88 ± 1.4 ^{d,e}	2.93 ± 0.01 ^{d}
	50	3.08 ± 0.3 ^{d}	0.50 ± 0.043	4.20 ± 0.05 ^{e}	0.37 ± 0.02 ^{d}	20.57 ± 1.3 ^{d,e}	3.48 ± 0.03 ^{b,c}
	100	3.46 ± 0.4d	0.75 ± 0.02 ^{c}	4.25 ± 0.02 ^{e}	0.38 ± 0.02 ^{d}	24.73 ± 1.7 ^{b,c}	3.57 ± 0.02 ^{b,c}

The means followed by a different letter in the same column were significantly ($p < 0.001$) different according to Duncan's multiple range test.

The effects of MT application on chickpea growth under salinity and non-salinity conditions were found to be statistically significant ($p < 0.001$). The findings of the study pointed out that foliar MT application improved the growth of the chickpea seedlings (Table 1). Compared with the control (non-MT treatment), the 100 µM exogenous MT treatment increased plant fresh weight by 32.9% and 45.9% at 75 and 150 mM NaCl, respectively, plant dry weight—by 40.3% and 66.7%, root fresh weight—by 44.2% and 39.8%, root dry weight—by 46.8% and 46.15%, plant height—by 17.6% and 18.4%, stem diameter—by 25.5% and 21.8%. In a study on *Arabidopsis*, it was determined that the high dose of MT had a negative effect, the low dose had no effect at all, whereas the moderate MT dose had the best effect in improving plant growth [38]. Moreover, on cotton seedlings, the most effective dose was determined to be 200 µM of MT [39]. It has been shown that the effect of MT is dose-dependent, and this effect varies from plant to plant [40]. Previous studies indicated that MT application could led to an increase in endogenous MT levels, enhancing turgidity and photosynthetic activity under water deficit conditions as a result [41,42]. Furthermore, MT application has been reported to stimulate germination, lateral root formation, hypocotyl elongation and growth under abiotic stress conditions [28,43]. MT is considered to be a natural biostimulating regulator which is widely involved in regulating growth, development, and yield of crops [19].

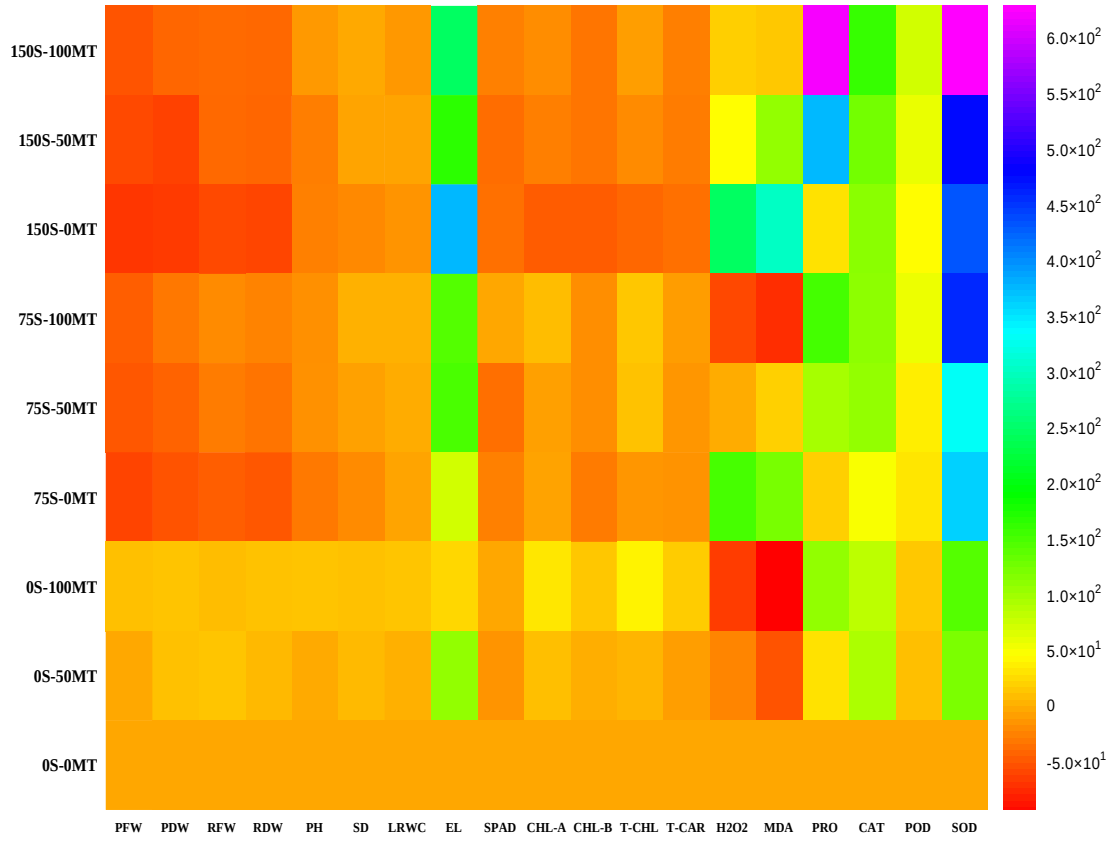

Figure 1. Heatmap analysis for percentage change (%) of the morphological, physiological, and biochemical parameters of chickpea with different treatments compared to the control. PFW: plant fresh weight, PDW: plant dry weight, RFW: root fresh weight, RDW: root dry weight, PH: plant height, SD: stem diameter, LRWC: leaf relative water content, EL: electrolyte leakage, SPAD: chlorophyll reading value, CHL-A: chlorophyll a, CHL-B: chlorophyll b, T-CHL: total chlorophyll, T-CAR: total carotenoid content, H_2O_2: hydrogen peroxide, MDA: malondialdehyde, PRO: proline, SUC: sucrose, CAT: catalase, POD: peroxidase, SOD: superoxide dismutase.

As can be seen in Table 2, salinity stress decreased the CRV, chlorophyll a, chlorophyll b, total chlorophyll and carotenoid content of chickpea. The CRV, chlorophyll a, chlorophyll b, total chlorophyll, and total carotenoid content decreased by 31.1%, 42.8%, 42.9%, 36.2%, and 31.1%, respectively, under 150 mM NaCl compared to the control. Salty conditions reduce magnesium (Mg^{+2}) and iron (Fe^{+2}) uptake in plants. Especially, Fe^{+2} and Mg^{+2} deficiencies cause a decrease in chlorophyll synthesis under salinity conditions [44]. Accumulation of Na^{+} and ROS in plants under salt stress may lead to negative effects and contribute to the reduction of photosynthetic pigments [45]. The decrease in chlorophyll a and b content with salt stress generally occurs due to the damage to the chloroplast membranes. The deterioration in pigments has been attributed to salt-induced weakening of the protein–pigment–lipid complex or to increased chlorophyllase enzyme activity [46]. On the other hand, MT treatments improved the chlorophyll content of the chickpea seedlings under salt stress (Table 2), which confirmed the crucial role of MT in the maintenance of pigment–protein complexes. An exogenous 100 µM MT application mitigated the adverse impact of salinity conditions on the chlorophyll content in radish [23]. Similarly, it was shown that a 100 µM MT application enhanced the chlorophyll content of cucumber under salinity conditions [47]. In our study, similar results were observed. Pretreatment with MT enhanced the salt tolerance in rice, enhancing photosynthetic activity [40].

Table 2. CRV, chlorophyll a, b, total chlorophyll, and total carotenoid content response in leaves of the chickpea seedlings under salinity stress to MT applications.

NaCl (mM)	MT (μM)	CRV (SPAD)	Chlorophyll a (mg g $^{-1}$)	Chlorophyll b (mg g^{-1})	Total Chlorophyll (mg g^{-1})	Total Carotenoid Content (mg g^{-1})
0	0	47.00 ± 2.2 ^{a}	0.75 ± 0.1 ^{c}	0.28 ± 0.03 ^{b}	0.94 ± 0.03 ^{c}	5.59 ± 0.12 ^{b}
	50	41.90 ± 2.4 ^{b}	0.85 ± 0.3 ^{a,b}	0.29 ± 0.02 ^{b}	1.01 ± 0.06 ^{b}	5.28 ± 0.15 ^{b}
	100	47.00 ± 2.4 ^{a}	1.02 ± 0.2 ^{a}	0.33 ± 0.01 ^{a}	1.34 ± 0.04 ^{a}	6.74 ± 0.17 ^{a}
75	0	36.40 ± 1.8 ^{c}	0.73 ± 0.07 ^{c}	0.21 ± 0.02 ^{d}	0.85 ± 0.03 ^{c}	4.96 ± 0.14 ^{c}
	50	32.13 ± 1.9 ^{d}	0.72 ± 0.04 ^{c}	0.24 ± 0.04 ^{c}	1.08 ± 0.07 ^{b}	5.03 ± 0.16 ^{c}
	100	46.77 ± 1.5 ^{a}	0.84 ± 0.03 ^{a,b}	0.24 ± 0.05 ^{c}	1.11 ± 0.05 ^{a,b}	5.25 ± 0.13 ^{b}
150	0	32.40 ± 1.1 ^{d}	0.43 ± 0.04 ^{f}	0.16 ± 0.06 ^{e}	0.60 ± 0.07 ^{d}	3.85 ± 0.15 ^{e}
	50	31.50 ± 1.7 ^{d}	0.58 ± 0.02 ^{d,e}	0.20 ± 0.04 ^{d}	0.79 ± 0.02 ^{c}	4.21 ± 0.18 ^{d}
	100	36.50 ± 1.3 ^{c}	0.64 ± 0.05 ^{d}	0.20 ± 0.04 ^{d}	0.89 ± 0.01 ^{c}	4.31 ± 0.11 ^{d}

The means followed by a different letter in the same column were significantly ($p < 0.001$) different according to Duncan's multiple range test.

Furthermore, 50–150 μM MT applications increased photosynthesis in cucumber under salinity conditions. Photosynthesis enhancement is modulated by elevated chlorophyll content. Overall, exogenous MT improves photosynthesis by significantly mitigating chlorophyll impairment and stomatal closure led by salinity conditions, thus improving salinity stress tolerance [47]. MT also increased the salt tolerance of rice seedlings by reducing the rate of chlorophyll impairment [48,49]. It was determined MT applications indirectly regulated root formation, modulating the ROS system. [50]. Exogenous MT treatments have been reported to cause chlorophyll recovery under water deficit conditions [42,43]. MT plays a protective role in preventing chlorophyll aging and increases the efficiency of photosynthesis. It promotes plant growth by preventing a decrease in photosynthesis due to drought stress [51]. MT application increased photosynthesis by 19% in salt-stressed maize [52]. Again, 100 mM leaf MT was applied against drought stress in maize, and it increased the photosynthetic activity and stomatal conductivity [53].

Salt stress statistically ($p < 0.001$) affected the EL and LRWC compared to those of the non-salt-treated plants. The LRWC decreased by 11.0% while the EL increased by 378.8% under 150 mM NaCl compared to the control (Figure 2). Since salinity causes an external osmotic stress, the LRWC was reduced (Figure 2). Damage in leaves caused by NaCl toxicity resulted in an increased leakage of electrolytes from cell membranes [10,12]. The value of EL decreased with 100 μM MT under 150 mM NaCl. The best results were achieved when 100 μM MT was applied under salt stress conditions. The LRWC of the 100 μM MT-treated chickpea seedlings increased by 12.7% compared to the non-MT-treated plants under 150 mM NaCl. Under 75 mM NaCl, the MT treatment of plants did not positively affect the membrane permeability and LRWC in the plants.

At 150 mM NaCl, the H_2O_2, MDA, proline content, CAT, POD, and SOD activities increased by 249.0%, 304.6%, 33.3%, 115.2%, 48.5%, and 434.1%, respectively, compared with the non-stressed plants (Table 3). Na^+ and Cl^- ions cause not only nutrient deficiency and nutritional imbalance, but also synthesis of reactive oxygen species (ROS) in plants under salinity stress conditions. The increase in ROS results in membrane function impairment, DNA damage, decrease in protein and chlorophyll syntheses [54]. As can be seen in Table 3, salt stress increases the H_2O_2 and MDA contents of chickpea. H_2O_2 is a strong oxidant that is synthesized from O_2 under stress conditions, and MDA is the major reactive aldehyde from the peroxidation of cell membranes. MDA is a product of lipid oxidation, and H_2O_2 is a typical ROS in plants [55].

The composition of lipids in cell membranes may change and/or irregularities in the hydrolysis of phospholipids may occur in crops under salinity conditions. The elevated levels of H_2O_2 due to the oxidative damage in the cell membranes increased the MDA content, which led to an increase in membrane permeability (EL) (Figure 2). As can be seen

in Table 3, the amount of proline increases under salinity stress since plants accumulate proline to increase their resistance to salinity stress [56,57]. Salinity conditions stimulate ROS accumulation (O_2^-, H_2O_2, OH^-, and $1O_2$) [58]. Excess ROS usually lead to cell damage and oxidative stress [59]; they also act as signaling molecules fundamentally involved in mediating salt tolerance [26]. Plants develop an antioxidant system to alleviate ROS-triggered damage, enzymatic and non-enzymatic systems [60].

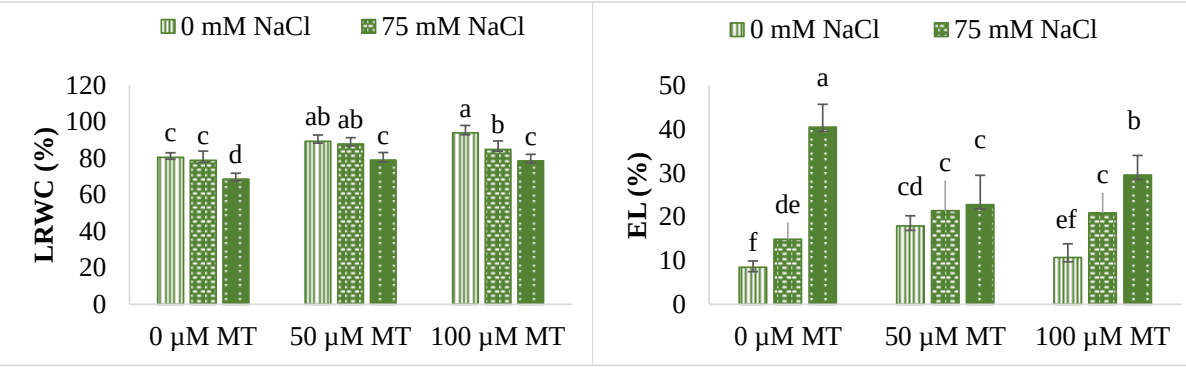

Figure 2. The LRWC and EL response in the leaves of the chickpea seedlings under salinity stress to MT applications. The means followed by a different letter on the bars were significantly ($p < 0.001$) different according to Duncan's multiple range test.

Table 3. H_2O_2, MDA, proline, sucrose, CAT, POD, and SOD activity response in the leaves of the chickpea seedlings under salinity stress to MT applications.

NaCl (mM)	MT (µM)	H_2O_2 (mmol kg^{-1})	MDA (mmol kg^{-1})	Proline (mmol kg^{-1})	CAT (eu g^{-1})	POD (eu g^{-1})	SOD (eu g^{-1})
0	0	4.55 ± 0.11 ^{e}	2.16 ± 0.14 ^{d}	0.09 ± 0.01 ^{f}	849.12 ± 17.65 ^{f}	10,284.93 ± 22.54 ^{f}	540.62 ± 10.44 ^{h}
	50	3.66 ± 0.14 ^{f}	1.13 ± 0.05 ^{e}	0.12 ± 0.03 ^{e}	1669.89 ± 18.13 ^{d}	11,651.22 ± 24.62 ^{e}	1211.77 ± 17.53 ^{g}
	100	1.78 ± 0.16 ^{h}	0.12 ± 0.02 ^{f}	0.19 ± 0.02 ^{c,d}	1592.38 ± 16.43 ^{d}	12,210.89 ± 25.77 ^{e}	1330.96 ± 16.40 ^{f}
75	0	11.56 ± 1.10 ^{b}	4.88 ± 0.11 ^{b}	0.11 ± 0.02 ^{e,f}	1299.62 ± 12.55 ^{e}	13,952.95 ± 29.48 ^{d,e}	2511.04 ± 15.90 ^{d}
	50	4.66 ± 1014 ^{e}	2.66 ± 0.06 ^{c}	0.18 ± 0.04 ^{c,d}	1790.30 ± 15.98 ^{c}	14,391.24 ± 24.85 ^{d}	2345.81 ± 18.56 ^{e}
	100	2.10 ± 0.12 ^{g}	0.67 ± 0.01 ^{g}	0.23 ± 0.04 ^{c}	1817.91 ± 19.23 ^{c}	16,441.12 ± 25.71 ^{b}	3029.99 ± 17.21 ^{b}
150	0	15.88 ± 1.14 ^{a}	8.74 ± 1.12 ^{a}	0.12 ± 0.01 ^{e}	1832.22 ± 13.86 ^{c}	15,268.32 ± 22.68 ^{c}	2890.17 ± 19.05 ^{c}
	50	6.79± 0.11 ^{c}	4.55± 0.23 ^{b}	0.43 ± 0.02 ^{b}	1944.96 ± 10.43 ^{b}	16,629.69 ± 30.19 ^{b}	3124.15 ± 14.88 ^{b}
	100	5.56 ± 0.13 ^{d}	2.56 ± 0.08 ^{c}	0.65 ± 0.04 ^{a}	2237.35 ± 11.77 ^{a}	18,074.17 ± 20.80 ^{a}	3930.75 ± 20.67 ^{a}

The means followed by a different letter in the same column are significantly ($p < 0.001$) different according to Duncan's multiple range test.

We have found that exogenous MT application reduced the EL, H_2O_2 and MDA contents of chickpea under salinity conditions (Figure 2 and Table 3). The data show that foliar MT treatment of the chickpea seedlings affected by salinity stress enhanced tolerance to this stress and that this improvement was accompanied by a decrease in oxidative markers (H_2O_2 and MDA) and increased CAT, SOD, and POD enzyme activities. These effects depended on the MT dose, and were the greatest at 100 µM MT. When 100 µM MT was applied under the 75 and 150 mM salinity conditions, the H_2O_2 and MDA contents were reduced by 81.8–64.9% and 86.2–70.7%, respectively, compared to the non-MT-treated plants (Figure 1 and Table 3). On the other hand, when MT was applied, the proline content of the chickpea plants increased since MT alleviated salinity stress. The MT content of plants increases under salt stress. Reactive oxygen species (ROS) clearance plays an important role in increasing photosynthetic efficiency and metabolite content. External application of MT can be effective in resisting salt stress and increasing the chance of survival of the plant. The improvement in physiological processes by means of MT application may result in indicate a better performance for plant growth and development under salinity conditions.

MT has been proven to reduce the adverse effects of salinity on plant growth in a variety of crops. MT is considered a plant hormone because it regulates growth, development, and responses to biotic and abiotic stresses in plants [1,26,61].

The highest SOD, CAT, and POD activities in the chickpea plants were found under salinity conditions (Table 3). Exogenous MT treatments further increased the SOD, CAT, and POD activities in saline and non-saline conditions. Furthermore, MT treatments significantly enhanced the activity of antioxidant enzymes of several plants under salinity conditions [52,62]. MT has been reported to interact with ROS by enriching their antioxidant activities [48]. MT applications were found to increase the AsA and GSH concentrations in cucumber seedlings 1.7 and 1.3 times, respectively, compared to the control [47]. Similar findings were found for maize under salinity conditions, where MT increased the AsA and GSH concentration [62,63]. Since MT is a strong water-soluble antioxidant that can mitigate stress-induced ROS damage through different mechanisms, a reduction in antioxidant enzyme activity in chickpea seedlings under salinity stress conditions was determined when MT was applied. It has been observed that there is a decrease in the amount of ROS and an increase in the content of amino acids, organic acids, and sugars in plants exposed to various abiotic stresses after melatonin application [64]. These findings show that MT induces enzymatic or non-enzymatic antioxidants to scavenge increased ROS in plants exposed to stress, thereby rendering the plants stress-tolerant. The mitigating role of MT in oxidative damage from salt stress has been demonstrated, but the role of MT has been little reported in the literature. Previous studies investigated defense mechanisms in plants under stress in terms of ROS scavenging by antioxidants [45]. The alleviation effects of MT on plant abiotic stress are usually associated with enhanced activity of antioxidant enzymes [21]. Catalase is involved in the dismutation (disproportionation) of H_2O_2 to H_2O and O_2 [65]. Generally, salinity conditions result in increased CAT activity. In this study, MT increased the activity of CAT under salinity conditions. Earlier studies indicated that MT increased the activity of CAT in cucumber [47], maize [62], and tomato plants [66]. SOD and POD are antioxidant enzymes that catalyze the conversion of O_2 and H_2O_2 to O_2 and H_2O (non-harmful molecules), respectively [45]. MT has been proven to enhance the activity of SOD and POD during stress conditions [61,65]. Similarly, we found that foliar MT application improved SOD and POD enzyme activity. Conversely, the opposite results (reduction in the SOD and POD activities) were determined for tomato [67] and wheat plants [68]. The findings of this study indicate that foliar application of MT provides plants with a certain ability to avoid oxidative stress, therefore improving plant salinity tolerance.

Salinity conditions significantly increased the Na^+ content of the chickpea leaves whereas the Ca^{+2} and K^+ content of the chickpea leaves under salinity condition significantly decreased (Figure 3). Furthermore, the MT-treated chickpea seedlings had a higher Ca^{+2} and K^+ content than the non-treated ones. Exogenous MT treatments also reduced the Na^+ content of the chickpea leaves under salinity conditions (Figure 3). It has also been reported in previous studies that excessive salinity in the soil solution limits the nutrient uptake of plants [11,36]. The K^+/Na^+ and Ca^{+2}/Na^+ ratios were significantly affected by salinity and MT treatments (Figure 4). Salt-stressed plants had lower K^+/Na^+ and Ca^{+2}/Na^+ ratios than the non-stressed ones. The K^+/Na^+ and Ca^{+2}/Na^+ ratios decreased by 92.8% and 92.5%, respectively, under salinity conditions. However, exogenous MT application increased the K^+/Na^+ (666.6–802.0%) and Ca^{+2}/Na^+ (430.8–674.5%) ratios under the 75 mM and 150 mM salt stress conditions (Figure 4). Salinity stress decreased the K^+/Na^+ and Ca^+/Na^+ ratios while MT treatments elevated these values under salinity stress conditions (Figure 4). Salinity induced a nutrition disorder via its physiological effects on nutrient availability, competitive uptake, transport, or partitioning within the cell, which influence plant performance and productivity. Studies reported that exogenous application of MT decreased Na^+ accumulation and increased the K^+ content in the above-ground parts of various species grown under saline conditions [69]. Melatonin plays a key role in maintaining ion homeostasis. The inward-rectifying channel AKT1 (*Arabidopsis* K^+ transporter 1) mediates the relative uptake rates of Na^+ and K^+ under high salinity [70].

This also explains why the LRWC increased with MT treatment since the K^+ uptake increased due to the decrease in the Na^+ uptake as an effect of MT, which regulated the water balance. Nevertheless, the specific ion and molecular processes by which MT regulates ion transport and controls ionic homeostasis in plants subjected to salt stress remain largely unexplored. It has been reported that K^+ plays a major role in maintaining water balance and controlling stomata movements in plants under stress conditions [71].

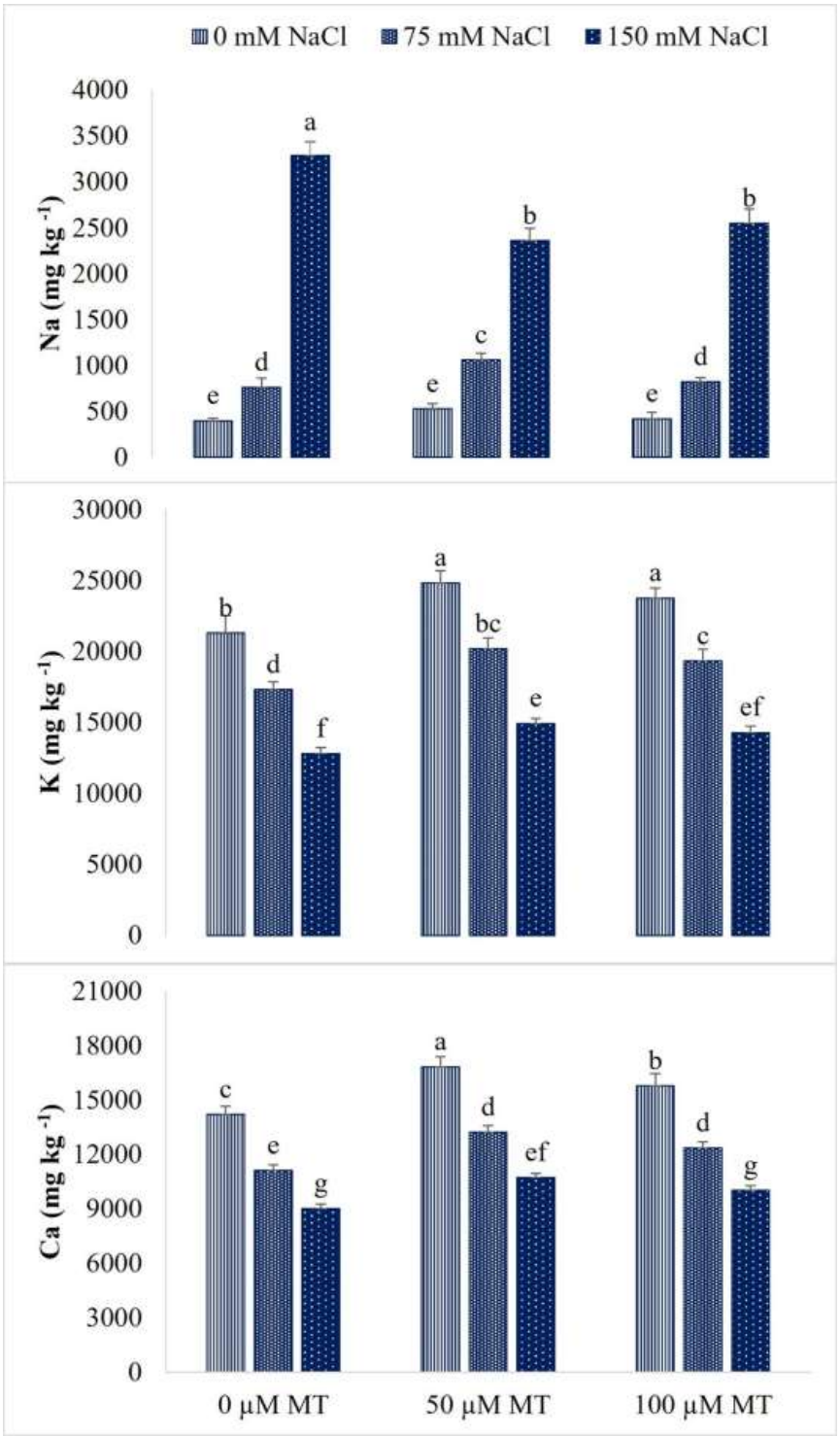

Figure 3. K, Na, and Ca content response in the leaves of the chickpea seedlings under salinity stress to MT applications.

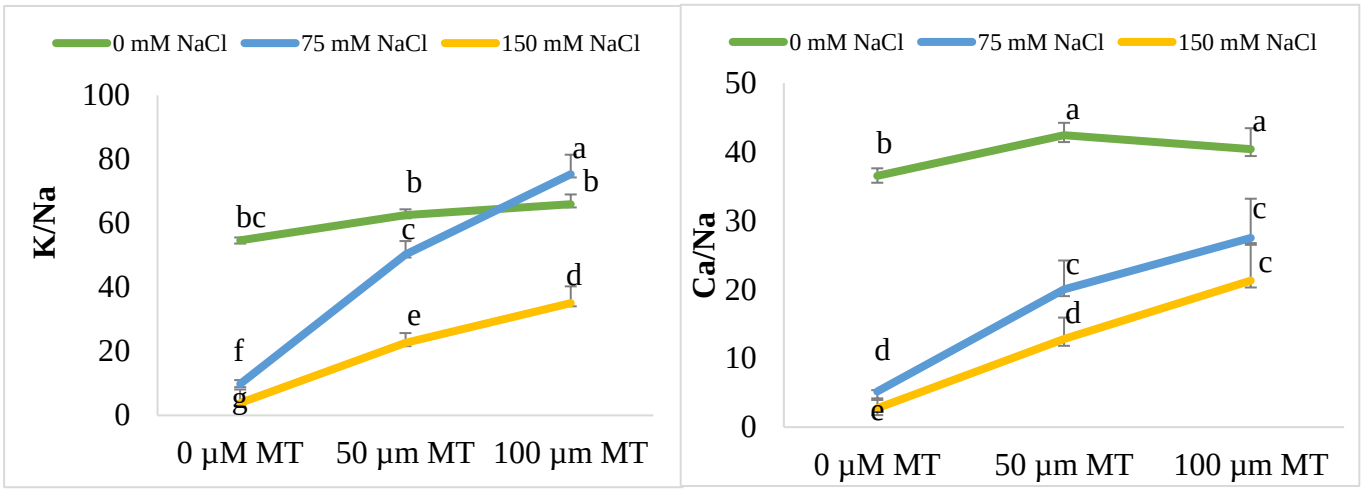

Figure 4. K/Na and Ca/Na ratio response in the leaves of the chickpea seedlings under salinity stress to MT applications.

Another strategy used by plants under salt stress is to maintain ion homeostasis and reduce ionic toxicity. An important way to increase salt tolerance is to keep the K^+/Na^+ ratio high in cells. [72]. In our study, MT application significantly enhanced the K^+ and Ca^{+2} content in leaves affected by salinity. This may also be due to the fact that MT application increases the concentration of IAA in roots and, thus, root development [73]. It has been reported that MT improves plant growth by increasing the Ca^{2+} and K^+ content in leaves and decreasing MDA and H_2O_2 in wheat plants under Cd stress [74]. MT can mitigate cold stress on plants with a higher Ca^{2+} ATPase activity [75].

4. Conclusions

The impact of MT in salinity conditions was studied by comparing the growth, biochemical and physiological responses in chickpea. Our data showed that the exogenous MT application counteracted the salt stress-induced growth inhibition of chickpea and improved plant growth significantly. With the MT treatment, salinity-induced oxidative damage decreased, resulting in an increase in photosynthetic pigments and a decrease in membrane permeability. As a result, it can be concluded that external application of MT to chickpea seedlings against salt stress can be used to increase tolerance to stress. In future studies, the effects of external MT application against salt stress on yield and quality in chickpea can be examined.

Author Contributions: E.D., M.T. and E.Y. designed the study. M.E. and E.D. performed the experiments. M.T. conducted the chemical analysis. S.A. revised the English composition. E.Y., M.E., M.T., S.A. and E.D. analyzed the experimental data. All authors discussed the results and commented on the manuscript. All authors have read and agreed to the published version of the manuscript.

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