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Synergistic Impact of Melatonin and Putrescine Interaction in Mitigating Salinity Stress in Snap Bean Seedlings: Reduction of Oxidative Damage and Inhibition of Polyamine Catabolism

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Abstract: While the individual influences of melatonin (MT) and polyamines (PAs) have been widely studied under various abiotic stresses, little is known about their interaction under salinity stress. In the present study, salt stress applied by 50 mM of sodium chloride (NaCl) on snap bean seedlings has been supplemented with 20 µM of MT and/or 100 µM of putrescine (Put) (individually and in combination). The results indicated that under salinity stress, the combination of MT + Put achieved the highest significant increase in shoot fresh and dry weight, chlorophyll (Chl *a*), Chl *a* + *b*, carotenoids, total soluble sugars, proline, K, Ca, and cell membrane stability index (CMSI), as well as catalase (CAT) and peroxidase (POX) activities. This improvement was associated with an obvious decrease in Na, Na/K ratio, and oxidative damage as indicated by reducing leaf contents of methylglyoxal (MG), hydrogen peroxide (H_2O_2), and the rate of lipid peroxidation (malondialdehyde; MDA). Moreover, the combination of MT + Put demonstrated a significant decrease in the activities of diamine oxidase (DAO) and polyamine oxidase (PAO) leading to the reduction of the rate of polyamine oxidation. Meanwhile, MT applied individually gave the highest significant increase in leaf relative water content (RWC), Chl *b*, superoxide dismutase (SOD), and ascorbate peroxidase (APX). Conclusively, the combination treatment of MT + Put could decrease the degradation of polyamines and enhance tolerance to salinity stress in snap bean seedlings.

Keywords: antioxidant enzymes; nutrient homeostasis; osmolytes; *Phaseolus vulgaris* L; polyamine oxidation; putrescine and saline conditions

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1. Introduction

Salinity stress is one of the biggest impediments to growth and crop production around the world. Each year, there is a significant increase in salt affected soil, which is estimated

at 2 million hectares (about 1%) of the world's agricultural lands [1]. Furthermore, soil salinization is predicted to increase in the future due to frequent climate change scenarios, i.e., rising of sea level, temperature, and global warming, leading to increased evaporation and further salinization [2]. This issue represents a major serious threat to sustainable agriculture and food security worldwide [3]. The deleterious effects of salinity stress on plants consist of: (1) the osmotic stress, which is related to the lower water potential of soil, as well as preventing a plant's water uptake from soils with high salt concentration; (2) the toxic effect or ionic stress, which is caused by particular ionic species such as Na^+ and Cl^-; (3) the oxidative stress that is a result of the excessive release of reactive oxygen species (ROS) i.e., superoxide anion, hydrogen peroxide, singlet oxygen, and hydroxyl radical; and (4) the combined influence of these stresses [4–7].

The snap bean (*Phaseolus vulgaris* L.) is considered a rich source of protein, fibers, vitamins, and micronutrients [8,9]. Its consumption as a human food source is estimated to be around 50% of the total legumes consumed worldwide [10]. Several previous studies have demonstrated that consumption of snap beans can reduce the risk of heart diseases [11,12] and cancers [13,14], leading to improved human health and longevity [15]. As a glycophyte, the snap bean is very salt-sensitive plant with threshold of 1 dS m^{-1} [16,17]. Therefore, there is an urgent need for research to develop plants that are tolerant under saline conditions.

Melatonin (N-acetyl-5-methoxytryptamine) is an auxin-like biomolecule due to the presence of an indole ring in its structure [18]. It is ubiquitous in all kingdoms including animals, algae, plants, and microorganisms [19,20]. In recent years, melatonin has been suggested as a new plant growth regulator with a wide spectrum of effects [21]. It plays an important role in seed germination [22], rhizogenesis [23], delaying leaf senescence [24], and alleviating the effects of biotic [25] and abiotic stresses [26]. In this context, melatonin has been found to protect plants against heat stress [27], chilling [28], heavy metals [29,30], drought [31,32], and salinity stress [1,33,34]. It can reduce chlorophyll degradation, protect photosynthetic machinery, and regulate the redox status of the salt-stressed plants [35,36]. Additionally, melatonin has the ability to adjust the osmotic potential and increase the antioxidant capacity of plants under saline conditions [37,38]. It has been shown that melatonin can play several roles in various signaling processes which are related to the evocation of systemic salt tolerance i.e., nitric oxide [39], calcium/calmodulin (Ca^{2+}/CaM) [40], and ROS [41].

Polyamines (PAs) are multifunctional polycationic plant growth regulators that affect several developmental, physiological, and biochemical aspects [42]. They can regulate DNA synthesis, cell division, seed germination, fruit set, and development [43,44]. In higher plants, putrescine (Put; diamine with two positive charges), spermidine (Spd; triamine with three positive charges), and spermine (Spm; tetraamine with four positive charges) are most abundant polyamines [45]. However, Put is considered the most common PA, and it is directly produced from the non-proteinogenic amino acid, ornithine, through the activity of ornithine decarboxylase (ODC), or indirectly produced from arginine through arginine decarboxylase (ADC) [46]. Under environmental stresses, the positive charges of PAs can serve in cell membranes to stabilize through binding to the membranes negatively charged macromolecules i.e., phospholipids and proteins [44]. Moreover, these positive charges can protect photosystem II (PSII) in the isolated thylakoid membranes under photoinhibition conditions [47]. PAs play a key role in signaling and ion homeostasis through affecting various cell membrane transporters [48]. Several lines of evidence suggest that PAs are involved in plant stress responses to various adverse environmental conditions i.e., heat stress ([49], heavy metals [50], drought [51], and salinity [52,53]. Additionally, PAs are responsible for the scavenging of free radicals [54], enhancement of antioxidant systems [55], accumulation of osmolytes [56], and regulation of gene expression [57].

Despite the fact that the influences of melatonin and PAs on plants have been widely and individually studied under various abiotic stresses, little is known about the possible link between melatonin and PAs under salinity stress in snap bean plants. Therefore, this

study was conducted to investigate the effect of melatonin and Put, either individually or in combination, on salt-stressed snap bean seedlings.

2. Materials and Methods

2.1. Plant Material and Growth Conditions

Phaseolus vulgaris L. (Colter HMX 2117 cv.) served as the study's plant material. Using 0.5% sodium hypochlorite (NaOCl; *w*/*v*) for 4 min, seeds were sterilized before being rinsed five times with distilled water. The seedlings were cultivated in black plastic pots with a diameter of 13 cm and a capacity of 700 cm^3, which contained an equivalent amount of pre-washed sand. The seedlings were consistently watered twice a week with 250 mL of Hoagland's solution at 1/2 strength [58]. After two weeks, pots were irrigated daily with a modified nutrient solution containing 20 µM melatonin (MT; Bio Basic, Markham, ON, Canada), and/or 100 µM putrescine (Put; Sisco Research Laboratories Pvt. Ltd., Mumbai, India). These concentrations of melatonin and putrescine were determined by doing an initial experiment to measure the concentration of malondialdehyde (MDA), which is used as a measure of lipid oxidation after treatment with several concentrations of melatonin and putrescine. The best results were (with a decrease in MDA concentration) at 20 µM and/or 100 µM putrescine Figure S1.

Additionally, salinity was applied using sodium chloride (NaCl) at 50 mM through the nutrient solution. The experimental design (Complete Randomized Design) included eight different treatments: (1) 1/2 strength Hoagland's solution as a control, (2) 1/2 strength Hoagland's solution + 100 µM Put, (3) 1/2 strength Hoagland's solution + 20 µM MT, (4) 1/2 strength Hoagland's solution + 100 µM Put + 20 µM MT, (5) 1/2 strength Hoagland's solution + 50 mM NaCl, (6) 1/2 strength Hoagland's solution + 50 mM NaCl + 100 µM Put, (7) 1/2 strength Hoagland's solution + 50 mM NaCl + 20 µM MT, and (8) 1/2 strength Hoagland's solution + 50 mM NaCl + 100 µM Put + 20 µM MT. The concentration of MT and Put was determined by a preliminary study (Supplementary 1). All pots were kept under greenhouse conditions (24.3 ± 5.3 day/night temperature and 73.4 ± 2.6% relative humidity recorded by a digital thermo-hygrometer placed in the middle of greenhouse, Art. No.30.5000/30.5002, TFA, Wertheim, Baden-Württemberg, Germany). One month after sowing, samples were taken to determine the different growth and biochemical parameters.

2.2. Plant Growth and Leaf Pigments

A digital balance was used to calculate the fresh weights of the shoot and the root. In accordance with Lichtenthaler and Wellburn [59], the concentration of chlorophyll (Chl) a and b, as well as carotenoids in fresh leaves were assessed spectrophotometrically. The fresh weight (0.2 g) of fully inflated leaves was ground in 80% acetone. The absorbance of the extract was measured versus a blank of pure 80% acetone at 663, 644, and 452.5 nm for Chl a, Chl b, and carotenoid contents, respectively. The results are expressed as mg/g FW.

2.3. Leaf Relative Water Content and Osmolytes

In accordance with Abd El-Gawad et al. [60], the relative water content (RWC) of the leaves was estimated. Six completely expanded leaf discs were weighed (FW), immediately submerged in distilled water for two hours at 25 °C, and their turgid weight (TW) was noted. After that, discs were properly dried for 24 h at 110 °C in an oven (DW). The following formula was used to determine relative water content (RWC):

$$RWC\ (\%) = \frac{FW - DW}{TW - DW} \times 100 \quad (1)$$

The ninhydrin assay was used, according Bates et al. [61], to spectrophotometrically measure proline at 520 nm. Using a method published by Chow and Landhäusser [62], total soluble sugars were measured at 490 nm using phenol and sulfuric acid.

2.4. Determination of Na, K, and Ca

Crushed samples weighing 10 g were weighed in porcelain crucibles. The samples were then dried for 5 h in an oven, charred on a hot plate, and then ash-dried for 3 h at an initial temperature of 100 °C, which automatically rose to the ultimate temperature of 500 °C. The results of the destruction were allowed to cool in the desiccator. A few drops of demineralized water were then dropped on the wall of the crucible's porcelain until wet, dissolved in 5 mL of nitric acid, put into a 100-mL volumetric flask, the crucible's porcelain was rinsed three times with 10 mL of demineralized water, put into the same volumetric flask, and diluted with demineralized water until the marking line. The atomic absorption spectrometer (AAS-Hitachi, Tokyo, Japan) was used to determine Na, K, and Ca using hollow cathode lamp at a wavelength 589.0, 766.5, and 422.7 nm respectively [62]. Results are expressed as mg/g DW.

2.5. Determination of Cell Membrane Stability Index and Oxidative Damage

With few adjustments, the cell membrane stability index (CMSI) was calculated as described by Abd Elbar et al. [63]. Eight leaf discs measuring 1.8 cm in diameter were shaken for 24 h in 10 mL of deionized water. Then, using an electrical conductivity meter (EC; DOH-SD1, TC-OMEGA, USA/Canada), the contents' electrical conductivity (EC1) values were determined. Then, samples were autoclaved at 120 °C for 20 min to determine the values of EC_2. Cell membrane stability index (CMSI) was calculated using the following equation:

$$CMSI = \left[1 - \left(\frac{EC1}{EC2}\right)\right] \times 100 \quad (2)$$

With certain changes, the amount of methylglyoxal (MG) was calculated in accordance with Hossain et al. [64]. Fresh leaves were homogenized in 3 mL of 0.5 M perchloric acid before being incubated on ice for 15 min. The mixture was centrifuged for 10 min at 10,000 rpm at 4 °C. After adding charcoal to the supernatant, it was de-colored, left at ambient temperature for 15 min, and then centrifuged at 10,000 rpm for 10 min. Before utilizing this supernatant for the MG assay, it was neutralized by allowing it to stand in a saturated potassium carbonate solution at room temperature for 15 min before centrifuging it once more for 10 min at 10,000 rpm. The MG was estimated using the neutralized supernatant. One milliliter of the reaction mixture, which included 250 µL of 7.2 mM 1, 2-diaminobenzene, 100 µL of 5 M perchloric acid, and 650 µL of the neutralized supernatant, was added first. A UV spectrophotometer was used to measure the absorbance at 335 nm after 25 min. The methodology outlined by Velikova et al. [54] was used to measure the hydrogen peroxide (H_2O_2) content. A total of 0.5 g of leaf tissues were thoroughly powdered in a 5 mL ice bath containing 0.1% (*w*/*v*) TCA. A total of 0.5 mL of a 10 mM potassium phosphate buffer (PH 7.0) containing 1 M KI was added to the extract. The amount of H_2O_2 was determined spectrophotometrically at 390 nm by reference to a standard curve prepared with H_2O_2 solution. Malondialdehyde (MDA) was used to measure the level of lipid peroxidation in the leaf tissues by reactions with thiobarbituric acid (TBA) [65].

2.6. Measurement of the Activities of Antioxidant Enzymes

Fresh leaves (0.5 g) were homogenized in 4 mL of 0.1 M sodium phosphate buffer (pH 7.0), containing 1% (*w*/*v*) polyvinylpyrrolidone (PVP), and 0.1 mM ethylenediaminetetraacetic acid (EDTA). The supernatant was then utilized for tests after centrifugation at 10,000× *g* for 20 min at 4 °C. The Bradford method was used to assess soluble proteins [66]. Using a spectrophotometer (UV-1601PC; Shimadzu, Tokyo, Japan), all investigated enzyme activity and protein content in the crude enzyme extract were determined as follows:

The superoxide dismutase (SOD) assay was developed using the Beyer and Fridovich [67] approach. A total of 100 L of crude enzyme, 50 mM phosphate buffer (pH 7.8), 75 mM nitroblue tetrazolium (NBT), 13 mM L-methionine, 0.1 mM EDTA, and 0.5 mM riboflavin were all included in the reaction mixture, which had a total volume of 3 mL. Riboflavin was added

to start the reaction, and then a 20 W fluorescent bulb was used to illuminate the mixture for 20 min. The quantity of enzyme needed to provide a 50% inhibition in the rate of NBT degradation at 560 nm was determined to be one enzyme activity unit.

In accordance with Cakmak et al. [68], the decrease in absorbance at 240 nm was used to evaluate the catalase (CAT) activity. A total of 15 mM H_2O_2 was present in the reaction mixture, which had a total volume of 3 mL containing 50 mM phosphate buffer (pH = 7). The addition of 50 µL of crude enzyme started the reaction. The activity was determined using the extinction coefficient for H_2O_2 (ε = 40 mM^{-1} cm^{-1}). The breakdown of one mole of H_2O_2 per minute was used to define one unit of enzyme activity.

In accordance with Nakano and Asada [69], ascorbate peroxidase (APX) activity was assessed. For three minutes, the decrease in absorbance at 290 nm was observed. A total of 100 µL of crude enzyme, 50 mM of phosphate buffer (pH 7), 0.1 mM of EDTA, 0.5 mM of ascorbic acid, and 0.1 mM of H_2O_2 made up the reaction mixture, which had a total volume of 3 mL. The reaction was started by the addition of H_2O_2. A unit of enzyme activity was established as the quantity of enzyme needed to oxidize 1 µmol of ascorbate per minute. The extinction coefficient (ε = 2.8 mm^{-1} cm^{-1}) was used to determine the rate of ascorbate oxidation.

With a few modest adjustments, the Dias and Costa [70] method was used to measure the peroxidase (POX) activity. The assay mixture (100 mL) contains 80 mL of 50 mM phosphate buffer (pH = 6.6), 10 mL of 0.3% H_2O_2, and 10 mL of 1% (v/v) guaiacol. To begin the reaction, 2.9 mL of the assay mixture was mixed with 100 µL of the crude enzyme. The absorbance at 470 nm was measured every 30 s for 3 min.

2.7. Determination the Activities of Polyamines Catabolism Enzymes

The method of Su et al. [71] was used to determine diamine oxidase (DAO) and polyamine oxidase (PAO). Briefly, leaf tissues were well ground in an ice bath with 0.1 mM potassium phosphate buffer (pH 6.5). The extract was centrifuged at 8000× g for 20 min at 4 °C. From the supernatant, 0.2 mL was combined with 2.5 mL of potassium phosphate buffer (100 mM, pH 6.5), 0.2 mL of 4-aminoantipyrine/*N*, *N*-dimethylaniline, and 0.1 mL horseradish peroxidase (250 U mL^{-1}). The addition of 15 mL of 20 mM putrescine as a substrate was used to measure the activity of DAO, and the addition of 15 mL of 20 mM spermidine as a substrate was used to measure the activity of PAO. One activity unit was defined as a change in absorbance at 555 nm of 0.01 in value.

2.8. Statistics

SAS [72] software was used to carry out a one-way ANOVA procedure. Three replicates' worth of means and standard deviations were generated, and a Duncan's multiple range test ($p \leq 0.05$) was used to identify any variations in means that were statistically significant.

3. Results

Data in Figure 1A,B indicate that all plant growth parameters were decreased by salinity stress. Under non-saline conditions, all treatments had increased shoot fresh weight and root fresh weight compared to the control. The best treatment was the dual application of MT and Put. Additionally, under saline stress, MT treatment alone or with Put also increased shoot fresh weight and root fresh weight compared to the control. However, the individual application of Put did not enhance shoot fresh weight and root fresh weight compared to the control. Our results in Figure 1C–E indicate that all leave pigments including chlorophyll-a, chlorophyll b, total chlorophyll, and carotenoids were decreased by salinity stress. Additionally, all leaf pigments were increased by all treatments compared to the control under non-saline conditions. In addition, all treatments increased leaf pigments compared to the control treatment under saline conditions. The best treatments were the application of MT alone and when combined with Put.

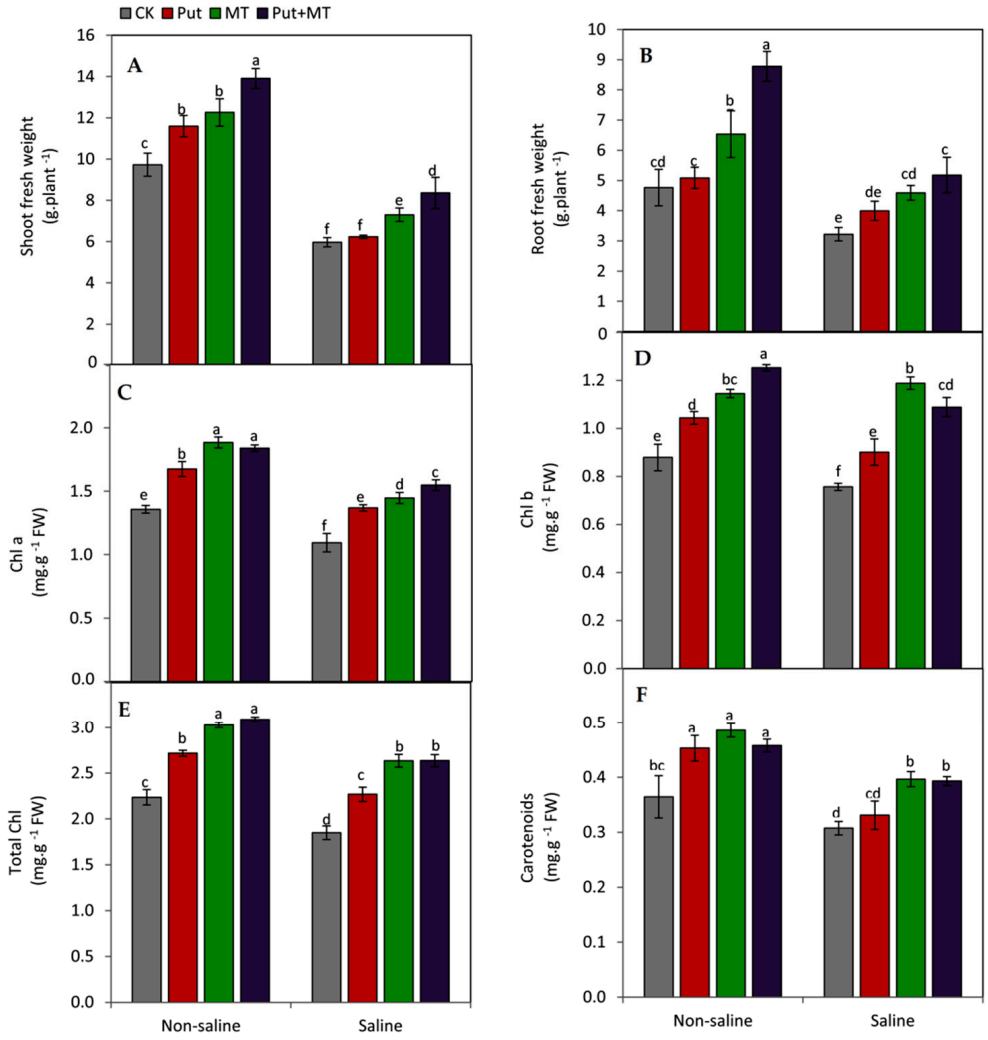

Figure 1. Effect of melatonin (MT; 20 µM), putrescine (Put; 100 µM), and their combination on the growth and leaf pigments of snap bean seedlings grown under control and salt stress (50 mM NaCl). (**A**) Shoot fresh weight, (**B**) shoot dry weight, (**C**) chlorophyll *a*, (**D**) chlorophyll *b*, (**E**) total chlorophyll, and (**F**) carotenoids. The upper error bar denotes standard deviation, while various lowercase letters above the bars denote significant differences according to Duncan's multiple range tests ($p \leq 0.05$).

Our results in Figure 2A indicate that RWC was decreased by salinity treatment. Under non-saline conditions, the RWC was not affected by any treatment compared with the control. However, under salinity stress all treatment significantly increased RWC compared to the control. Additionally, both the MT and MT + Put treatments showed higher RWC than the Put treatment. Figure 2B shows that the total soluble sugars were increased by salinity stress. Under both saline and non-saline conditions, all treatments (without significant difference between them) significantly increased total soluble sugars compared to the control treatment. Proline content increased under salinity stress compared to non-saline conditions (Figure 2C). However, under non-saline conditions, no changes in proline content were observed in all treatments. However, under a salinity stress condition, all treatment significantly increased the content of proline compared to the control treatment. Additionally, the dual application of MT and Put had a higher content of proline compared with the Put and control treatments.

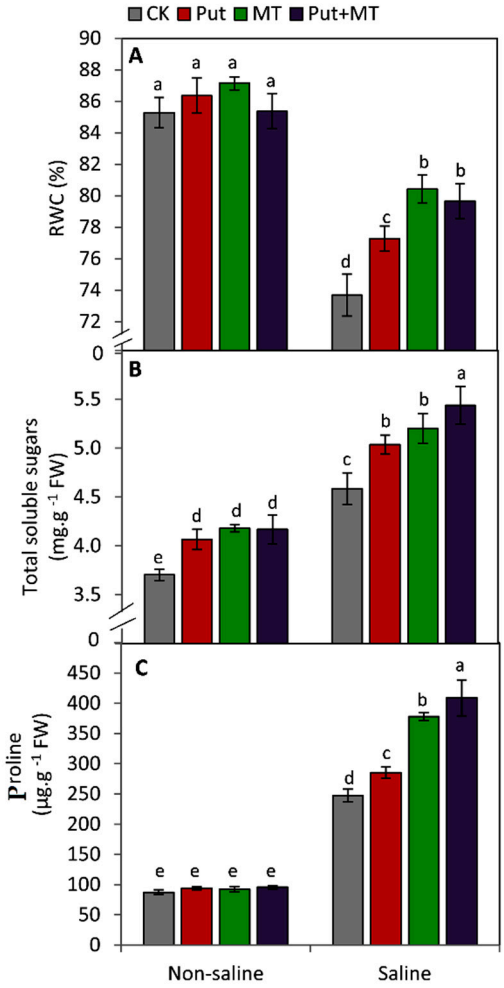

Figure 2. Effect of melatonin (MT; 20 µM), putrescine (Put; 100 µM), and their combination on the leaf relative water content (RWC) and osmolytes of snap bean seedlings grown under control and salt stress (50 mM NaCl). (**A**) RWC, (**B**) total soluble sugars, and (**C**) proline. The upper error bar denotes standard deviation, while various lowercase letters above the bars denote significant differences according to Duncan's multiple range tests ($p \leq 0.05$).

The effect of salinity and treatments on Na, K, Na/K, and Ca contents is presented in Figure 3A–D. As expected, Na content and Na/K ratio increased under saline conditions compared with normal conditions (Figure 3A,C). All treatments had no effect on Na content and Na/K ratio under non-saline conditions. However, under saline conditions, all treatments had significantly decreased Na content and Na/K ratio compared to the control treatment. Our results in Figure 3B show that K content was decreased by saline treatment compared with non-saline conditions. Under non-saline conditions, all treatments had significantly increased K content compared to the control treatment. However, under saline conditions, both MT and MT + Put treatments enhanced the content of K compared with other treatments. Additionally, the individual application of Put did not affect the content of K.

The results in Figure 3D show that Ca content was decreased by saline treatment compared with non-saline conditions. Under non-saline conditions, all treatments had significantly increased Ca content compared to the control treatment. Additionally, under saline conditions, all treatments (without significant difference between them) enhanced the content of Ca compared with other treatments.

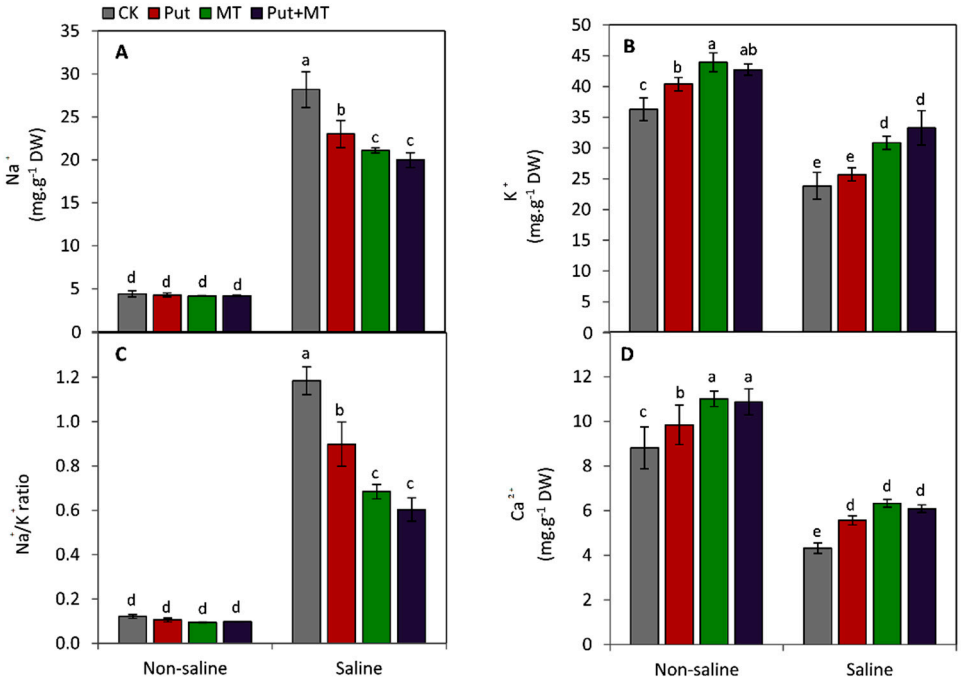

Figure 3. Effect of melatonin (MT; 20 µM), putrescine (Put; 100 µM), and their combination on the nutrient homeostasis of snap bean seedlings grown under control and salt stress (50 mM NaCl). (**A**) Na, (**B**) K, (**C**) Na/K ratio, and (**D**) Ca. The upper error bar denotes standard deviation, while various lowercase letters above the bars denote significant differences according to Duncan's multiple range tests ($p \leq 0.05$).

The results in Figure 4A show that CMSI was decreased by saline treatment. Under normal conditions, all treatments (without significant difference between them) had increased CMSI compared to the control treatment. The same trend of results was observed under saline conditions. Figure 4B shows that MG was increased by salinity stress. No significant difference in MG was observed among all treatments (except the MT treatment) compared to the control under non-saline conditions. However, all treatments had a significantly decreased content of MG compared to the control. Moreover, the dual application was the most effective treatment.

The content of H_2O_2 was increased by salinity treatment (Figure 4C). MT treatment showed a lower H_2O_2 content compared to all treatments under non-saline conditions. However, under saline conditions, all treatments significantly decreased the H_2O_2 content compared to the control, with the superior treatment being the combination of MT + Put. The content of MDA was increased by saline stress (Figure 4D). Moreover, there were no significant differences among all treatments with regards to MDA content under non-saline conditions. However, under saline stress, all treatments significantly decreased the content of MDA compared to the control treatment.

As expected, the activity of all tested antioxidant enzymes (SOD, CAT, POX, and APX) was increased by salinity stress (Figure 5A–D). Generally, under non-saline conditions, there were no significant differences between all of the treatments. However, all treatments showed a higher activity of all antioxidant enzymes under saline conditions compared to the control. The applications of MT + Put and MT were more effective than the Put treatment.

The results in Figure 6A,B show that DAO and PAO contents were increased by salinity stress. There was no significant difference between all treatments in DAO and PAO contents under non-saline conditions. However, under saline stress, all treatments significantly decreased the contents of DAO and PAO compared to the control treatment.

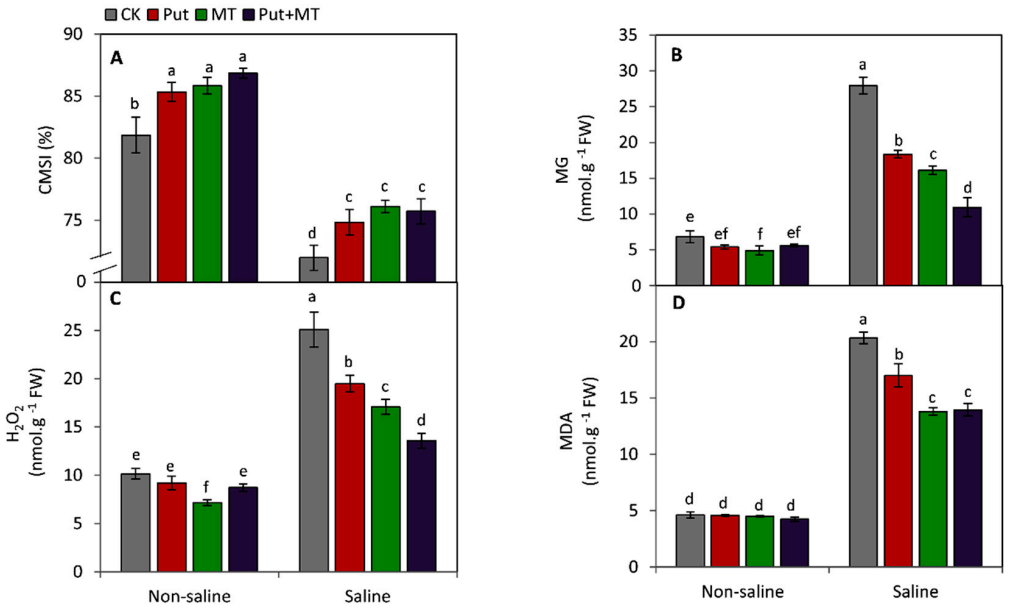

Figure 4. Effect of melatonin (MT; 20 µM), putrescine (Put; 100 µM), and their combination on the membrane stability and oxidative damage of snap bean seedlings grown under control and salt stress (50 mM NaCl). (**A**) Cell membrane stability index (CMSI), (**B**) methylglyoxal (MG), (**C**) H_2O_2, and (**D**) malondialdehyde (MDA). The upper error bar denotes standard deviation, while various lowercase letters above the bars denote significant differences according to Duncan's multiple range tests ($p \leq 0.05$).

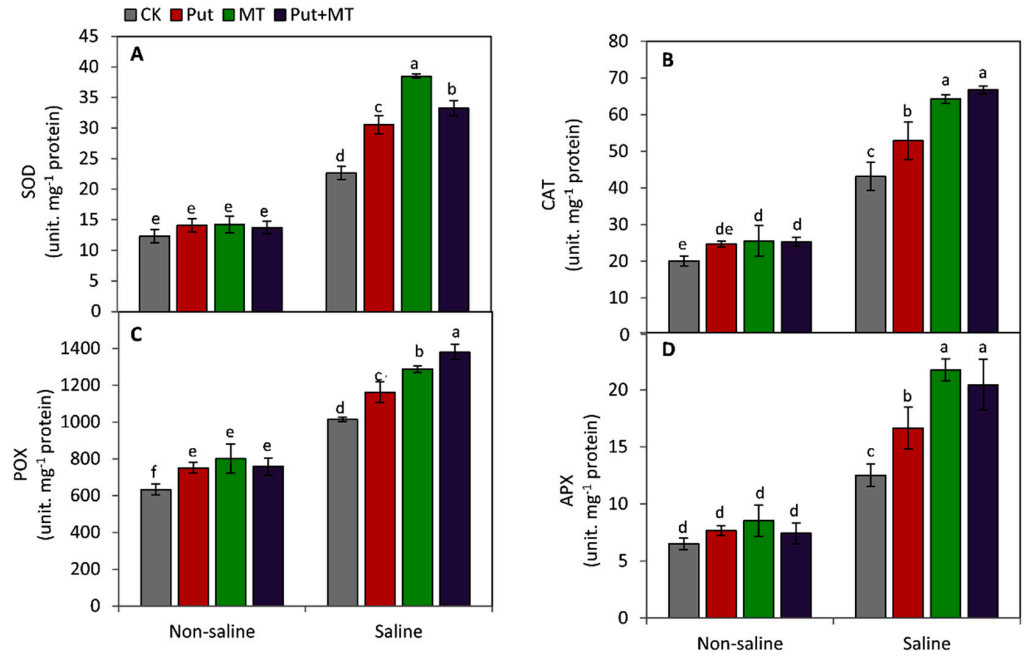

Figure 5. Effect of melatonin (MT; 20 µM), putrescine (Put; 100 µM), and their combination on the activities of antioxidant enzymes of snap bean seedlings grown under control and salt stress (50 mM NaCl). (**A**) Superoxide dismutase (SOD), (**B**) catalase (CAT), (**C**) peroxidase (POX), and (**D**) ascorbate peroxidase (APX). The upper error bar denotes standard deviation, while various lowercase letters above the bars denote significant differences according to Duncan's multiple range tests ($p \leq 0.05$).

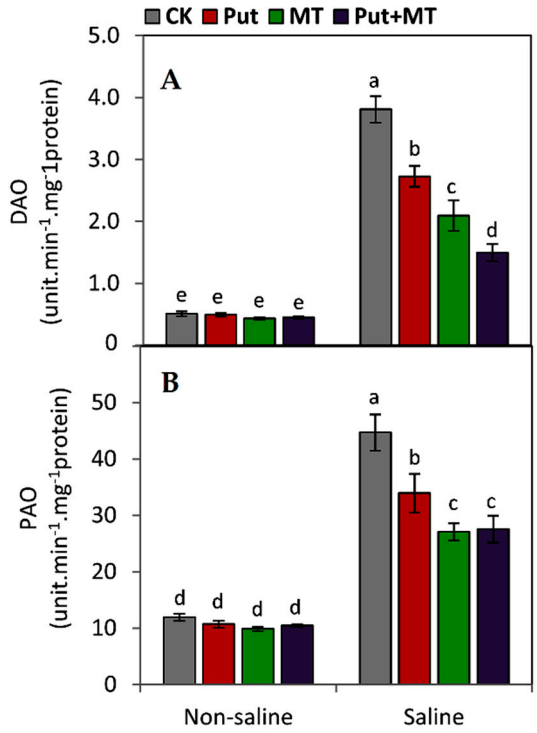

Figure 6. Effect of melatonin (MT; 20 μM), putrescine (Put; 100 μM), and their combination on the oxidation of polyamines of snap bean seedlings grown under control and salt stress (50 mM NaCl). (**A**) Diamine oxidase (DAO) and (**B**) polyamine oxidase (PAO). The upper error bar denotes standard deviation, while various lowercase letters above the bars denote significant differences according to Duncan's multiple range tests ($p \leq 0.05$).

4. Discussion

Green bean plants are considered one of the most saline-sensitive crops [73]. By limiting water intake and ion toxicity, salinity stress has an impact on the water status of the tissue, metabolic processes, and plant growth [74]. Salinity stress dramatically reduces growth by lowering the amount of chlorophyll and the rate of photosynthesis [26]. Likewise, our results showed that growth parameters such as the fresh weight of shoots and roots were decreased by salinity stress. In the current study, salinity stress decreased the leaf pigments, cell division, water uptake, and nutrient homeostasis [7,75,76]. In this study, MT enhanced growth and leaf pigments under saline conditions. Melatonin can regulate plant growth, photosynthetic machinery, and antioxidant capacity on the one hand, while delaying leaf senescence and suppressing ABFs-mediated abscisic acid (ABA) biosynthesis and chlorophyll degradation on the other hand [35,77,78]. Under salinity stress, MT has been shown to have positive effects on growth, chlorophyll content, photosynthesis process, and stomatal conductance in common bean crops [37] and other crops i.e., naked oat [77] and rice [79].

The low molecular weight polycations known as polyamines (PAs) are present in all living organisms and are involved in and/or regulate a wide range of physiological processes, including cell division, plant growth, gene expression control, cell proliferation, modulation of cell signaling, and membrane stabilization [80]. Some previous works have shown that treating different crops with polyamines reverses the damaging effects of salt stress and improves their tolerance [53,81,82].

In our study, the individual application of Put was not effective for enhancing growth under saline conditions (Figure 1A,B). In contrast with our results, Zhao and Qin [83] found that exogenous application of Put enhanced the root growth in barley seedlings under salt stress. This difference might be due to the differences between plant types and application

method. The dual application of MT and Put had superior effect. Thus, the enhancement might be mainly related to MT application.

Preserving chlorophyll activity is critical for preventing leaf senescence, which reduces yields. Photosynthetic activity depends on the photosynthetic pigments such as chlorophylls and carotenoids. In this study, the Put treatment increased leaf pigments under normal and saline conditions (Figure 1C–F), which was similar to the results of previous studies conducted on pumpkins [84]. This enhancement of leaf pigments by Put application may be due to the role of Put in preventing the synthesis of ethylene leads to inhibit the degradation of chlorophyll, resulting in an increase in photosynthetic pigments [85]. These effects could also be attributed to the positive effects of Put on chlorophyll levels via thylakoid membrane protection at the site of the chlorophyll–protein complex [86].

Our results indicated that MT enhanced leaf pigments (chlorophyll *a*, chlorophyll *b*, total chlorophyll, and carotenoids) under normal and saline conditions. Similarly, Erdal [87] found that chlorophyll contents in maize seedlings were increased by MT application compared with the control. In accordance with our results, Arnao and Hernández-Ruiz [88] found that exogenous MT treatment protected chlorophyll from degradation in barley. Melatonin's role in chlorophyll preservation was later demonstrated in other crops such as tomatoes [89]. These results could be due to the fact that MT enhanced the gene expression encoding two photosystem I subunits, two photosystem II elements, and ferredoxin PetF, while decreasing the expression of genes encoding the chlorophyll-degrading enzyme chlorophyllase [90]. In agreement with our results (Figure 1F), it has been previously reported that MT application also preserves the carotenoid content [91].

It has been well known that abiotic stresses, such as salinity, affect the metabolism of some compounds including carbohydrates and amino acids [34]. The proline mostly consists of carbohydrates [37]. In this study, and previous studies [37,92], we found that salinity increased the content of proline and total soluble sugars in bean plants (Figure 2B,C). A previous study has shown the role of proline in the resistance of plants to salinity stress by controlling the activity of enzymes and controlling the permeability of the cell membrane [93]. A previous study [37] and our study found that MT treatment increased the levels of proline and total soluble sugars in salinity-stressed plants. This shows the role of MT in combatting salinity by increasing total soluble sugars and proline. In the current study, Put supplementation improved proline synthesis compared to the control. Similarly, previous studies observed enhanced proline content when treated with Put under salt stress in some crops [94,95]. Additionally, Put application under abiotic stresses, such as salinity, increases endogenous Put levels in the plant, increasing plant resistance to saline stress [48].

Relative water content (RWC) is a vital physiological parameter for water status that supports the ability of plants to survive in stressed conditions. In this study, RWC was decreased by saline stress (Figure 2A) which could be explained by the negative effect of salinity on water absorption and availability [96]. Our results also indicated that MT treatment increased RWC under salinity stress. Some previous works reported that Put application enhanced RWC under salt stress [97,98].

All abiotic stresses negatively affect the cell membrane and enzymatic systems by raising ROS levels, including H_2O_2, and change their balance [95]. The exposure of plants to salinity leads to damage to the cell membrane, which causes an increase in the amount of MDA content [99]. In this study, we recorded the same previous results that H_2O_2, MDA, and MG were increased under salinity stress. In this study, MT treatment resisted the negative effects of salinity by decreasing H_2O_2 and MDA content by raising transcription levels [100] and stimulating the activity of antioxidant enzymes (Figure 5A–D). The same results were recorded previously [37,99].

It has been well known that PAs has a role in scavenging ROS under abiotic stresses, in addition to its function as an antioxidant [101]. In this study, the application of Put caused a significant reduction in H_2O_2, MDA, and MG. In accordance with our results,

previous works indicated that Put applications reduced MDA and MG toxicity in mung bean plants [102,103].

Antioxidant enzymes work to resist the harmful effect caused by ROS inside the plant cells. For example, SOD converts O_2^- to H_2O_2, while CAT converts H_2O_2 to H_2O and molecular oxygen inside plant cells. Additionally, APX converts H_2O_2 to monodehydroascorbate [104]. The results in this study showed that MT treatment alleviated the harmful effects of salinity by increasing the activity of antioxidant enzymes. Our results are in agreement with Li et al. [105] who found that MT treatment mitigates the negative effect of salinity by increasing the activity of SOD and CAT enzymes. In this study, MT treatment enhanced the activity of all antioxidant enzymes. Previous works indicated the positive role of MT in ROS-scavenging by increasing the activity of antioxidant enzymes [37,104–107].

The data in Figure 5A–D indicated that Put application increased the activities of the antioxidant enzymes. Put application has been shown to induce POX activity under saline conditions, and this activation can be interpreted as a conformational change caused by polyamine binding [94]. Additionally, exogenous application of Put improved the activity of CAT in pepper seedlings grown under salt stress conditions, according to Ekinci et al. [108].

Our results in Figure 3A–D show that MT treatment decreased the Na content and Na/K ratio while there were increased K and Ca contents under saline stress. Our results are in agreement with Li et al. [105] who found that MT treatment decreased the Na content and Na/K ratio in rice plants under salinity conditions. Additionally, Qu et al. [109] found that MT treatment increased the content of K, which might be due to the improvement of stomatal conductance by MT treatment under salinity stress [37].

The results in Figure 3A–D show that Put treatment reduced Na uptakes. Similarly, previous studies indicated that Put applications inhibit or reduce the uptake of Na from the soil in some crops such as rice and mung beans [52,103] due to the role of Put for controlling the balance of cation and anion [84]. Additionally, when exogenous Put was added, there was a decrease in Na and an increase in K, which was associated with root growth promotion [110]. Ca content was clearly reduced in the salt-stressed seedlings (Figure 3D). In this work, Put application alleviated the harmful effect of saline stress and enhanced Ca content in plant. This result is in agreement with Nahar et al. [103] who found that Put application increased Ca content in mung bean shoots and roots.

Our findings showed salinity-induced increases in DAO and PAO activity. By regulating H_2O_2 signalling, which is created by stress-induced PAO activity leading to spermidine, spermine, and spermine oxidation, plant polyamine oxidases (DAO and PAO) play key roles in various stress tolerance and programmed cell death (PCD) processes [111]. In contrast, the treatments of MT and MT + Put diminished the activities of DAO and PAO. Melatonin can accelerate the biosynthesis of PAs through increasing their metabolic flow from the precursor amino acids arginine and methionine [1]. Moreover, this response has been found to be correlated with the suppression of polyamine oxidase (PAO) and diamine oxidase (DAO) activities [1].

5. Conclusions

The present study revealed that the combination treatment of MT + Put mitigated the salinity-induced oxidative damage of snap bean seedlings. This effect was correlated with suppressing the activties of polyamine oxidases (DAO and PAO) and enhancing several features of tolerance to salinity stress. These aspects included the improvement of growth, leaf pigments, CMSI, RWC, osmolytes, and antioxidant enzymes. Conversely, there was an obvious decrease in the oxidative damage as indicated by the reduction in the leaf content of methyglycoxal, H_2O_2, and MDA. Further future studies at the molecular level are required to understand the crosstalk between melatonin and polyamines in regulating plant tolerance to salinity stress.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae9020285/s1. Figure S1. Effect of different concentrations of melatonin and putrescine on the membrane lipid oxidation of snap bean seedlings grown under control and salt stress (50 mM NaCl).

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