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Beneficial Effects of Exogenous Melatonin on Overcoming Salt Stress in Rice Seedlings (*Oryza sativa*)

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ABSTRACT

Salinity negatively impacts the growth and development of rice during early growth stages that can be lessened through melatonin (MT), a hormone found naturally in plant and animal cells, which acts as a potential exogenous biostimulant on plants since it aids to fight against various abiotic stresses. The current research explored the potential impact of exogenouly applied MT on promoting early-stage rice growth under salinity conditions. At seedling stage, rice seedlings were exposed to 100 mM NaCl or maintained under normal conditions for 15 days and also pretreated with or without 100 µM MT. The findings of this study revealed that NaCl-stress hampered the growth of rice seedlings including shoot and root growth, reduced photosynthetic capacity. Additionally, exposure to salinity induced osmotic stress in rice, as indicated by increased proline accumulation, and oxidative stress, as evidenced by elevated hydrogen peroxide (H_2O_2) levels, increased lipid peroxidation, and reduced antioxidant enzymatic activities. However, application of MT markedly enhanced the overall growth performance and biomass under salinity condition. Plants treated with MT and salt stress showed augmented photosynthetic pigment content, decreased proline content and reduced H_2O_2 content contributing to reduction of oxidative damage. Moreover, MT application reduced the membrane damage under salinity conditions as reflected by remarkably reduced amounts of malondialdehyde in salt-exposed rice seedlings. The protective effect of MT against oxidative stress was associated with increased activities of antioxidant enzymes, including catalase, ascorbate peroxidase, and peroxidase. Our study reveals that the application of exogenous MT develops the resilience of rice seedlings to salinity stress by improving antioxidant defense systems which suggests that applying MT could be a dynamic tool for rice cultivation in saline-affected areas.

1. Introduction

The human population is predicted to reach 9 billion in the next 40 years which means crop productivity needs to increase 70-110% by 2050, but in true sense it is difficult to increase the crop productivity due to many adverse climatic conditions (Ganie et al. 2019; Molotoks et al. 2021; Ben Ayed and Hanana 2021). Abiotic stresses create adverse effects that directly connected to the reduction of crop yield worldwide (Kopecká et al. 2023). A statistic shows that more than 6% of the world's area is salt affected, which accounts for more than 800 million hectares of land all over the world (Gupta and Shaw 2021).

Plant growth and development are interfered by the salinity (Tabassum et al. 2021; Rodríguez Coca et al. 2023). Physiological changes including root and shoot length, root and shoot dry weight reduction are seen in both sensitive and tolerant plants, whereas those that are sensitive to salinity have a higher growth decrease than tolerant plants (Rasel et al. 2020; Tabassum et al. 2021). Salt stress can lead to several adverse effects on plants by the generation of reactive oxygen species (ROS) or free radicals such as superoxide, singlet oxygen and hydrogen peroxide (H_2O_2) (Singh 2022; Kesawat et al. 2023). It results the oxidative stress and induces lipid peroxidation and nucleic acid oxidation, phytotoxic reactions in plants and also caused protein damage (Mano 2012; Hasanuzzaman et al. 2020). Excessive oxidative stress enhances the production of toxic malondialdehyde in plants by triggering peroxidation of

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polyunsaturated fatty acids (Mano 2012). Moreover, plant photosynthesis and nutrient transport efficiency can be changed due to salt ions in soil, which cause ion imbalance, osmotic stress, and oxidative damage (Tufail et al. 2018; Tahjib-Ul-Arif et al. 2019; Shahid et al. 2020). Under salt stress conditions, plants resist oxidative damage by overcoming ROS through cellular level protection systems such as enzymatic and non-enzymatic antioxidant defense systems (Hasanuzzaman et al. 2020; Kesawat et al. 2023). Antioxidant enzymes such as catalase (CAT), peroxidase (POD), ascorbate peroxidase (APX) etc. are involved in the enzymatic antioxidant defense system, on the other hand, glutathione, flavonoids, phenols, free proline, ascorbic acid, tocopherols etc. are the non-enzymatic antioxidant that encounter or depollute the ROS effects (Hasanuzzaman et al. 2020; Kesawat et al. 2023).

Melatonin (MT, *N*-Acetyl-5-Methoxytryptophan, also known as pinealin, is a tryptophan byproduct with a low molecular weight indole structure that is found in all organisms (Ke et al. 2018; Arnao et al. 2022). Application of MT, either exogenously or endogenously, helps plants fight against abiotic stresses such as drought, salinity, heat, etc. (Gao et al. 2019; Nawaz et al. 2020; Hoque et al. 2021). It is also known as an antioxidant molecule (Siddiqui et al. 2019), and it acts as a ROS scavenger, which is thought to protect plants from various oxidative stresses (Florido et al. 2022). A major role of MT is to protect of chlorophyll deprivation and preserve membrane integrity (Jiang et al. 2021; Jafari et al. 2022). Moreover, MT application can counterbalance ROS by stimulating the antioxidant system in *Brassica napus* (Zeng et al. 2018); mitigate osmotic stress in *Zea mays* (Ren et al. 2020) and cucumber (*Cucumis sativus)* (Wang et al. 2016); ion toxicity in *Malus hupehensis* (Sun et al. 2023), develop stomatal characteristics, protect chloroplast structure, grana lamellae in cotton (*Gossypium hirsutum*) (Jiang et al. 2021) under salinity stress conditions.

The food security of Bangladesh largely depends on rice (*Oryza sativa*) production (Islam et al. 2021). Various degrees of soil salinity in the coastal districts of Bangladesh affect the cultivable area (Bhuyan et al. 2023). According to recent research, approximately 0.83 million hectares of land in Bangladesh are affected by NaCl in 1973, which has now increased to 1.2 million hectares (Habiba et al. 2013; Rabbani et al. 2019). Rice is unable to perpetrate their life cycle under high salt concentration which increase from 3.1 dS m^{-1} to 5.12 dS m^{-1} reducing 40% rice production (Grattan et al. 2002; Selim et al. 2021). Rice is the foremost food for Bangladeshi people also need more production to feed the increasing population, so it is necessary to cultivate rice in the coastal region. Under the saline condition, it is difficult to cultivate rice in coastal area, hence management of soil salinity is required to improve productivity. Our study showed the potential effect of MT on plants to improve its salt tolerance at seedling stage conditions. To observe the efficiency of exogenous MT, we evaluated several parameters related to growth including fresh and dry weight of root and shoot, length of root and shoot, photosynthetic efficiency, oxidative stress such as raising H_2O_2 antioxidant system (CAT, APX, POD) exposed on salinity with and without MT supplementation. Our results revealed that MT plays a prospective role in the improvement of growth of rice under salinity.

2. Materials and Methods

2.1. Layout of experiment

The experiment was performed using a salt-susceptible and high-yielding rice cultivar, *BRRI dhan29.* First, the rice seeds were sterilized by soaking in 5% sodium hypochlorite + 2% Tween-20 for 25 min and then washed with distilled water for five times. Sterilized seeds were imbibed in distilled water for one day and subsequently incubated in the dark condition for germination at 37 ^{o}C. Around 150 sprouted rice seeds were sown in each cylindrical shape plastic pot with a diameter of 9 cm and height of 4 cm. Blotting papers were placed on each plastic pot moistened with distilled water and the germinated seeds were allowed to grow for 3 days. From day four after sowing, rice seedlings were maintained in a modified Hoagland nutrient solution (Tahjib-Ul-Arif et al. 2018a). From 20th days of sowing, rice seedlings in pots were divided by the following treatments: control (C), nutrient solution only; salt stress (S), 100 mM NaCl; sprayed 100 μM melatonin (MT); and 100 μM MT + 100 mM NaCl (MT+S). Approximately 30 mL of MT was foliar sprayed on each pot every time. MT was applied 2 days before initiating salt stress and was repeated every three days interval. On the 22nd day after sowing, rice seedlings were subjected to 100 mM NaCl stress by supplying NaCl with Hoagland solution. On 35th day of sowing, different morpho-physiological and biochemical parameters were measured. The research was done in completely randomized design with three replications per treatment.

2.2. Determination of growth parameters

The growth parameters were measured on the 35th day of planting. Shoot length (SL) and root length (RL) were measured from shoot initiation point to the highest shoot tip and from root base to the root tip respectively by using a scale. Approximately 20 seedlings were taken to determine the fresh weight. Fresh weight (FW) of shoot (SFW) and root (RFW) was taken separately by weight machine. The dry weight (DW) of root (RDW) and shoot (SDW) was determined after four days of oven drying at 60 ^{o}C.

2.3. Determination of hydrogen peroxide (H_2O_2) content

The contents of H_2O_2 were determined based on the method of Kadir et al. (2024). Fresh leaves (0.5 g) were homogenized in a mortar and pestle at 4 ^{o}C with 5 mL of 0.1% trichloroacetic acid (TCA) and the homogenate was centrifuged at 11,500×g for 12 min at 4 ^{o}C. Then 0.5 mL of supernatant was added to 0.5 mL of 10 mM potassium phosphate buffer (pH 7.0) and 1 mL of 1.0 M potassium iodide. The reaction mixture was kept in a dark condition for 60 min. After incubation, the absorbance was recorded at 390 nm using a UV-VIS spectrophotometer (T-80, PG-instrument, China) and H_2O_2 content was calculated by using extinction coefficient of 0.28 $\mu M^{-1}cm^{-1}$.

2.4. Measurement of malondialdehyde (MDA) content

MDA content was measured according to the method of Kadir et al. (2024). Fresh leaf tissue (0.1 g) was homogenized in 1 mL of 5% TCA using a pre-chilled mortar and pestle at 4 °C. The homogenate was centrifuged at 11,500 × g for 15 min at 4 °C, and the resulting supernatant was mixed with 20% TCA containing 0.5% thiobarbituric acid (TBA). This mixture was incubated at 95 °C for 15 min, rapidly cooled, and centrifuged again at 11,500 × g for 12 min. Absorbance was then measured at 532 nm using a UV–VIS spectrophotometer (T-80, PG Instruments, China).

2.5. Measurement of proline content

Proline content was determined following the method of Bates et al. (1973) with slight modifications. Fresh leaf tissue (0.05 g) was homogenized in 4 mL of 3% sulfosalicylic acid. The homogenate was centrifuged at 4000 × g for 10 min, and 2 mL of the supernatant was mixed with 2 mL of acid ninhydrin reagent and 2 mL of glacial acetic acid in a screw-cap test tube. The reaction mixture was incubated at 100 °C for 1 h and then cooled to room temperature. The developed chromophore was extracted with 4 mL of toluene, and its absorbance was measured at 520 nm using a UV–Vis spectrophotometer (T-80, PG Instruments, China).

2.6. Antioxidant enzyme activity measurement

Antioxidant enzyme activities of catalase (CAT; EC 1.11.1.6), peroxidase (POD; EC 1.11.1.7), and ascorbate peroxidase (APX; EC 1.11.1.11) were assayed. Leaf tissue (50 mg) was homogenized in an ice-cold mortar and pestle with 3 mL of 50 mM phosphate buffer (pH 8.0). The homogenate was centrifuged at 12,000 × g for 10 min at 4 °C, and the resulting clear supernatant was collected for enzyme activity assays.

Catalase activity was determined according to the method of Aebi (1984). The reaction mixture contained 2.1 mL of 50 mM phosphate buffer, 0.3 mL of EDTA, and 0.3 mL of H_2O_2 in an Eppendorf tube. The reaction was initiated by adding 0.3 mL of plant extract, and CAT activity was calculated using an extinction coefficient of 40 M^{-1} cm^{-1}. Ascorbate peroxidase and peroxidase activities were measured following the method of Nakano and Asada (1981), using extinction coefficients of 2.8 M^{-1} cm^{-1} and 26.6 M^{-1} cm^{-1}, respectively. For APX, the reaction mixture consisted of 1.8 mL of 50 mM phosphate buffer, 0.3 mL of EDTA, 0.3 mL of H_2O_2, and 0.3 mL of ascorbate. For POD, the mixture contained 1.8 mL of 50 mM phosphate buffer, 0.3 mL of EDTA, 0.3 mL of H_2O_2, and 0.3 mL of guaiacol. In both assays, the reactions were initiated by adding 0.3 mL of enzyme extract. Changes in absorbance were recorded at 240 nm, 290 nm, and 470 nm for CAT, APX, and POD, respectively, at 30-second intervals for 2 minutes using a UV–Vis spectrophotometer (T-80, PG Instruments, China).

2.7. Measurement of chlorophyll content

Chlorophyll content was determined following the method of Coombs et al. (1985). Fresh leaf samples (0.05 g) were placed in small screw-cap tubes containing 10 mL of 80% acetone and wrapped with aluminum foil to prevent light exposure. The tubes were stored in the dark for 7–10 days to allow complete extraction. Absorbance was measured at 645 nm, 663 nm, and 480 nm using a UV–Vis spectrophotometer (T-80, PG Instruments, China).

2.8. Statistical analysis

Data were analyzed using a two-way analysis of variance (ANOVA) in R version 4.1.2. Statistically significant differences between treatments ($p<0.05$) were determined using Fisher's least significant difference (LSD) test, with different letters indicating significant differences. All data presented in the figures are means ± standard error (n = 3).

3. Results

3.1. Exogenous melatonin improved plant growth under saline condition

The effects of salt, MT, and their combined treatments varied across different growth parameters, including shoot length (SL), root length (RL), shoot fresh weight (SFW), root fresh weight (RFW), shoot dry weight (SDW), and root dry weight (RDW) of the plants (Fig. 1A-G). Under 100 μM MT treatment, shoot and root growth of plants was comparable to that of control plants (Fig. 1B-G). On the other hand, in response to NaCl-stress, SL and SFW decreased significantly while RL, RFW, SDW and RDW exhibited insignificant alteration compared to non-stressed control conditions (Fig. 1B-G). However, combined supplementation of 100 μM MT with NaCl stress diminished the negative effect of salt by enhancing SL and SFW of plants compared to NaCl stress only (Fig. 1B & 1D).

3.2. Exogenous MT enhanced chlorophyll and carotenoid contents under saline conditions

The effects of MT, salt or combined treatments on the chlorophyll and carotenoid contents were investigated. Exposure to MT treatment caused significant increases in chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*) and total chlorophyll (Chl *a*+*b*) levels compared to control conditions (Fig. 2A-C). Under NaCl stress, Chl *a*, Chl *b*, Chl (*a*+*b*), and carotenoid contents were significantly reduced compared to control plants. However, exogenous MT supplementation under salt stress significantly enhanced Chl *a*, Chl *b*, Chl (*a*+*b*), and carotenoid contents compared to salt-stressed rice seedlings (Fig. 2A–D).

3.3. Exogenous MT suppressed MDA and H_2O_2 content in rice seedlings under saline condition

The MDA, H_2O_2 and proline content were significantly modified due to exposure to different treatments (Fig. 3A-C). In the case of MT treatment, MDA and H_2O_2 levels were almost stable, while proline content enhanced significantly compared to controls. Exposure to NaCl stress resulted in a marked increase in the contents of MDA, H_2O_2, and proline (Fig. 3A–C).

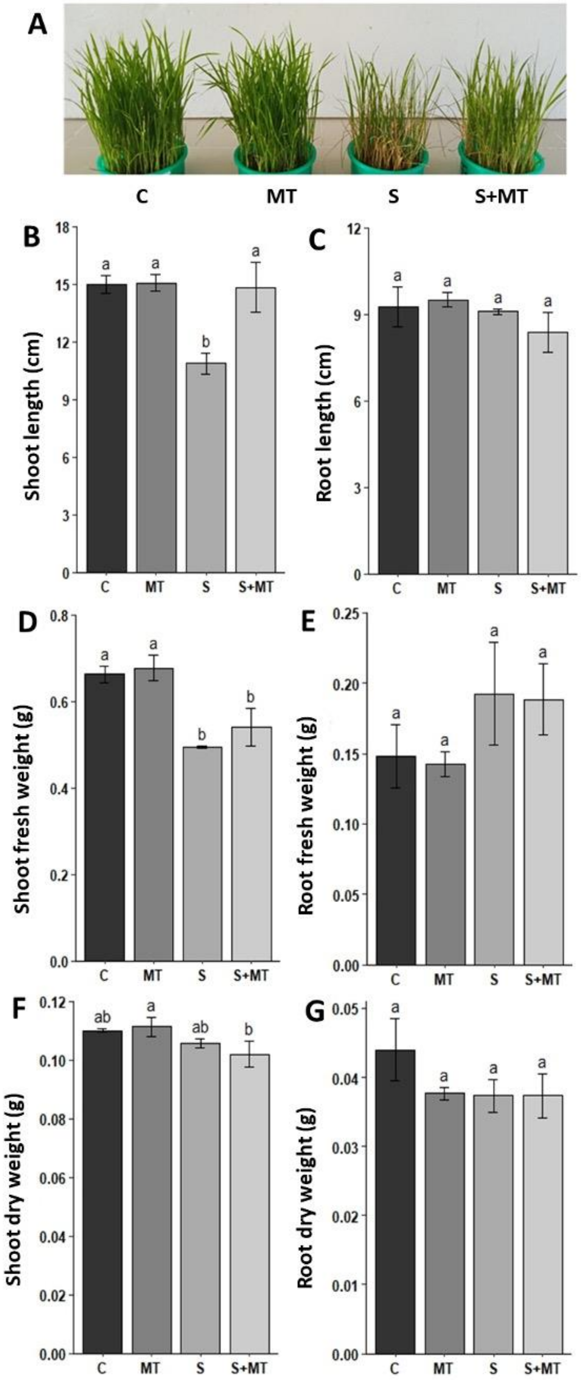

Figure 1. Impact of supplemented melatonin (MT) on (A) overall growth, (B) shoot length, (C) root length, (D) shoot fresh weight, (E) root fresh weight, (F) shoot dry weight, and (G) root dry weight of rice seedlings under control and salt stress conditions. Rice seedlings were grown under four different treatment conditions viz. "C", (Control), 0 mM NaCl + 0 µM MT; "MT", 100 µM MT; "S", 100 mM NaCl; and "S+MT", 100 mM NaCl + 100 µM MT. Different letter(s) indicate statistically significant differences based on Fisher's LSD test at $p < 0.05$

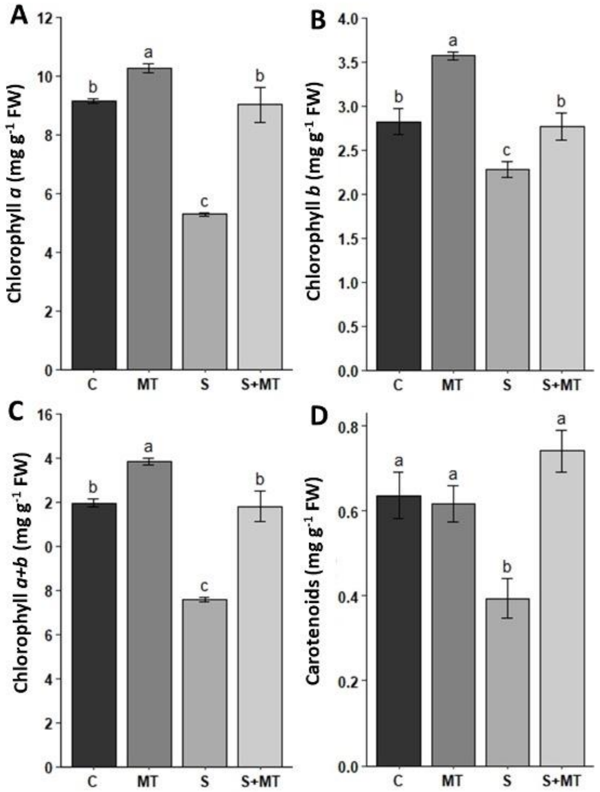

Figure 2. Effect of exogenous MT on (A) chlorophyll *a*, (B) chlorophyll *b*, (C) chlorophyll *a*+*b*, and (D) carotenoid contents in rice seedlings grown in the presence and absence of salt stress. Rice seedlings were grown under four different treatment conditions viz. "C", (Control), 0 mM NaCl + 0 3M MT; "MT", 100 µM MT; "S", 100 mM NaCl; and "S+MT", 100 mM NaCl + 100 µM MT. Different letters indicate statistically significant differences based on Fisher's LSD test at $p < 0.05$

On the contrary, the application of salt stress jointly with MT declined the MDA and H_2O_2 content compared to salt stress alone and restored at a level comparable to controls (Fig. 3A-B). Proline content also declined under salt and MT combined treatment than only salt stress condition, however, plants preserved its level significantly higher than that under controls (Fig. 3C).

3.4. Exogenous MT assisted in preserving the antioxidant enzymatic activity under saline conditions

Exogenous supplementation of MT did not affect the CAT and POD activities but it reduced APX activity compared to controls (Fig. 4A-C). Conversely, NaCl stress triggered significant reductions in CAT and POD activities but no impact on APX activity contrasted to non-stressed controls. (Fig. 4A-C). However, under NaCl stress supplemented with MT, CAT activity elevated and APX activity maintained, while POD activity yet suppressed compared to under salt stress only (Fig. 4A-C).

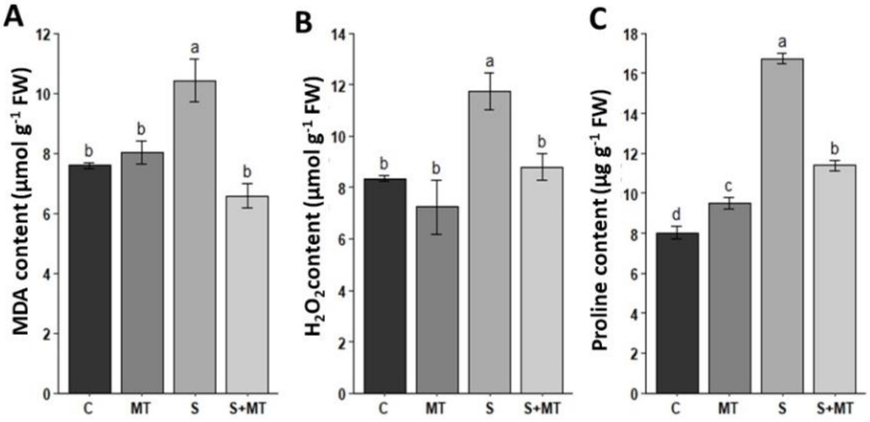

Figure 3. Impact of supplemented MT on (A) malondialdehyde (MDA), (B) hydrogen peroxide (H_2O_2) and (C) proline content in rice seedlings grown in the presence and absence of salt stress. Rice seedlings were grown under four different treatment conditions viz. "C", (Control), 0 mM NaCl + 0 µM MT; "MT", 100 µM MT; "S", 100 mM NaCl; and "S+MT", 100 mM NaCl + 100 µM MT. Different letter(s) indicates statistically significant differences based on Fisher's LSD test at $p < 0.05$

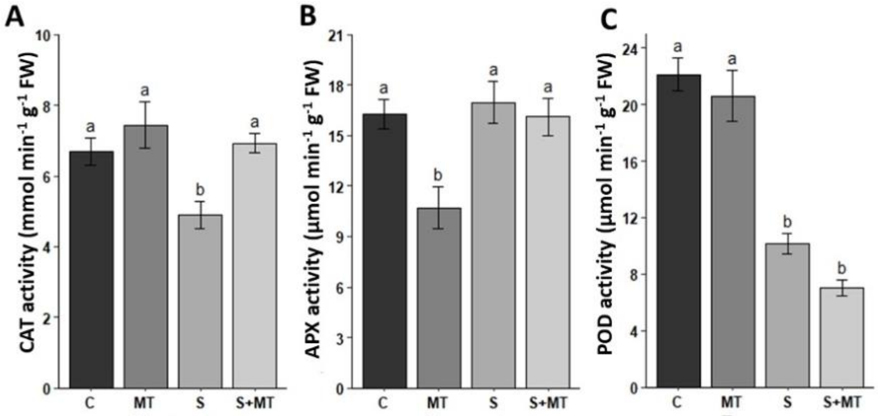

Figure 4. Effect of exogenous MT on the activity of (A) CAT, (B) APX and (C) POD in rice seedlings grown under non-stress and salt stress conditions. Rice seedlings were grown under four different treatment conditions viz. "C," (Control), 0 mM NaCl + 0 µM MT; "MT", 100 µM MT; "S", 100 mM NaCl; and "S+MT", 100 mM NaCl + 100 µM MT. Different letter(s) indicates statistically significant differences based on Fisher's LSD test at $p < 0.05$

4. Discussion

Soil salinity is one of the most detrimental factors that minimize the production of various economically important crops, including rice, maize, wheat, etc. (Ahmed Kalhoro et al. 2016; Chen et al. 2018; Tahjib-Ul-Arif et al. 2018b). The use of exogenous stimuli on a variety of important crops has demonstrated significant impacts in alleviating the unfavorable consequences of salinity (Akram et al. 2020; Bukhat et al. 2020; Hassan et al. 2021). In this experiment, we assessed the probable contributions of MT in the alleviation of salt stress by evaluating the growth and antioxidant defense responses of salt-stressed rice seedlings.

Morphological parameters such as growth and weight determination are used to appraise the hostile effects of various abiotic stresses (Akram et al. 2020; Khaleduzzaman et al. 2021). In the present study, a significant reduction was observed in shoot growth such as SL and SFW under NaCl stress, while SL and SFW increased in response to NaCl stress supplemented with MT (Fig. 1B & 1D). This result suggests that exogenous MT contributed to overcoming the harmful effects of salinity and restored plant growth. However, root growth was relatively unaffected in NaCl-stressed plants (Fig. 1C, 1F and 1G). Alleviation of salt stress-induced inhibition of plant growth by exogenous MT was also observed in earlier reports on rice as well as on maize, sweet corn, tomato, etc. (Ahmad et al. 2021; Yan et al. 2021; Wei et al. 2022; Wang et al. 2023; Khan et al. 2024).

The growth of a plant is largely dependent on photosynthesis where chlorophyll is a key pigment (Simkin et al. 2022). The chlorophyll content is directly related to plant healthiness since chlorophyll a and chlorophyll b are the principal components of the light energy harvesting systems for photosynthesis (Pareek et al. 2017; Jiménez-

Lao et al. 2021). Carotenoids also contribute to photosynthesis by harvesting light energy (Jiménez-Lao et al. 2021). In our experiment, chlorophyll and carotenoid contents were significantly reduced under salt stress (Fig. 2). This reduction in photosynthetic pigments might directly impact photosynthesis and result in retarded plant growth. However, the application of exogenous MT significantly enriched chlorophyll and carotenoid levels in plants exposed to saline conditions (Fig. 2). This outcome is consistent with the findings of prior research on rice as well as on tomato, maize, cotton, canola etc. (Altaf et al. 2020; Javeed et al. 2021; Ahmad et al. 2021; Shen et al. 2021; Khan et al. 2024). This result suggests the potential impact of MT on enhancing photosynthetic pigment levels, thereby promoting plant growth in response to salinity.

To cope with salt stress, plants accumulate compatible solutes such as proline to adjust cytoplasmic osmotic pressure and facilitate water absorption (Van Zelm et al. 2020; Balasubramaniam et al. 2023). Our investigation also observed a sharp increment in proline content due to salinity indicating that plants initiated an adaptive mechanism to tolerate salinity (Fig. 3C). After supplementation of MT with salt stress, proline accumulation significantly declined than salt stress alone but was still higher than controls (Fig. 3C). This outcome is not aligned with several reports where significant raises in proline level were found in plants exposed to salt stress with MT compared to salt stress only (Wang et al. 2023). Nevertheless, the earlier reports on canola, cotton and maize supported our findings expressing that proline content in salt-stressed plants treated with exogenous MT declined but still sustained higher than in control (Javeed et al. 2021; Ahmad et al. 2021; Shen et al. 2021). Huang et al. (2019) reported that both root irrigation and leaf spraying of MT lowered proline levels in maize under drought stress. Therefore, their results indicate that MT may maintain a positive turgor pressure to meet the water balance.

Salinity spawns oxidative stress due to the generation of ROS such as H_2O_2 which are highly cytotoxic and can cause lipid peroxidation, protein denaturation, etc. by reacting with important biomolecules (Yan et al. 2021). Melatonin, a bio-stimulator of plant growth, is known to play a vital role in the improvement of plant's tolerance against various environmental stresses by maintaining redox equilibrium, removing ROS, improving photosynthesis and encouraging plant development (Ahmad et al. 2023; Sharma et al. 2024). In our experiment, salinity stress increased the accumulation of H_2O_2 and MDA (Fig. 3A-B). However, the levels of H_2O_2 and MDA decreased under salt stress when exogenous MT was applied (Fig. 3A-B). These findings are supported by previous reports representing that MT contributes to mitigating adverse effects of salt stress by suppressing ROS accumulation and membrane injury (Chen et al. 2020; Wei et al. 2022; Wang et al. 2023; Khan et al. 2024).

The enhancement of salt tolerance in plants is acclaimed to be associated with increased ROS-scavenging activities by antioxidant enzymes (Zhao et al. 2020; Balasubramaniam et al. 2023). During the stress condition, CAT performs in the conversion of H_2O_2 into H_2O and O_2, POD scavenges the H_2O_2 located in the extra-cellular space and APX converts H_2O_2 to water by utilizing ascorbate as an electron donor (Rajput et al., 2021). In the current study, a significant reduction in CAT and POD activities, but a persistent level in APX activity was observed in response to salinity compared to control conditions (Fig 4A-C). However, MT supplementation under NaCl stress facilitated plants to maintain elevated CAT and APX activities comparable to non-stressed controls (Fig. 4A-B). A significant elevation in CAT, POD and APX activities was reported in salt-stressed rice, canola and okra plants when MT were supplemented (Javeed et al. 2021; Wei et al. 2022; Wang et al. 2023). Similarly, in another report, CAT activity in salt-treated rice plants was observed to accelerate due to MT treatment (Khan et al. 2024). Therefore, the findings suggested that exogenous MT application might play a role in inducing elevated antioxidant enzyme activities in response to salinity stress. The suppression of ROS accumulation and the reduction in lipid peroxidation are likely due to the enhanced antioxidant defense system induced by exogenous MT.

5. Conclusion

This study exhibited that salinity caused a severe decline of growth and biomass. On the other hand, application of MT in rice seedlings accelerated antioxidant enzyme activities, thereby enhancing their growth and biomass under salinity. It also downregulated lipid peroxidation, leading to lower MDA and H_2O_2 levels, and improved photosynthetic efficiency in rice seedlings. We therefore suggest that exogenous MT supplementation could be an effective strategy to mitigate the adverse effects of salinity stress on growth, physiological processes, and the antioxidant defense system in rice seedlings.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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