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Enhancing salt tolerance in rice genotypes through exogenous melatonin application by modulating growth patterns and antistress agents

Mohammad Ubaidillah[1], Muhammad Farooq[2] & Kyung-Min Kim[2]✉

Melatonin is a bioactive molecule with an important role in plants responding to various abiotic and biotic stresses. This study aims to determine the role of melatonin in rice under salt stress. This study used a factorial completely randomized design. The first factor was local rice varieties (IR64 and Silaun), and the second factor was plant treatments (control, 1 µM melatonin, 150 mM NaCl, 150 mM NaCl + 1µM melatonin). This study shows that exogenous melatonin can increase plant growth, such as plant height, root length, stem length, leaf length, leaf area, and plant biomass under salt stress compared to treatment without melatonin. Exogenous melatonin can increase the total chlorophyll content, relative water content, and proline content, reduce the total sodium content, and increase potassium absorption under conditions of salinity stress. Melatonin is also able to scavenge ROS in plants, resulted the decrease in ROS and MDA content. In terms of gene expression, *OsAPX1* and *cytosolic APX* exhibited the highest expression in IR64 under combined salt and melatonin treatment, while *GPOD*, *Mn-SOD*, and *Cu/Zn-SOD* were upregulated under various conditions in both varieties. Additionally, *OsLEA* showed high expression in both varieties under control conditions, and *CAT* was significantly upregulated under salt stress. Our findings indicate that exogenous melatonin has the potential to enhance various factors under salt stress and helping in the recovery of rice plants from sodium (Na+) damage.

Keywords Salt stress, Antioxidant genes, Melatonin, Rice, Oxidative stress

Salinity is one of the major abiotic stresses which could impose plant growth. In soil, salinity is an environmental factor that affects seed germination, crop growth, and productivity. This condition severely affects all physiological and biochemical processes in plant growth phases, including germination phase[1]. Salt stress will cause plants to experience ion and osmotic stress. These conditions occur as a result of plants poisoned by Na^+ and Cl^-. Excessive Na^+ amounts can inhibit K^+ uptake from the environment resulting in an ion imbalance[2]. Osmotic stress is caused by an increase in salt content which affects high osmotic pressure thereby inhibiting the absorption of water and elements that take place through the process of osmosis and will cause disruption of physiological and biochemical processes in plants[3]. When under salinity stress, plants will produce reactive oxygen species (ROS) and reactive nitrogen species (RNS) overload. Excessive ROS content in plants will cause cell damage that occurs because ROS is a damaging free radical[4].

Initially melatonin was discovered by two groups of workers in 1995; they have been studied several physiological aspects of plants, including cytoprotector, circadian regulator, growth promotor, stress protector, and organogenic agent[5,6]. Many researcher have include these aspects, including with others to establish the role of melatonin in plants[7,8]. Melatonin is a bioactive molecule with a physiological function and a very important role in helping plants respond to various abiotic and biotic stresses[9]. Melatonin has been detected in large enough quantities in both horticultural and food crops. In increasing plant resistance to salinity stress, melatonin works indirectly by increasing antioxidant enzyme activity, photosynthetic efficiency, metabolite content, and by regulating transcription factors that regulate stress[10].

[1]Study program of Agrotechnology Faculty of Agriculture, University of Jember, Jl Kalimantan, 37 Kampus Tegal Boto, Jember, East Java, Indonesia. [2]Department of Applied Biosciences, Kyungpook National University, Daegu 41566, Republic of Korea. ✉email: kkm@knu.ac.kr

Melatonin an indolamine, exhibits diverse role in plant physiology and mainly act as a growth promoter, rooting agent, and antioxidant particularly in stress responses[11]. Melatonin was initially found in the unicellular alga *Lingulodinum polyedrum* Stein by[12]. Since then, it's been discovered in various plants, including cereals, vegetables, fruits, and roots[13–15]. Interestingly, plants tend to have higher melatonin level than animals, fluctuating from pictograms to micrograms per gram of fresh weight (FW)[16–18]. Melatonin has been identified in the leaves, flowers, fruits, seeds and roots of many numerous plant species[19–21]. Previous study reported the addition of 1μM of exogenous melatonin was confirmed to increase salt stress resistance in *Zea Mays*, indicated by higher leaf area, biomass, and plant photosynthesis compared to plants with no exogenous melatonin[22]. Based on this study, exogenous melatonin can increase biomass, leaf area, plant height, root length and while reducing oxidative stress and the amount of ROS under salinity stress. It is essential to confirm the role of melatonin in enhancing the salt resistance of rice plants, especially during the germination phase. This study aims to determine the effect of salinity stress on the germination phase of rice plants and the role of melatonin in improving germination resistance under conditions of saline conditions.

Materials and methods

Rice preparation and experimental treatment

This research was conducted at the Agrotechnology Laboratory of Agriculture Faculty, University of Jember. This study used a factorial completely randomized design. The first factor were two different local rice varieties, IR64 (Indica) and Silaun (East Indonesia Germplasm). The second factor was plant treatments (control, 1 μM melatonin, 150 mM NaCl, 150 mM NaCl + 1 μM melatonin). The seeds of IR64 and Silaun rice variety were soaked in distilled water and placed in a dark room for 3 days and the seeds were sown in paper and homogenous seeds transferred to pot trays filled with sand media. After the emergence of the roots, seedlings were irrigated with ABMix solution. Rice seedlings at 3 DAP were treated 1 μM Melatonin, 150 mM NaCl, 150 mM NaCl + 1 μM Melatonin and control (without treatment). The observation was conducted at 8 DAP.

Evaluation of rice plant morphology

Plant morphology including height, root length, stem length, leaf length, leaf area, fresh weight, and dry weight was observed based on samples collected 8 days after treatment. Leaf area was determined using Image J software. Fresh weight was determined by weighing fresh samples. Dry weight was obtained by drying samples in the oven at 70 °C for 72 h and then determined the weight using an analytical balance.

Total chlorophyll content

Leaf samples (100 mg) were crushed and homogenized with 5 ml of 95% ethanol solution then samples were centrifuged at 10,000-rpm for 10 min. Total chlorophyll was measured by absorbance readings using UV-Vis spectrophotometer at 664 nm and 649 nm wavelengths then calculated using appropriate extinction coefficient[23].

Chlorophyll A (μg/mL) = (13.36 × *Abs*. 664) – (5.19 × *Abs*. 649).
Chlorophyll B (μg/mL) = (27.43 × *Abs*. 649) – (8.12 × *Abs*. 664).
Total Chlorophyll (μg/mL) = (5.24 × *Abs*. 664) – (22.24 × *Abs*. 649).

Relative water content (RWC)

The fresh weight (FW) of the isolated leaves was measured immediately and then immersed in deionized water in test tubes for 24 h. The water on the leaf surface was dried after the swollen leaf was removed from the test tubes, and the turgid weight (TW) was calculated. Later, these leaves were dried at 70 °C for 24 h, and their dry weight (DW) was recorded. RWC was calculated according to[24] formula :

$$\mathrm{RWC} = \frac{\mathrm{FW} - \mathrm{DW}}{\mathrm{TW} - \mathrm{DW}} \times 100$$

Proline content

The 0.5 mg rice leaves were subjected to leached in 10 ml of 3% sulfosalicylic acid solution, followed by filtered paper. A ninhydrin acid solution was prepared by dissolving 1.25 g of ninhydrin in a mixture of 30 ml of glacial acetic acid and 20 ml of warm 6M H_3PO_4 until complete dissolution and stored at 40 °C. Subsequently, 2 ml of the filtrate was combined with 2 ml of ninhydrin acid solution and 2 ml of glacial acetic acid in a test tube. This mixture was incubated at 100 °C for 1 h, and the reactions were terminated by immersion in an ice bath. The solution was thoroughly mixed after adding 4 mL of toluene. The light absorbance of the toluene phase was measured at 520 nm, and the proline content was evaluated using a standard proline curve. The concentration of proline was estimated in micromoles per gram of fresh weight (μmol g^{-1} FW). (Bates, 1973)

$$\mu\,\mathrm{mol\ proline/g\ leaf} = \frac{\mu\,\mathrm{g\ proline/ml\ \ (ppm)} \times \mathrm{\ vol\ toulene/115{,}5}\ \mu\,\mathrm{g\ proline/umol}}{\mathrm{sample\ mass/5}}$$

Sodium and potassium accumulation

A 0.5 mg portion of crushed rice leaf sample was placed in porcelain crucible and subjected to carbonization on a hot plate. Subsequently, the carbonized sample was ashed in a furnace, with a gradual temperature increase from an initial temperature of 100 to 500 °C, ascending in intervals of 25 °C every 5 min. The ashing process spanned 14 h, following which the resultant ash was transferred to a desiccator for further treatment. The ashes were moistened with 10 drops of distilled water, and 3–4 ml of nitric acid (HNO_3) were cautiously introduced and were added carefully. Dissolution of the ashes was facilitated by adding 5 ml HNO_3 until a clear solution

was obtained. This solution was then carefully transferred into a 100 ml volumetric flask for subsequent analysis. Meanwhile, the residual sample remaining in porcelain crucible was rinsed three times with distilled water, collected, and filtrated. This filtrate was designated as the sample solution for the analysis of sodium content. The determination of sodium content involved measuring the absorbance of the digested sample solution using an atomic absorption spectrophotometer at wavelength of 590 nm, while potassium content was assessed at 766.5 nm. The analysis was conducted utilizing an air-acetylene flame, as described by[25].

Accumulation of ROS and MDA content

Rice leaves (100 mg) were homogenized in 1 mL of 0.1% trichloroacetic acid (TCA) solution. The H_2O_2 content was determined according to the method of Christou et al. (2014), with the H_2O_2 absorbance measured using a spectrophotometer at 390 nm wavelength. MDA Content was determined using[26] approach. The result was examined using a spectrophotometer at wavelengths 532 nm and 600 nm. The absorption values were calculated using the formula proposed by[27].

$$\text{MDA level} = \Delta\ (\text{A}\,532\,\text{nm} - \text{A}\,600\,\text{nm})/1.56 \times 105.$$

The absorption coefficient for MDA calculations is 156 $\text{mmol}^{-1}\ \text{cm}^{-1}$.

Molecular analysis

Gene expression analysis stages following the procedure of[28] consist of RNA isolation, cDNA synthesis, and PCR. Total RNA was extracted following the procedure Ribospin™ Plant Kit (GeneAll), and cDNA synthesis followed the procedure ReverTra Ace® qPCR RT Master Mix (Toyobo). Quantitative real-time Polymerase chain reaction (qRT-PCR) was performed with a total volume of 20 µL and analyses was carried out using the Steponeplus Real-time PCR System, Life Technologies Holdings Pte.Ltd (Singapore). Primers for the target genes (listed in Table S1) were used for amplification. The expression levels were normalized using *OsActin* (gene id. 4333919) as the internal reference gene, ensuring accurate quantification of gene expression.

Data analysis

Data were analyzed using Analysis of Variance (ANOVA). Significant data were analyzed using 5% DMRT (*Duncan Multiple Range Test*). Gene expressions were analyzed using a two-way ANOVA followed by Tukey's multiple comparisons test was performed, with a minimum of three replicates for each condition. Statistical significance was determined at p values < 0.05. Data are presented as the mean ± SE.

Results

Effect of NaCl and melatonin treatment on the morphological characteristics of rice plants

Compared to the control, the addition of exogenous melatonin under normal conditions did not affect the growth of rice plant (Fig. 1). Under NaCl treatment, the stress could significantly inhibit rice plant growth in both varieties, including plant height, root length, stem length, leaf length, leaf area and plant biomass. Under salinity stress, a significant decrease in both varieties was shown in plant height with a decrease of 51.6% in IR64 and 46% in Silaun (Fig. 1C).

Quantitative assessment of growth parameter under NaCl and melatonin treatment in rice genotypes

Root length exhibited a significant reduction in both rice varieties, with IR64 showing a significant decrease of 59.5% while Silaun showed a comparatively smaller reduction of 37%. Similarly, stem length followed a comparable trend, with IR64 demonstrating a 56.6% reduction in length, whereas Silaun showed a 34.2% decline. When analyzing leaf length, IR64 presented a moderate decrease of 51.6% contrasting with Silaun, which exhibited a more substantial reduction of 71.4%. The leaf area also showed a considerable decrease in both varieties, with IR64 showing a 73.8% reduction and Silaun showing a decrease of 69.6% (Fig. 2A–D). The results showed that the differential impact on growth parameters between the two rice varieties, revealing that both varieties were affected, but to varying extents, across root, stem, leaf and leaf area. Additionally, exogenous melatonin treatment under salinity stress significantly increased the morphological response as indicated by plant height with an increase of 28.6% in IR64 and 20.5% in Silaun compared to control treatment; root length by the increase of 42.2% in IR64 and 23.5% in Silaun; stem length by 37% in IR64, but did not show significant change in Silaun; leaf length by 28.7% in IR64 and 31.7% in Silaun; leaf area under salt stress conditions by the increase of 44.6% in IR64 and 57.8% in Silaun (Fig. 2A–D).

Melatonin increases biomass under salt stress in IR64 and Silaun

Plant biomass measurements revealed significant reductions under salt stress. Fresh weight decreased by 54.5% in IR64 and 56% in Silaun, while dry weight decreased by 40.3% in IR64 and 48% in Silaun (Fig. 3A,B). However, the application of exogenous melatonin led to significant enhancements. Fresh biomass increased by 44% in IR64 and 43% in Silaun, and dry weight increased by 18.1% in IR64 and 37.7% in Silaun under salt stress conditions (Fig. 3A,B).

Influence of NaCl and melatonin on rice plant chlorophyll content

Chlorophyll content in rice plants decreased under salinity stress treatment. The reduction in chlorophyll "a" content was insignificant in both varieties: IR64 (11.9%) and Silaun (28.9%). The addition of exogenous melatonin did not significantly increase chlorophyll "a" content in rice plants under salinity stress conditions with a slight increase of 4.19% in IR64 and 20.34% in Silaun (Fig. 4A). In terms of chlorophyll "b" (Fig. 4B),

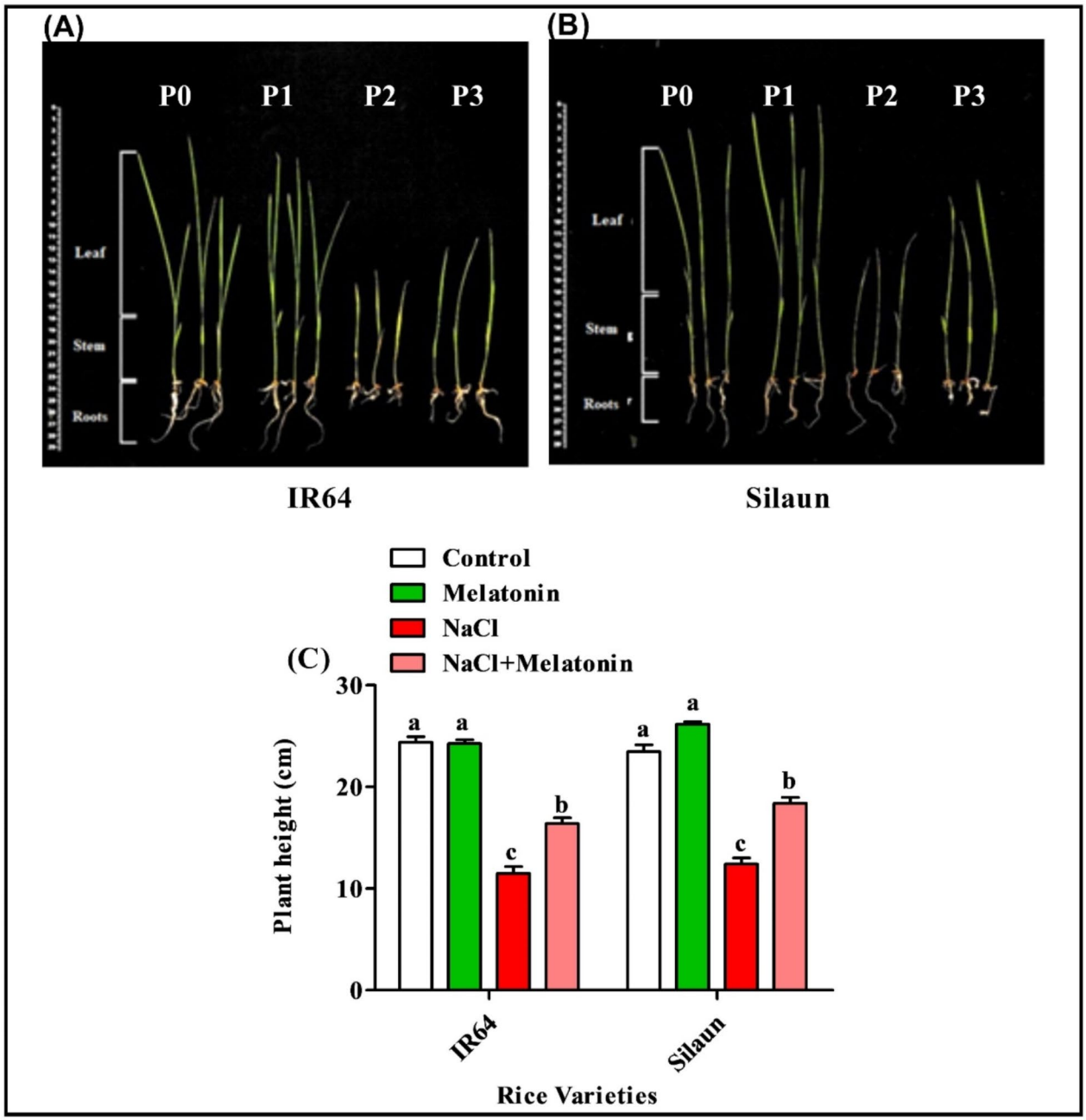

Fig. 1. The effect of NaCl and melatonin treatment on rice plant growth after 8 days of treatment. P0: Control, P1: Melatonin, P2: NaCl, P3: NaCl + Melatonin (**A**,**B**). Effect of NaCl and melatonin treatment on rice plant height after 8 days of treatment. Different letters showed significantly different results at 5% DMRT test (**C**).

shows a significant decrease in chlorophyll "b" under the salinity stress in both IR64 (34%) and Silaun (43%). However, the addition of exogenous melatonin did not significantly increase the chlorophyll "b" content in rice plants under salinity stress, resulting in a modest increase of 24.9% IR64 and 23% in Silaun. Total chlorophyll content exhibited a significant reduction in both varieties under salinity stress, leading to a decrease of 24.9% in IR64 and 36.6% in Silaun. However, exogenous melatonin significantly increased the overall Chlorophyll content in a rice plant under salinity stress conditions, with an 15.9% in IR64 and 21.6% in Silaun (Fig. 4C).

Impact of NaCl and melatonin on rice plants relative water content

Relative water content (RWC) showed a significant decrease in both varieties of rice plants under salt stress. The salt stress treatment reduced relative water content (RWC), by 12.6%, in IR64 and 29% in Silaun compared to

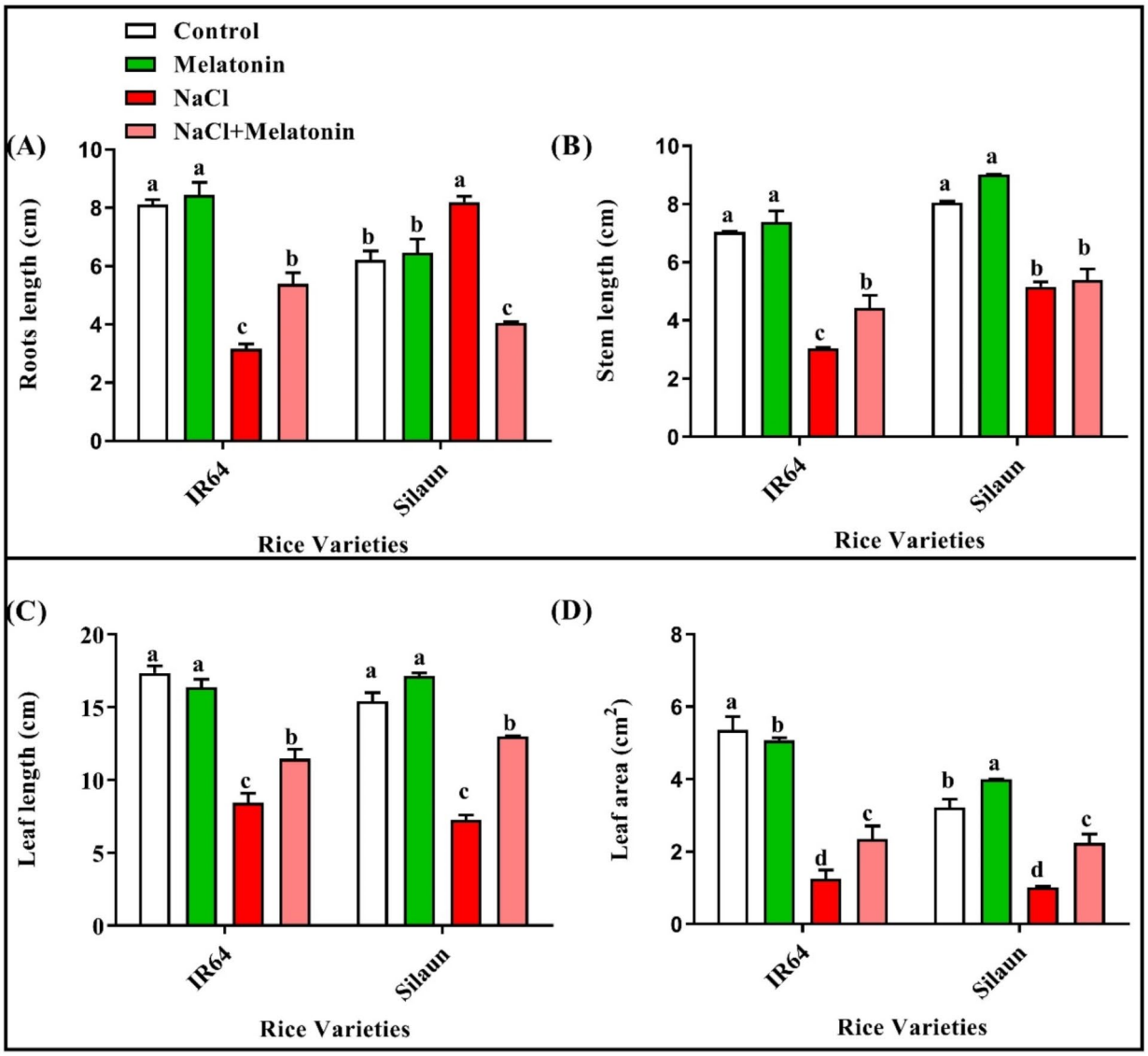

Fig. 2. The effect of NaCl and melatonin treatment on (**A**) root length, (**B**) stem length, (**C**) leaf length, and (**D**) leaf area after 8 days of treatment. Different letters indicate significantly different results based on a 5% significance level using Duncan's Multiple Range Test (DMRT). Error bars represent the mean ± standard error of mean (SEM) calculated from 3 replicates. For each condition, 14 plants were used from per replicate.

the control. Exogenous melatonin was able to increase the relative water content (RWC) of leaves under salinity stress, with an increase up to 12.9% in IR64 and up to 25% in Silaun (Fig. 5A).

Effect of NaCl and melatonin treatment on the proline content of rice plants

Proline is an important osmotic substance that can maintain turgor pressure in a cell. Compared to the control, proline content significantly increased under salinity stress condition. There was a significant increase in the proline content parameter for both varieties of rice plants treated with salinity stress. In the IR64 variety, the salinity stress treatment experienced an increase of 40.8% compared to the control, while Silaun experienced an increase of 73.3% compared to the control. Exogenous melatonin can increase proline content under conditions for both varieties in IR64 (33.5%) and Silaun (15.4%) (Fig. 6A).

Effect of NaCl and melatonin treatment on sodium and potassium content of rice plants

There was a significant increase in the total sodium content parameter (Fig. 7A) for both varieties of rice plants under salinity stress conditions. The total sodium content increased by 90.2% in IR64 and 90.1% in Silaun compared to control. Exogenous melatonin under salinity stress was able to reduce the total sodium content by 0.8% in IR64 and 1.9% in Silaun. In the total potassium content (Fig. 7B) there was a significant decrease in IR64

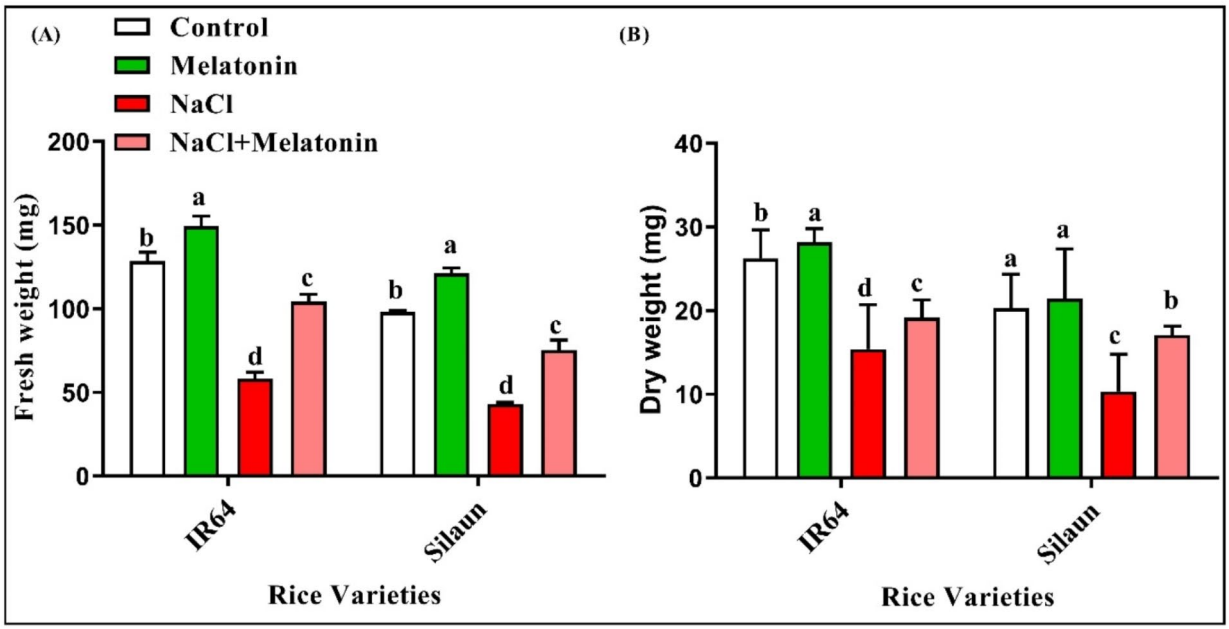

Fig. 3. The effect of NaCl and melatonin treatment on (**A**) fresh weight and (**B**) dry weight after 8 days of treatment. Different letters indicate significantly different results based on 5% significance level using Duncan's Multiple Range Test (DMRT). Error bars represent the mean ± standard error of the mean (SEM) calculated from replicates. For each condition, 14 plants were used from per replicate.

of 26.3%, whereas the total potassium content in Silaun increased 2.9% compared to the control. Exogenous melatonin under salinity stress conditions was able to increase the total potassium content in both varieties. The increase in the total potassium content in IR64 increased by 10.9% and in Silaun it increased by 26.1%.

Effect of NaCl and melatonin treatment on $H_2O_2$and malondialdehyde (MDA) levels in rice plants

In the IR 64 variety, salinity stress treatment increased the H_2O_2 content (Fig. 8A) by 5.83% compared to the control treatment, while in Silaun, it increased by 5.12%. Exogenous melatonin, when applied under salt stress, significantly reduced H_2O_2 content by 2.18% in IR64 and 2.56% in Silaun. The MDA parameter (Fig. 8B) showed salt stress treatment significantly increased the MDA value by 18.2% in IR64 and 46.9% in Silaun compared to the control treatment. However, exogenous melatonin application under salt stress can significantly reduce MDA values in rice plants by 9.1% in IR64 and 31.5% in Silaun.

Effect of NaCl and melatonin treatment on antioxidant gene expression in rice

In our study, we investigated the expression of antioxidant genes in two rice varieties, IR64, and Silaun, under different treatment conditions. Our results showed that the highest expression level of *OsAPX1* and *Cytosolic APX* genes were observed in IR64 followed by 150 mM NaCl + 1 µM melatonin treatment (Fig. 9A,C). In contrast, under 150 mM NaCl stress, the genes *OsAPX1*, *OsCATA*, and *cytosolic APX* exhibited elevated expression levels (Fig. 9A–C), highlighting a differential response in these antioxidant defense mechanisms across the two treatments. Our results revealed that *GPOD* was highly expressed in the IR64 variety under 150 mM NaCl and 150 mM NaCl + 1 µM melatonin treatment. *Mn-SOD* also showed elevated expression in IR64 (Fig. 9D,E), while in the Silaun variety, its expression was notably higher in response to melatonin treatment only. On the other hand, *OsAB13* exhibited similar expression in both control and 150 mm NaCl treatments in IR64. However, in Silaun, the expression of *OsAB13* significantly increased under 150 mM NaCl stress (Fig. 9F). In our findings, *Cu/ZnSOD* exhibited similar expression levels in IR64 under both 150 mM NaCl, and 150 mM NaCl + 1 µM melatonin, while in Silaun, its expression was significantly higher under 150 mm NaCl stress (Fig. 9G). The gene *OsLEA* was highly expressed in both IR64 and Silaun under control condition, whereas *CAT* also showed strong expression in both varieties under 150 mM NaCl stress (Fig. 9H,I). These findings suggest the complexity of antioxidant responses and the potential of melatonin to enhance stress tolerance in rice varieties.

Discussion

Salinity is the main factor that triggers decreases in rice plant growth by adversely changing morphological structures, which also undergo physiological changes as depicted in (Fig. 1). Seedling stages are most susceptible to salinity during the entire plant's life cycle. Both rice varieties under salinity stress show the inhibition of plant growth, which can be identified through their morphological characteristics. Our study elucidates how salt

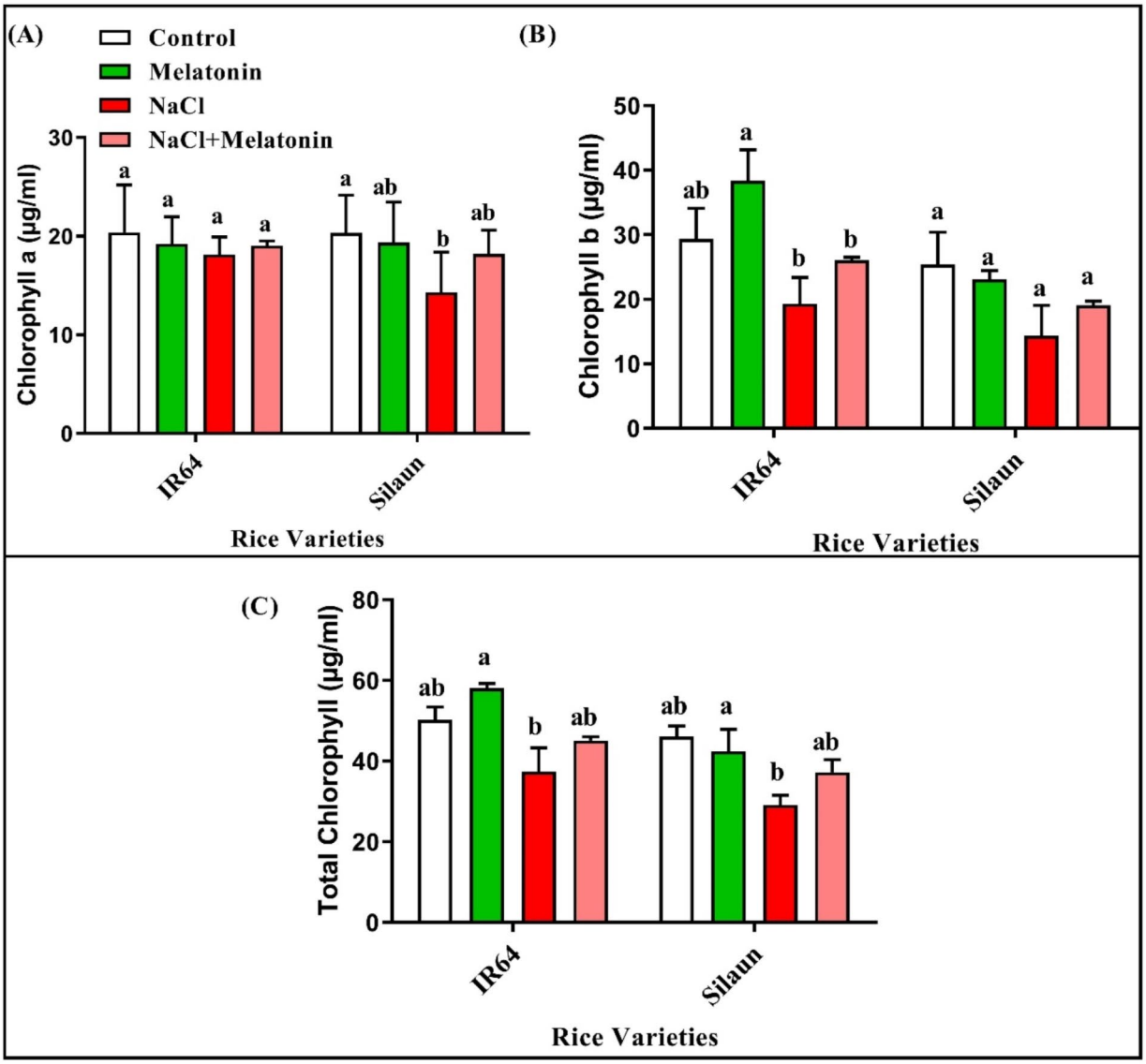

Fig. 4. The effect of NaCl and melatonin treatment on (**A**) chlorophyll a content, (**B**) chlorophyll b content, and (**C**) total chlorophyll content after 8 days of treatment. Different letters indicate significantly different results based on a 5% significance level using Duncans Multiple Range Test (DMRT). Error bars represent the mean ± standard error of the mean (SEM) calculated from 3 replicates. For each condition, 14 plants were used from per replicate.

stress diminishes various growth parameters, including but not limited to plant height, root length, stem length, leaf length, and leaf area in rice plants subjected to saline conditions (Fig. 2B–D). These consequences arise from the saline soil ability to limit water uptake by plants, which in turn leads to reduced capacity for nutrient absorption[29]. This water deficiency disrupts plants' physiological and molecular activities, which could affect plant growth by reducing plant development due to dehydration and shrinkage. It also inhibits photosynthesis, resulting in a lack of resources to promote cell division[30]. The observed reduction in the canopy area may be considered as an avoidance mechanism by reducing leaf area and stomatal closure which minimizes water loss by transpiration[31]. Under salt stress conditions, the IR64 variety exhibited a reduction in root length compared to normal conditions (Fig. 2A). This is because salinity stress exposure affected cell division and elongation, which significantly reduced roots growth[32]. In the Silaun variety, root elongation occurs under conditions of salinity stress. Plants that are tolerant to salinity stress can survive by growing more extended roots system to penetrate deeper layers of soil to acquire water and nutrients[30]. This study significantly showed reduction in plant biomass, including fresh and dry weight, under salt stress (Fig. 3A,B). This condition is due to decreased water potential in cells resulting in stomatal closure and reduced carboxylation process in the chloroplast, which could limit photosynthetic product in the thylakoid of the plant[33].

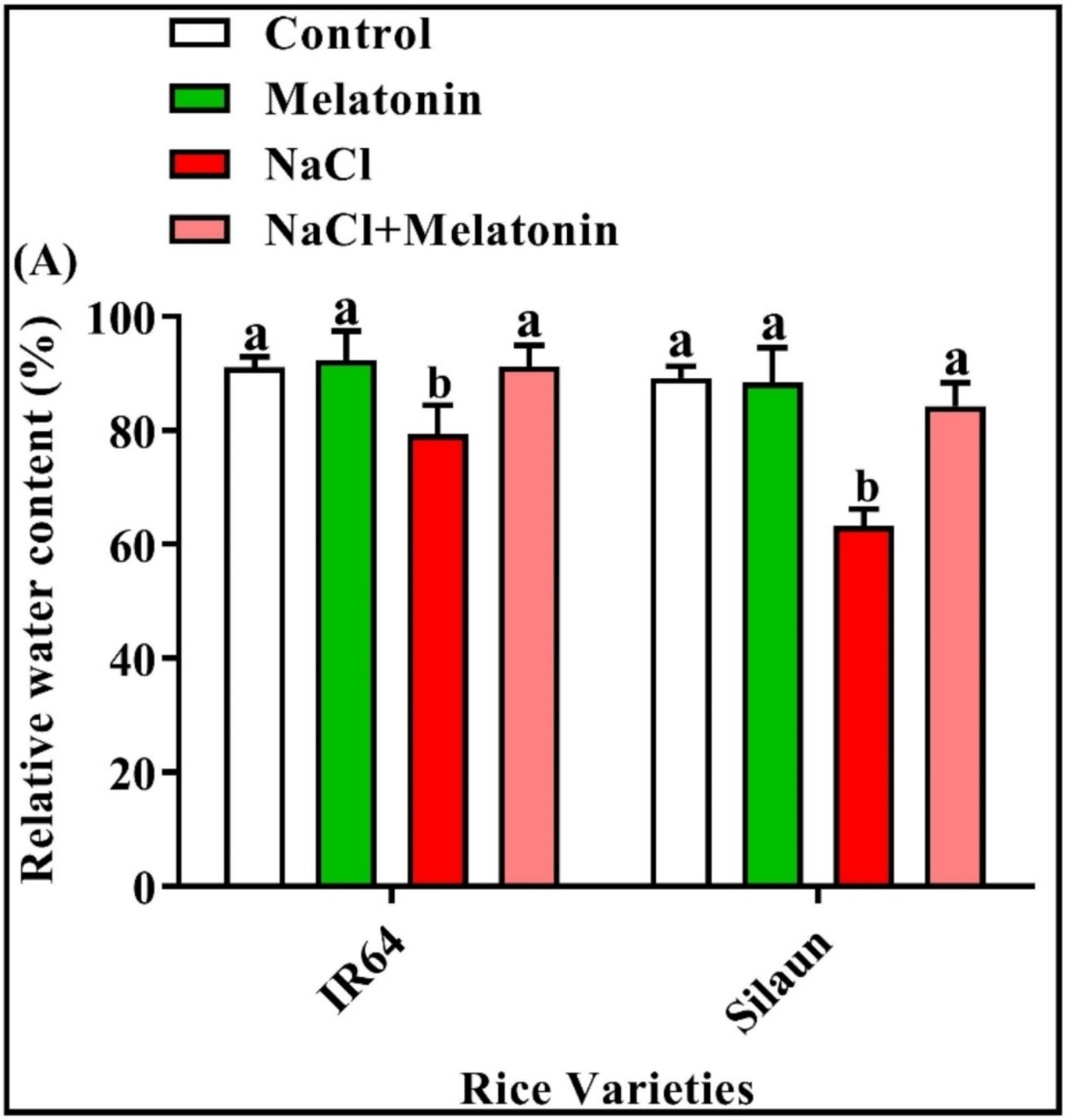

Fig. 5. The effect of NaCl and melatonin treatment on (**A**) relative water content after 8 days of treatment. Different letters indicate significantly different results based on a 5% significance level using Duncans Multiple Range Test (DMRT). Error bars represent the mean ± standard error of the mean (SEM) calculated from 3 replicates. For each condition, 14 plants were used from per replicate.

Research has shown that giving melatonin can increase plant growth under salinity stress conditions. Exogenous melatonin can help plants increase nutrient absorption in roots and impact plant growth and the leaf photosynthetic capacity[10]. Melatonin is a phytohormone act as a plant growth regulator, which regulates plant development and alleviates the damage caused by both abiotic and biotic stresses, including salt stress[34]. In previous studies, it was confirmed that giving melatonin with a concentration of 1 µM could increase the growth of various types of plants[22].

Among different physiological processes, photosynthesis is a vital physiological attribute related to plant growth and development affected by salt stress. Photosynthetic pigments are essential for light harvesting and, hence, for photosynthesis and plant growth. The decrease in total chlorophyll content in salt stress occurs due to the excessive accumulation of toxic ions, which will damage mesophyll cells and inhibit enzymes involved

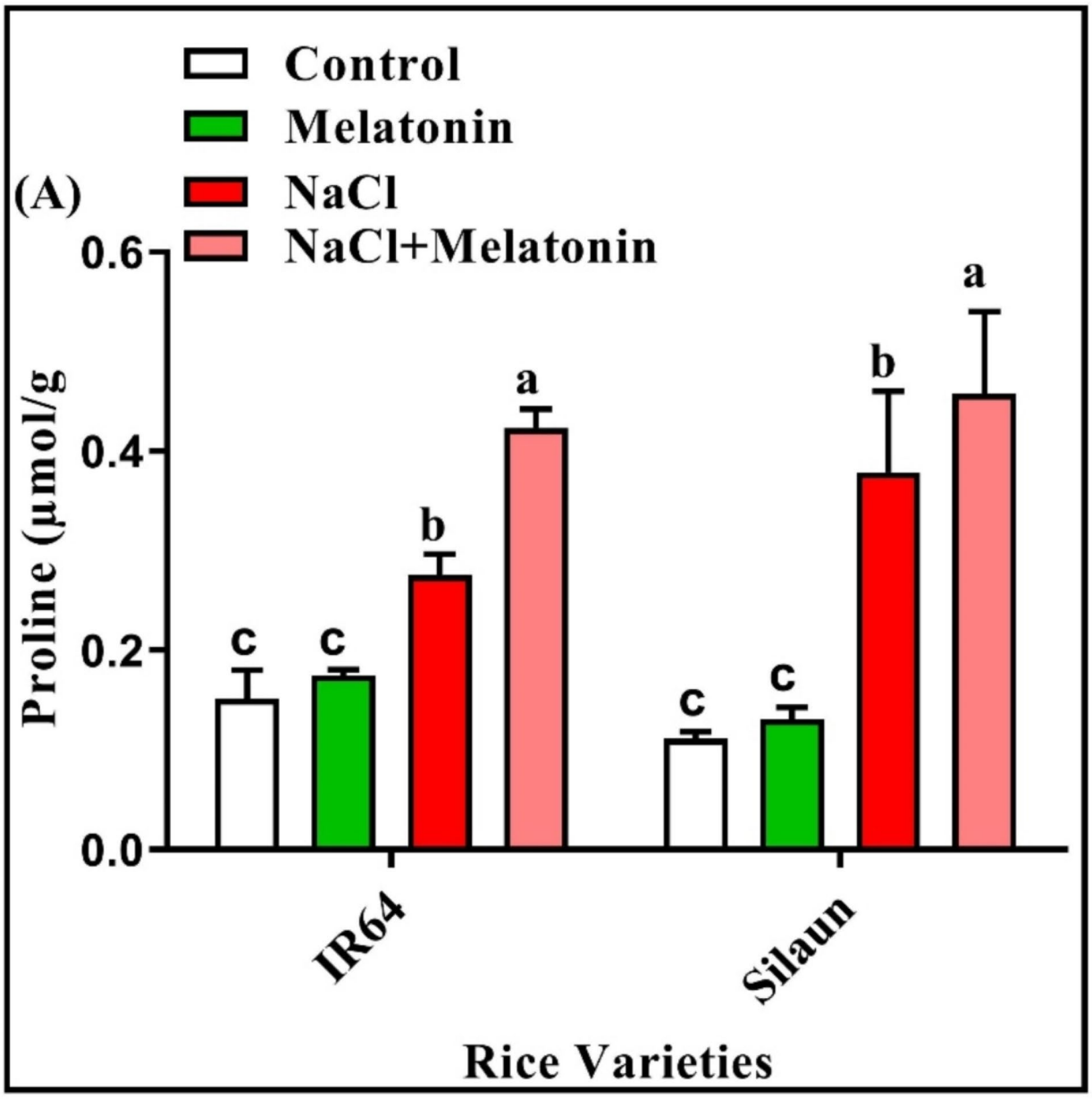

Fig. 6. The effect of NaCl and melatonin treatment on (**A**) proline content after 8 days of treatment. Different letters showed significantly different results at a 5% significance level using Duncans Multiple Range Test (DMRT). Error bars represent the mean ± standard error of the mean (SEM) calculated from 3 replicates. For each condition, 14 plants were used from per replicate.

in carbohydrate metabolism and several stages of chlorophyll synthesis[35]. During the germination stage of rice plants, salt stress treatment causes a decrease in the total chlorophyll content in rice plant sprouts due to disruption of chlorophyll biosynthesis and increased chlorophyll degradation, resulted the sprouts cannot grow and develop optimally[36]. This occurs due to the adaptation of rice plants by reducing or preventing ROS content under salinity stress. Our findings align with similar results reported in the literature[37], indicating that exogenous melatonin can increase total chlorophyll content by reducing chlorophyll degradation, protecting photosystems, and regulating photosynthesis in plant leaves. This effect enhances plant tolerance to salinity stress (Fig. 4A–C).

A decrease in leaf relative water content (RWC) indicates a loss of turgor pressure in leaves which causes limited water availability to be used for cell expansion processes. Differences in RWC content between rice varieties grown under salinity stress are related to differences in plant capacity to absorb water or the ability of stomata to reduce water loss. In this study, salinity stress reduced the RWC value of rice plants (Fig. 5A).

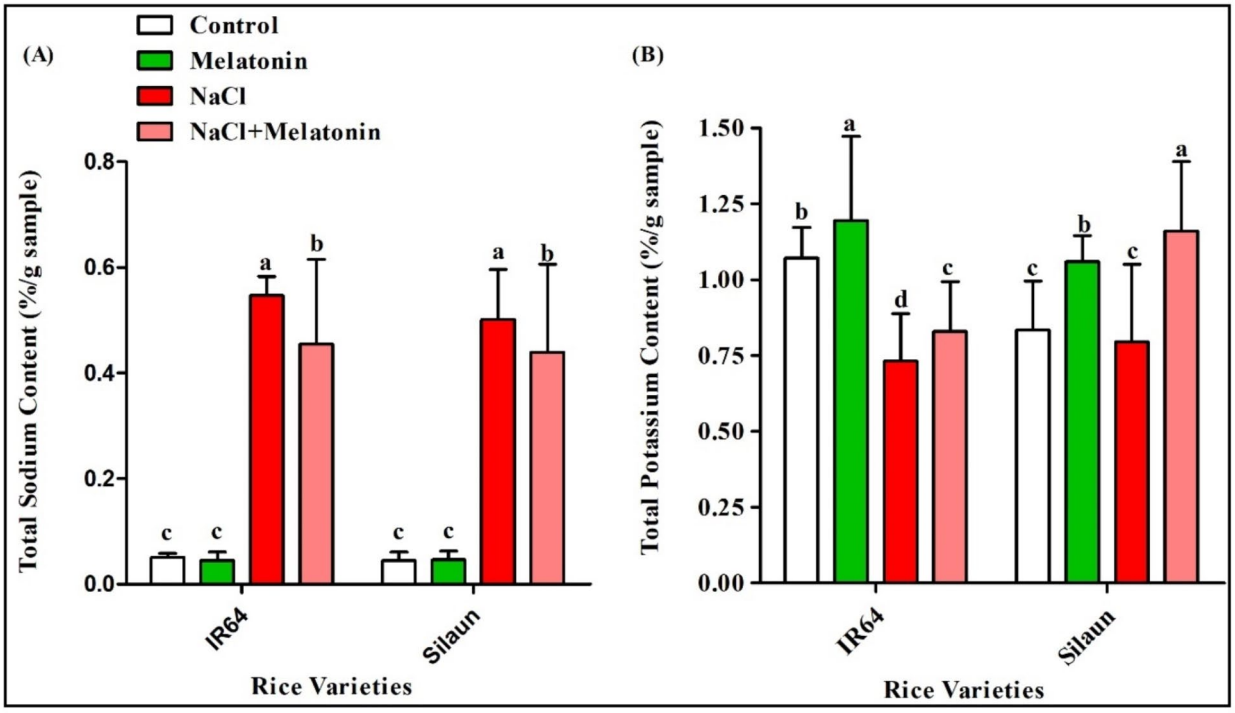

Fig. 7. The effect of NaCl and melatonin treatment on (**A**) total sodium content and (**B**) total potassium content after 8 days of treatment. Different letters indicate significantly different results based on a 5% significance level using Duncan's Multiple Range Test (DMRT). Error bars represent the mean ± standard error of the mean (SEM) calculated from 3 replicates. For each condition, 14 plants were used per replicate.

Exogenous melatonin can increase the RWC value, and this is related to a better water absorption capacity[38]. It was demonstrated the exogenous melatonin could enhanced water absorption by thickening plant's cuticle which limits water loss and stabilize cell's turgor[39].

Proline is a multifunctional amino acid that accumulates in plants and acts as protective membrane solute to respond to abiotic stresses. Plants accumulate proline to function as an osmoprotectant due to osmotic stress by maintaining water balanced[40]. This study results on rice plants under salinity stress increased the proline content, and exogenous melatonin treatment increased the proline content (Fig. 6A). This is due to the role of melatonin as an antioxidant signaling prevent proline degradation. Higher proline content is an adaptive response of plants during salt stress. The effect of melatonin treatment to enhanced proline content under salt stress was confirmed in *Cucumber melon* L[41].

Salinity stress will cause plants to experience an increase in sodium content due to the high NaCl content in the media. Sodium absorption competes with potassium by inhibiting specific potassium transporters in under salt stress[42]. Decreasing the potassium concentration in plant cells will reduce the ability of plants to regulate enzyme activity, osmotic pressure, and plant turgor. Some enzymes involved in plant metabolism are activated by potassium and cannot be replaced by other ions. In addition, potassium nutrients play an essential role in cell osmotic regulation. The ability of plants to maintain potassium concentrations at sufficient levels could maintain ion homeostasis and regulate osmotic balance under salt stress[43]. Giving melatonin can reduce sodium content and increase potassium absorption in rice plants (Fig. 7A,B). Increased potassium uptake in melatonin-treated plants was confirmed in apple plants (*Malus sp.*) tested under salinity stress[44].

Under salinity stress conditions, plants produce a considerable amount of ROS (*Reactive Oxygen Species*). ROS is a free radical present in plant tissue in large quantities and will damage the plant cells. Under normal conditions, plants can maintain low ROS levels due to a balance between the production of antioxidant enzymes and ROS production. Low concentration of H_2O_2 content can provide benefits for the plant, but high H_2O_2 content will leads to oxidative stress which can damage the plants cell[45]. ROS accumulation is associated with MDA. The increase in MDA values results from lipid peroxidation in plant tissues under salinity stress conditions. The increase in MDA in rice under salt stress conditions indicates that salinity stress causes an imbalance between ROS production and antioxidant enzymes, causing plants to experience oxidative stress (Fig. 8A,B). Giving melatonin to rice under salinity stress conditions can reduce the content of H_2O_2 and MDA values in plants through increased activity of antioxidant enzymes[46]. Increased antioxidant activities help to reduce salinity-mediated damage to membranes, proteins, nucleic acids and hence maintaining functional and physiological stability[47].

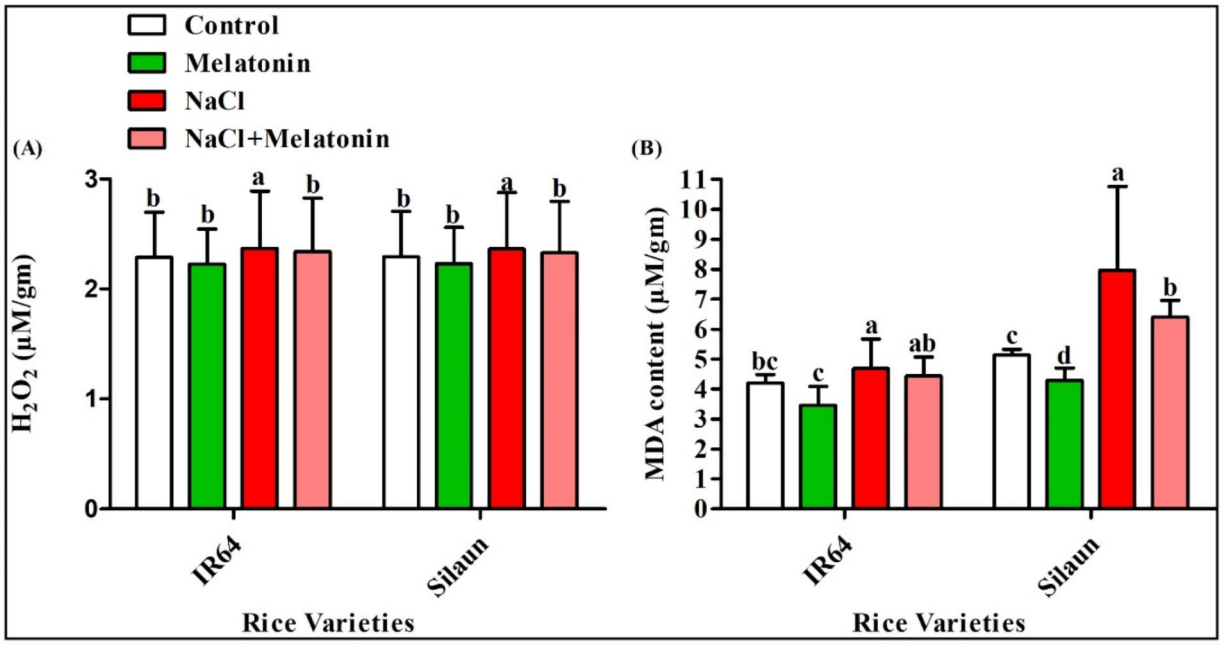

Fig. 8. The effect of NaCl and melatonin treatment on total H_2O_2 and MDA (**A,B**), content after 8 days of treatment. Different letters showed significantly different results based on a 5% significance level using Duncan´s Multiple Range Test (DMRT). Error bars represent the mean ± standard error of the mean (SEM) calculated from 3 replicates. For each condition, 14 plants were used per replicate.

To avoid excessive accumulation of ROS, plants have evolved defense systems that include ROS-scavenging enzymes, such as superoxide dismutase (SOD), ascorbate peroxidase (APX), and catalase (CAT). Superoxide dismutase (SOD) is the first line of defense in the antioxidant enzyme system, which functions to catalyze the conversion of O_2 to H_2O_2 so that it will reduce the amount of excessive superoxide anion contained in toxic cells (S1)[48,49]. Based on the gene expression in this study, two groups of SOD were observed, namely Mn-SOD and Cu/Zn-SOD. The difference between the two types of SOD is that Mn-SOD is located in the mitochondria, while Cu/Zn-SOD is located in the chloroplast, peroxisomes, and cytosol[50]. In our study, the expression levels of both Mn-SOD and Cu/Zn-SOD (Fig. 9E,G) in the rice varieties IR64 and Silaun indicated that salt stress, combined with exogenous melatonin treatment, led to higher expression compared to other treatment. Specifically, Mn-SOD was highly expressed in IR64, and Cu/Zn-SOD exhibited a significant increase in Silaun under 150 mM NaCl stress. These results suggest that exogenous melatonin enhance the activity of SOD as an antioxidant enzyme, particularly in IR64, where its activity was significantly increased compared to the control group (Fig. 9G).

The second antioxidant enzyme, APX or ascorbate peroxidase, catalyzes reactions that change plants' H_2O_2 to H_2O and O_2. The antioxidant enzyme APX is in peroxisomes, mitochondria, cytosol, and chloroplasts. Several categories of APX antioxidant enzymes exist, including APX or cytosolic APX. Under stress conditions, the plant will produce H_2O_2, where H_2O_2 has the property to diffuse resulted in high concentration accumulation in the cytosol. The role of APX is to respond optimally to the H_2O_2 content in the cytosol so that it can later be converted to H_2O. In the rice plant, *OsAPX1* functions as a signaling agent in plant tissue to encode the enzyme ascorbate peroxidase (APX). Our results demonstrate that *OsAPX1* and *cytosolic APX* were highly expressed in IR64 under salt stress, particularly in the presence of exogenous melatonin. This indicates that melatonin enhances the antioxidant capacity of *APX*, helping to regulate cytosolic H_20_2 levels and protect the plant from oxidative damage (Fig. 9A,C). Furthermore, *guaiacol peroxidase* (*GPOD*), another antioxidant enzyme, was highly expressed in IR64 under salt stress (Fig. 9D), while melatonin treatment significantly increased *GPOD* expression in Silaun under salt stress, indicating that *GPOD* plays a key role in mitigating oxidative stress induced by salinity (Fig. 9D). These findings suggest the idea that melatonin modulates the antioxidant response differentially across the two varieties.

Similarly to *APX*, catalase (*CAT*) also plays a vital role in detoxifying H_2O_2 but unlike APX, CAT does not require a reducing agent. One of the key catalyse genes in rice, *OsCATA*, is generally expressed at higher levels in leaves and young seeds of rice plants[51]. In this study, *OsCATA* (Fig. 9B) showed high expression in both IR64 and Silaun under salt stress, with exogenous melatonin further enhancing its expression, this indicates that melatonin amplifies the plants ability to neutralize oxidative damage caused by H_2O_2, acting through both APX and CAT pathways (Fig. 9H). There were two types of resistance genes analyzed, *OsABI3* and *OsLEA*. ABI3 is a transcription factor from the protein part that contains B3. The role of *OsABI3* is to assist adaptation by plants under abiotic stress by maintaining ABA-inducible gene expression during the rehydration period[52]. Our results

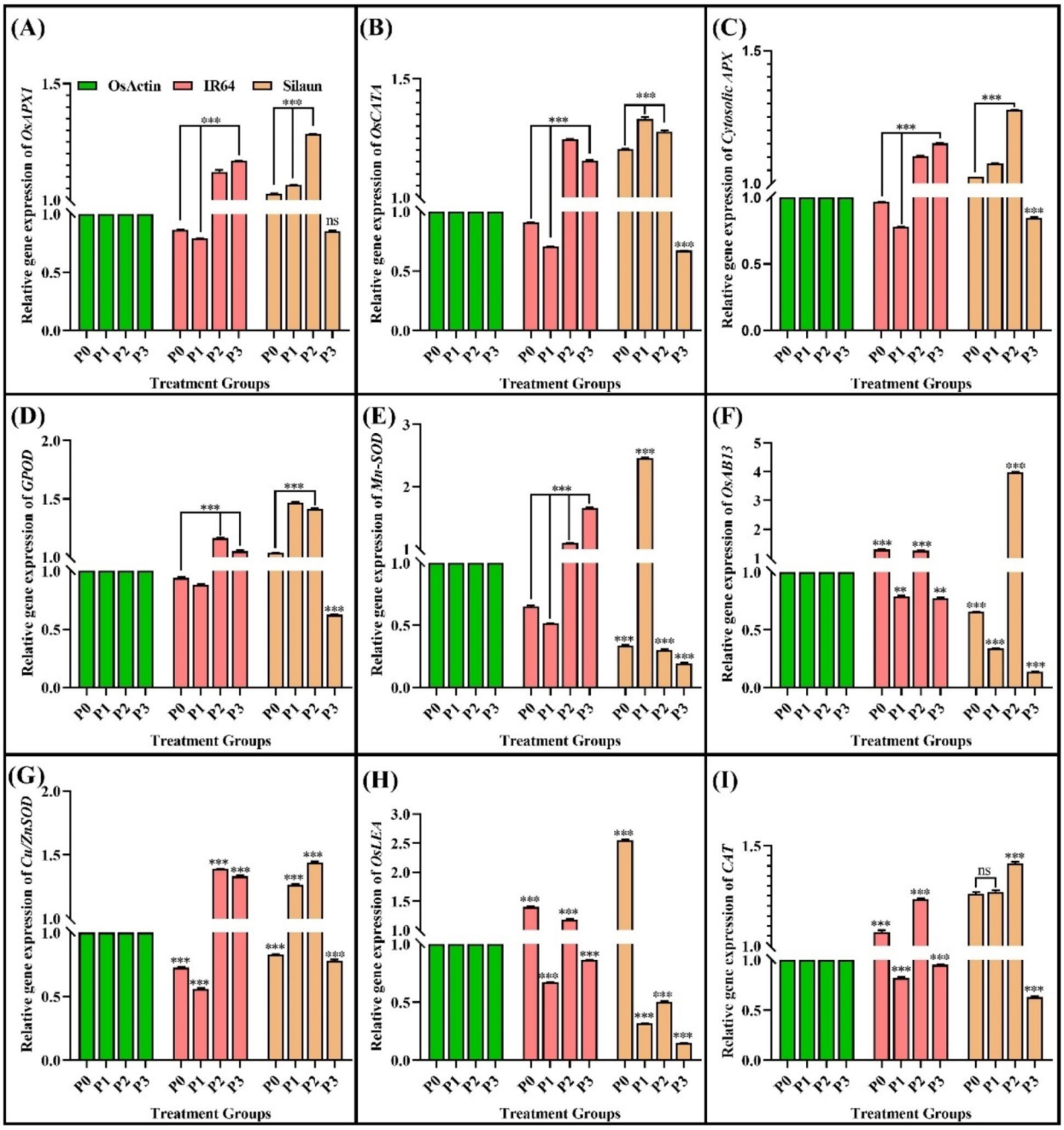

Fig. 9. Gene expression was analyzed using a two-way ANOVA followed by Tukey's multiple comparison test, with a minimum of three biological replicates for each condition. Statistical significances were determined at p values < 0.05 with asterisks denoting significant difference between groups, (**A**); P0: Control, P1: Melatonin, P2: NaCl, and P3: NaCl + Melatonin. Data are presented as the mean ± SE.

showed that *OsAB13* expression was similar in both control and salt stress conditions in IR64, but significantly increased under 150 mM NaCl in Silaun (Fig. 9F). This suggests a more pronounced stress response in Silaun under saltconditions. The *OsLEA* gene has an essential function in increasing the tolerance of rice plants under conditions of salinity stress, which will later correlate with abscisic acid and other hormones, which are a form of signaling to stress conditions[53]. The high expression of *OsLEA* (Fig. 9H) across both varieties further emphasizes the importance of this gene in improving rice plant resilience to salinity stress.

Conclusion

In conclusion, this study demonstrates that exogenous melatonin application enhances salt tolerance in rice genotypes by promoting morphological growth, increasing chlorophyll content, and maintaining water balance. Melatonin supplementation also mitigates oxidative stress by scavenging ROS, reducing MDA levels, and upregulating antioxidant gene expression. Furthermore, melatonin-treated rice plants exhibit improved sodium-potassium balance, elevated proline content, and enhanced activity of antioxidant enzymes, ultimately enhancing their resilience to salinity stress. These findings suggest the potential of melatonin as a promising strategy to boost salt tolerance in rice crops, offering insights for agriculture practices in salt-affected regions.

Data availability

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

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Author contributions

M.-U., M.-F. conducted the experimental study, performed data analysis, and wrote the manuscript. K.-K.-M. evaluate and edited the final version of the manuscript.

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Competing interests

The authors declare no competing interests.

Statement of adherence of the study to IUCN guidelines

The present study adheres to the IUCN Policy Statement on Research Involving Species at Risk of Extinction and adheres to the guidelines set forth by the Convention on international Trade in Endangered Species of Wild Fauna and Flora.

Additional information

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Correspondence and requests for materials should be addressed to K.-M.K.