

Exogenous application of melatonin alleviates salt stress-induced decline in growth and photosynthesis in *Glycine max* (L.) seedlings by improving mineral uptake, antioxidant and glyoxalase system

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Abstract: Soybean plants grown under NaCl were foliar sprayed twice with melatonin (MLT). Results revealed that salt stress reduced growth, biomass accumulation, photosynthesis, mineral uptake, the content of δ -aminolevulinic acid, chlorophylls, carotenoids and PSII efficiency. However, MLT application enhanced pigment synthesis and PSII activity. MLT up-regulated the antioxidant system and glyoxalase function resulting in reduced accumulation of reactive oxygen species (ROS). Reduced ROS in MLT-treated plants protected membrane functioning by reducing lipid peroxidation, electrolyte leakage and lipoxygenase activity. Nevertheless, MLT application reduced methylglyoxal accumulation while increased the content of reduced glutathione and ascorbic acid. It could be concluded that exogenous MLT mitigated the salt stress damage in soybean plants by improving photosynthesis, antioxidant systems, controlling ion homeostasis and minimising excessive ROS accumulation.

Keywords: salinity tolerance mechanisms; osmolyte; malondialdehyde; phytohormone; secondary metabolites; ascorbate

Salinity stress is one of the destructive environmental factors (Soliman et al. 2020a). Approximately 7% of the world's land is affected by salt, which negatively affects both the growth and development of plants (Ruiz-Lozano et al. 2012). Saline soils make most

agricultural areas either unproductive or even less efficient (Egamberdieva et al. 2017).

High levels of toxic salt ions have been recorded to minimise water absorption and discourage root growth (Rasool et al. 2013). Salinity stress induces

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osmotic and ionic stress resulting in impeding the major cellular functions of plants (Munns and Tester 2008, Plesa et al. 2018). A reduction in growth and biomass production of most crop plants with increasing salinity levels were reported (El-Lethy et al. 2013, Dawood et al. 2014). Salt stress decreases chlorophyll and carotenoid contents, which could be explained by the deterioration of the cells' membranes followed by disturbed enzyme activity (Rady et al. 2015, Sadak and Abdelhamid 2015). In addition, enhanced oxidative stress (Rafiq et al. 2017), limited transpiration (He et al. 2007), lowered relative water capacity and chlorophyll biosynthetic pathway (Soliman et al. 2020b) collectively reduces plant growth and biomass (Ahanger and Agarwal 2017, Soliman et al. 2020a).

One of the main reasons for these harmful effects is the excessive generation of reactive oxygen species (ROS) and methylglyoxal (MG), which damage key macromolecules, including proteins, lipids, nucleic acids, etc. (Ahanger and Agarwal 2017, Jahan et al. 2019a, Hasan et al. 2020a). In order to avoid the toxic effects of ROS and MG, plants have protection mechanisms that keep their concentrations much lower. These mechanisms include the antioxidant and glyoxalase systems, which respectively eliminate ROS and MG (Hasanuzzaman et al. 2014, Nahar et al. 2016, Hasan et al. 2018, 2020b).

Melatonin (N-acetyl-5-methoxytryptamine) was first discovered in plants in 1995 and subsequently in animals and has been reported in oxidative stress alleviation (Dubbels et al. 1995, Meng et al. 2014, Wei et al. 2015). MLT regulates flowering, seed germination, leaf resistance, root development and photosynthesis. Its involvement in the regulation of tolerance mechanisms against abiotic and biotic stresses has been reported in certain crops (Shi et al. 2015a,b, Zhao et al. 2017, Naghizadeh et al. 2019). Exogenous MLT improves endogenous MLT synthesis and enhances the antioxidant potential (Zhao et al. 2017, Jahan et al. 2019b, Kaya et al. 2019). In addition, it has been reported to influence the gene expression of important proteins like ion channels, antioxidants etc. (Shi et al. 2015a,b, Zhao et al. 2017, Jahan et al. 2020). Therefore, to further strengthen the understandings of its involvement in plant stress tolerance, the present study was conducted to examine the involvement of MLT in the modulation of antioxidant and osmolyte metabolism for growth and photosynthetic protection under salinity stress in soybean plants.

MATERIAL AND METHODS

Experimental procedures. Healthy *Glycine max* L. seeds were surface sterilised for 5 min using 5% NaOCl and subsequently washed by double distilled water (DDW). Seeds were sown in earthen pots containing soil and vermicompost (4:2). Ten days after sowing (DAS), seedlings were thinned, and four plants per pot were left. Pots were irrigated with Hoagland nutrient solution supplemented with 0 or 100 mmol/L NaCl starting from 14 DAS and lasted till 28 DAS.

The experiment comprised two factors. The first factor included two salt levels, i.e., 0 or 100 mmol/L NaCl. The second factor involved four MLT levels i.e. 0, 0.01, 0.05 or 0.10 mmol/L. The experimental layout was factorial arranged in a completely randomised design with three replicates. MLT was applied as a foliar spray, twice, at 14 DAS and 21 DAS. All spray treatments were done during the early morning before 9:00 a.m. After 28 DAS, plants were harvested and analysed for different parameters.

Determination of photosynthetic pigments, δ -aminolevulinic acid, photosynthesis and maximal photochemical efficiency. Chlorophylls and carotenoids (Caro) were extracted by homogenising fresh 100 mg leaf tissue by pestle and mortar using acetone (80%). After centrifugation for 20 min at 3 000 g, optical density (OD) of supernatant was taken at 480, 645 and 663 nm (Arnon 1949). δ -aminolevulinic acid (ALA) content was estimated according to the modified method of Turan and Tripathy (2015). For measurement of net photosynthetic rate (P_n) and photochemical efficiency (F_v/F_m) infra-red gas analyser (CID-340, Photosynthesis System, Bioscience, New York, USA) and modulated chlorophyll fluorometer (PAM 2500; Walz, Germany) was used.

Estimation of proline (Pro) and glycine betaine (GB). The Pro content was determined by Bates et al. (1973) method. For the determination of GB, the method of Grieve and Grattan (1983) was employed.

Determination of membrane stability index (MSI) and lipid peroxidation. Leaf discs (0.1 g) were thoroughly washed in running tap water and DDW and, after that, placed in 10 cm³ of DDW at 40 °C for 30 min, and their electrical conductivity was recorded (C_1). Subsequently, the same samples were placed in a boiling water bath (100 °C) for 10 min and their electrical conductivity recorded (C_2). The MSI was measured according to Sairam et al. (1997) using the following formula:

$$MSI = [1 - (C_1/C_2)] \times 100$$

Lipid peroxidation was determined by measuring the content of malonaldehyde (MDA) formation by the method of Heath and Packer (1968).

Determination of hydrogen peroxide (H_2O_2) and superoxide ion ($O_2^{\cdot-}$). For estimation of H_2O_2 , fresh tissue was extracted in 0.1% trichloroacetic acid (TCA). The mixture containing supernatant, potassium phosphate buffer (pH 7.0) and KI was read at 390 nm (Velikova et al. 2000). Estimation of $O_2^{\cdot-}$ was done using the method described by Yang et al. (2011).

Glyoxalase I activity and content of methylglyoxal. For the determination of glyoxalase I (EC 4.4.1.5) activity, the protocol of Hasanuzzaman et al. (2011) was adopted. MG content was estimated by following Wild et al. (2012) protocol.

Assay of antioxidant enzymes. Fresh tissue was extracted in cold 50 mmol phosphate buffer (pH 7.0) containing 1% polyvinylpyrrolidone (PVP) and 1 mmol ethylenediamine tetraacetic acid (EDTA) using pre-chilled pestle and mortar. The extract was centrifuged for 20 min at 15 000 g at 4 °C, and the supernatant was used as an enzyme source. Superoxide dismutase (SOD, EC 1.15.1.1) activity was assayed according to Beyer and Fridovich (1987) method. Luck's method (Luck 1974) was used for catalase (CAT, EC 1.11.1.6) assay. Ascorbate peroxidase (APX, EC 1.11.1.11) was measured following Nakano and Asada (1981). Glutathione reductase (GR; EC 1.6.4.2) activity was assayed as the change in optical density at 340 nm in accordance with Foyer and Halliwell (1976).

Determination of ascorbate (AsA) and reduced glutathione (GSH). For the determination of AsA content, the protocol of Mukherjee and Choudhuri (1983) was adopted. Extraction of GSH was done in phosphate buffer (pH 8.0), and the supernatant was mixed with 5,5-dithiobis-2-nitrobenzoic acid. After 10 min OD was taken at 412 nm (Ellman 1959).

Determination of phenols, flavonoids and total antioxidant activity. Total phenol content was measured in leaf methanolic extracts (Singleton and Rossi 1965). Flavonoid content was determined according to Jia et al. (1999). The total free radical scavenging activity was determined according to Shimada et al. (1992) by reacting methanolic extract with 0.1 mmol 1,1-diphenyl-2-picrylhydrazyl (DPPH). After 30 min of dark incubation, OD was taken at 517 nm.

Estimation of mineral ions. Nitrogen (N), potassium (K), calcium (Ca), and sodium (Na) were determined in fresh samples of leaves. Total N was determined using the micro-Kjeldahl method. K was determined using a flame photometer (ELE Flame

Photometer, Leighton Buzzard, Bedfordshire, UK). Ca and Na concentrations were determined by atomic absorption spectrophotometer (Chapman 1965). Cl was estimated by titration method against $AgNO_3$ using $KCrO_4$ as an indicator.

Statistical analysis. Data were subjected to an analysis of variance (ANOVA) for the homogeneity of error variances using Levene's test and testing for normality distribution (Shapiro and Wilk 1965). Statistically significant differences were compared at $P \leq 0.05$ using Tukey's *HSD* (honestly significant difference) test. Correlation coefficient r was calculated between total dry weight yield and each of the physiological and biochemical traits.

RESULTS AND DISCUSSION

Changes in physiological characteristics. Effect of MLT on TC, ALA, P_n and F_v/F_m in soybean plants grown under NaCl are shown in Table 1. Salinity stress significantly reduced TC, ALA, P_n and F_v/F_m , while MLT application significantly increased those traits. MLT at 0.05 mmol/L resulted in the highest values compared to control and increased these traits by 46.9, 40.0, 36.2, and 26.5% in TC, ALA, P_n , and F_v/F_m , respectively compared to control. Increases in these traits were by 36.8, 30.1, 31.9, and 27.1% in TC, ALA, P_n , and F_v/F_m , respectively, compared to control salinity and MLT. However, 0.05 mmol/L MLT application under salinity stress resulted in the highest values for all these traits, and the increase was 65.9, 44.7, 43.6, and 25.5% in TC, ALA, P_n , and F_v/F_m , respectively compared to MLT application under salinity stress. Salinity stress reduces the synthesis of chlorophyll pigments by influencing the functioning of enzymes (Wei et al. 2015, Chen et al. 2018). In the present study, MLT application ameliorated the decline in TC synthesis, which may be attributed to increased uptake of Mg and N as well as reduced TC degradation. Increased photosynthesis and PSII activity due to MLT application have been reported by Kaya et al. (2019). Salinity has been reported to reduce the Rubisco synthesis and uptake of essential elements required for photosynthetic regulation by maintaining the synthesis of important proteins (Najar et al. 2019). Reduced photosynthesis results from the cumulative effects of stresses on chlorophyll synthesis, gas exchange and PSII functioning (Hu et al. 2016, Hasan et al. 2019). Earlier, MLT application has been reported to mitigate temperature and salinity mediated decline in P_n and PSII activity in tomato

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Table 1. Effect of melatonin (MLT) on total chlorophylls (TC), δ -aminolevulinic (ALA), net photosynthesis (P_n) and maximal PSII efficiency (F_v/F_m) in soybean plants grown under salt stress

| Treatment | MLT (mmol/L) | TC (mg/g FW) | ALA (mg/g FW) | P_n ($\mu\text{mol CO}_2/\text{m}^2/\text{s}$) | PSII (F_v/F_m) | |
|---------------|-----------------|--------------------|--------------------|---|-----------------------|--------------------|
| NaCl (mmol/L) | 0 | 0.950 ^a | 98.4 ^a | 15.3 ^a | 0.775 ^a | |
| | 100 | 0.576 ^b | 53.7 ^b | 9.2 ^b | 0.530 ^b | |
| MLT (mmol/L) | 0.00 | 0.647 ^c | 65.3 ^d | 10.7 ^d | 0.585 ^d | |
| | 0.01 | 0.656 ^c | 67.1 ^c | 10.9 ^c | 0.605 ^c | |
| | 0.05 | 0.950 ^a | 91.3 ^a | 14.5 ^a | 0.740 ^a | |
| | 0.10 | 0.801 ^b | 80.5 ^b | 13.0 ^b | 0.680 ^b | |
| | 0.00 | 0.842 ^c | 85.2 ^c | 13.5 ^c | 0.700 ^c | |
| NaCl (mmol/L) | 0 | 0.01 | 0.845 ^c | 86.9 ^c | 13.7 ^c | 0.720 ^c |
| | | 0.05 | 1.152 ^a | 117.1 ^a | 17.8 ^a | 0.890 ^a |
| | | 0.10 | 0.962 ^b | 104.2 ^b | 16.2 ^b | 0.790 ^b |
| | | 0.00 | 0.451 ^f | 45.3 ^f | 7.8 ^g | 0.470 ^e |
| | | 0.01 | 0.467 ^f | 47.2 ^f | 8.1 ^f | 0.490 ^e |
| NaCl (mmol/L) | 100 | 0.05 | 0.748 ^d | 65.6 ^d | 11.2 ^d | 0.590 ^d |
| | | 0.10 | 0.640 ^e | 56.7 ^e | 9.8 ^e | 0.570 ^d |

Mean values within the same column with the same lower case letter are not significantly different according to Tukey's honestly significant difference (HSD) test at $P \leq 0.05$. FW – fresh weight

(Yang et al. 2018) and cucumber (Wang et al. 2016). Stresses damage photosynthetic apparatus by causing increased ROS generation, leading to structural and functional damage to the chloroplast (Li et al. 2015, Wei et al. 2015). In the present study, MLT-induced enhancement in photosynthesis may be attributed to the maintenance of low ROS concentrations by up-regulating the antioxidant system.

Changes in reactive oxygen species and related traits. Table 2 shows the effect of MLT on H_2O_2 , $\text{O}_2^{\bullet-}$, MDA and MSI. Salinity stress significantly increased H_2O_2 , and $\text{O}_2^{\bullet-}$, which resulted in a significant increase in MDA and a significant decline in MSI. MLT application significantly decreased H_2O_2 and $\text{O}_2^{\bullet-}$, which resulted in a significant reduction in MDA and a significant increase in MSI. Foliar MLT application at 0.05 mmol/L resulted in the best results compared to other MLT levels. MLT at 0.05 mmol/L resulted in a reduction by 37.7% and 31.0% in H_2O_2 and $\text{O}_2^{\bullet-}$ and decreased MDA by 27.0%, and an increase by 14.9% in MSI compared to control MLT. The interaction effects were significant in all mentioned traits, and foliar MLT application at 0.05 mmol/L under control salinity behaved significantly better compared to other treatments. MLT at 0.05 mmol/L under control salinity resulted in a reduction by 42.1% and 36.9% in H_2O_2 , and $\text{O}_2^{\bullet-}$ respectively and

consequently decreased MDA by 27.1%, and finally an increase by 11.0% in MSI compared to control MLT. However, 0.05 mmol/L MLT under salinity caused a reduction by 36.6% and 28.6% in H_2O_2 , and $\text{O}_2^{\bullet-}$ respectively and consequently decreased MDA by 27.0%, and finally, an increase by 20.3% in MSI compared to control MLT (Table 2). Salinity stress resulted in a significant increase in H_2O_2 , $\text{O}_2^{\bullet-}$ and LOX activity. Similar to our findings, Rasool et al. (2013) demonstrated increased ROS generation due to salinity lead to lipid peroxidation and membrane damage. Reduced ROS accumulation and LOX in MLT-treated seedlings depicts the beneficial role of MLT in protecting the membranes. Recently, in *Dracocephalum moldavica*, Naghizadeh et al. (2019) have demonstrated that MLT reduced ROS generation and LOX activity resulting in declined MDA.

Changes in lipoxygenases, glyoxalase I (Glo-I) and methylglyoxal (MG). The effects of MLT on LOX, Glo-I and MG are shown in Table 3. Salinity stress significantly reduced the activities of LOX, Glo-I, and increased MG content. MLT (0.05 mmol/L) resulted in the lowest values compared control MLT as MLT reduced LOX and MG by 33.0%, and 24.9%, respectively. However, 0.05 mmol/L MLT significantly increased Glo-I by 13.7% compared to control MLT. 0.05 mmol/L MLT at control salinity caused

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Table 2. Effect of melatonin (MLT) on hydrogen peroxide (H_2O_2), superoxide ($O_2^{\bullet-}$), lipid peroxidation (MDA) and membrane stability index (MSI) in soybean plants grown under salt stress

| Treatment | | MLT (mmol/L) | H_2O_2 | $O_2^{\bullet-}$ (nmol/g FW) | MDA | MSI (%) |
|---------------|------|-----------------|-------------------|---------------------------------|-------------------|-------------------|
| NaCl (mmol/L) | 0 | | 10.0 ^b | 5.5 ^b | 4.3 ^b | 85.1 ^a |
| | 100 | | 39.4 ^a | 13.7 ^a | 9.8 ^a | 63.8 ^b |
| MLT (mmol/L) | 0.00 | | 30.1 ^a | 11.3 ^a | 8.0 ^a | 69.3 ^d |
| | 0.01 | | 27.6 ^b | 10.5 ^b | 7.4 ^b | 72.2 ^c |
| | 0.05 | | 18.8 ^d | 7.8 ^d | 7.0 ^c | 79.6 ^a |
| | 0.10 | | 22.3 ^c | 8.7 ^c | 5.8 ^d | 76.9 ^b |
| | | 0.00 | | 12.1 ^e | 6.5 ^e | 4.8 ^e |
| NaCl (mmol/L) | 0 | 0.01 | 11.9 ^e | 6.4 ^e | 4.7 ^e | 82.5 ^c |
| | | 0.05 | 7.0 ^e | 4.1 ^g | 3.5 ^g | 89.9 ^a |
| | | 0.10 | 9.1 ^f | 4.9 ^f | 4.1 ^f | 86.9 ^b |
| | | 0.00 | 48.1 ^a | 16.1 ^a | 11.1 ^a | 57.5 ^f |
| | | 0.01 | 43.2 ^b | 14.6 ^b | 10.0 ^b | 61.8 ^e |
| NaCl (mmol/L) | 100 | 0.05 | 30.5 ^d | 11.5 ^d | 8.1 ^d | 69.2 ^d |
| | | 0.10 | 35.6 ^c | 12.5 ^c | 9.8 ^c | 66.8 ^d |

Mean values within the same column with the same lower case letter are not significantly different according to Tukey's honestly significant difference (HSD) test at $P \leq 0.05$. FW – fresh weight

a decrease in LOX and MG by 30.4% and 18.3%, respectively. MLT (0.05 mmol/L) under salinity resulted in a higher reduction in LOX and MG by

34.1% and 27.8%, respectively, compared to salinity only without MLT application. MG detoxification depends on the concentration of GSH (Mostofa et

Table 3. Effect of melatonin (MLT) on lipoxygenases, glyoxalase I and methylglyoxal in soybean plants grown under salt stress

| Treatment | | MLT (mmol/L) | Lipoxygenase (mmol/min/mg protein) | Glyoxalase I | Methylglyoxal (μ g/g FW) |
|---------------|------|-----------------|---------------------------------------|--------------------|----------------------------------|
| NaCl (mmol/L) | 0 | | 9.9 ^b | 0.112 ^b | 7.6 ^b |
| | 100 | | 20.4 ^a | 0.200 ^a | 15.8 ^a |
| MLT (mmol/L) | 0.00 | | 18.4 ^a | 0.146 ^c | 13.2 ^a |
| | 0.01 | | 15.9 ^b | 0.149 ^b | 12.5 ^b |
| | 0.05 | | 12.3 ^d | 0.165 ^a | 9.9 ^d |
| | 0.10 | | 14.0 ^c | 0.166 ^a | 11.1 ^c |
| | | 0.00 | | 11.2 ^e | 0.109 ^f |
| NaCl (mmol/L) | 0 | 0.01 | 10.7 ^e | 0.109 ^f | 8.1 ^e |
| | | 0.05 | 7.8 ^g | 0.121 ^e | 6.7 ^g |
| | | 0.10 | 9.8 ^f | 0.110 ^f | 7.3 ^f |
| | | 0.00 | 25.5 ^a | 0.182 ^d | 18.2 ^a |
| | | 0.01 | 21.0 ^b | 0.189 ^c | 16.9 ^b |
| NaCl (mmol/L) | 100 | 0.05 | 16.8 ^d | 0.209 ^b | 13.1 ^d |
| | | 0.10 | 18.2 ^c | 0.221 ^a | 14.9 ^c |

Mean values within the same column with the same lower case letter are not significantly different according to Tukey's honestly significant difference (HSD) test at $P \leq 0.05$. FW – fresh weight

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al. 2015), and in the present study, MLT resulted in increased GSH content, which may have further regulated the glyoxalase system.

Changes in the antioxidant defense system. Table 4 shows the effect of MLT on SOD, CAT, APX, GR, and DPPH• activity, AsA, GSH, TPh, Flav and Car. Salinity stress significantly increased SOD, CAT, APX, GR, DPPH, GSH, TPh, Flav while reduced AsA and Car. Exogenous MLT (0.05 mmol/L) gave highest values compared all other MLT rates and increased SOD, CAT, APX, GR, DPPH, AsA, GSH, TPh, Flav, and Car by 31.0, 20.4, 41.2, 33.3, 19.2, 21.0, 22.1, 21.3, 21.9 and 31.7% respectively compared to control MLT. Interaction effects were significant in all mentioned traits. Application of MLT (0.05 mmol/L) compared to control MLT under salinity stress further enhanced SOD, CAT, APX, GR, DPPH, AsA, GSH, TPh, Flav, and Car by 40.0, 24.0, 36.5, 35.2, 18.7, 32.1, 52.0, 31.6, 30.3, and 34.0%, respectively (Table 4). In the present study, salt-stressed increased antioxidant functioning confirmed other findings (Hasanuzzaman et al. 2011, Rasool et al. 2013). Increased antioxidant functioning

prevents the vulnerability of photosynthetic electron transport to $O_2^{\bullet-}$ in the PSII reaction center, which otherwise results in irreversible oxidation of D1 protein (Krieger-Liszkay et al. 2008, Vass and Cser 2009). Zhao et al. (2017) demonstrated increased activities and transcript levels of SOD and APX due to MLT application resulting in enhanced salinity tolerance. Improved AsA and GSH accumulation due to MLT application may have significantly contributed to salinity tolerance. Both AsA and GSH act as signaling molecules to protect photosynthesis from oxidant environments (Foyer and Shigeoka 2011). Increased synthesis of AsA and GSH concomitant with up-regulation of APX and GR significantly contributes to the maintenance of NADP concentration for smooth photosynthetic electron transport. In salinity-stressed cucumber, MLT up-regulated the antioxidant activities and synthesis of AsA and GSH (Wang et al. 2016). Similar to our results, increased accumulation of TPh and Flav due to MLT application has been reported in *Dracocephalum moldavica* (Naghizadeh et al. 2019).

Table 4. Effect of melatonin (MLT) on enzymatic antioxidants (SOD – superoxide dismutase; CAT – catalase; APX – ascorbate peroxidase; GR – glutathione reductase), antioxidant activities (DPPH• – radical scavenging activity), and non-enzymatic antioxidants (AsA – ascorbate; GSH – reduced glutathione; TPh – total phenols; Flav – flavonoids; Car – carotenoids) in soybean plants grown under salt stress

| Treatment | MLT (mmol/L) | SOD | CAT | APX | GR | DPPH• (%) | AsA (nmol/g FW) | GSH | TPh (mg/g DW) | Flav | Car (mg/g FW) |
|----------------------|--------------|-------------------|--------------------|-------------------|-------------------|-------------------|-------------------|------------------|--------------------|-------------------|--------------------|
| | | (U/mg protein) | | | | | | | (mg/g DW) | | |
| NaCl (mmol/L) | | | | | | | | | | | |
| | 0 | 1.52 ^b | 25.6 ^b | 1.44 ^b | 0.59 ^b | 52.3 ^b | 251 ^a | 219 ^b | 1.07 ^b | 0.86 ^b | 0.508 ^a |
| | 100 | 2.98 ^a | 43.2 ^a | 2.71 ^a | 1.01 ^a | 68.3 ^a | 212 ^b | 302 ^a | 1.58 ^a | 1.26 ^a | 0.352 ^b |
| MLT (mmol/L) | | | | | | | | | | | |
| | 0.00 | 1.97 ^d | 31.8 ^c | 1.77 ^d | 0.71 ^d | 55.4 ^d | 213 ^c | 239 ^d | 1.20 ^d | 0.96 ^d | 0.380 ^c |
| | 0.01 | 2.13 ^c | 33.0 ^{bc} | 1.85 ^c | 0.75 ^c | 57.9 ^c | 217 ^c | 245 ^c | 1.26 ^c | 1.01 ^c | 0.386 ^c |
| | 0.05 | 2.58 ^a | 38.3 ^a | 2.50 ^a | 0.94 ^a | 66.0 ^a | 258 ^a | 292 ^a | 1.46 ^a | 1.17 ^a | 0.500 ^a |
| | 0.10 | 2.32 ^b | 34.6 ^b | 2.17 ^b | 0.82 ^b | 61.8 ^b | 239 ^b | 264 ^b | 1.37 ^b | 1.09 ^b | 0.454 ^b |
| NaCl (mmol/L) | | | | | | | | | | | |
| | 0.00 | 1.44 ^g | 24.8 ^e | 1.21 ^g | 0.53 ^g | 48.2 ^g | 240 ^c | 200 ^f | 1.04 ^f | 0.83 ^f | 0.451 ^c |
| | 0.01 | 1.45 ^g | 24.9 ^e | 1.19 ^g | 0.54 ^g | 49.1 ^g | 239 ^c | 205 ^f | 1.03 ^f | 0.84 ^f | 0.458 ^c |
| | 0.05 | 1.66 ^e | 28.5 ^d | 1.82 ^e | 0.69 ^e | 57.8 ^e | 270 ^a | 249 ^d | 1.12 ^e | 0.92 ^e | 0.587 ^a |
| | 0.10 | 1.53 ^f | 24.3 ^e | 1.52 ^f | 0.61 ^f | 53.9 ^f | 256 ^b | 221 ^e | 1.07 ^{ef} | 0.85 ^f | 0.535 ^b |
| | 0.00 | 2.50 ^d | 38.8 ^c | 2.33 ^d | 0.88 ^d | 62.5 ^d | 186 ^e | 279 ^c | 1.36 ^d | 1.09 ^d | 0.309 ^f |
| | 0.01 | 2.80 ^c | 41.0 ^c | 2.51 ^c | 0.95 ^c | 66.7 ^c | 195 ^e | 285 ^c | 1.49 ^c | 1.19 ^c | 0.313 ^f |
| | 0.05 | 3.50 ^a | 48.1 ^a | 3.18 ^a | 1.19 ^a | 74.2 ^a | 246 ^{bc} | 336 ^a | 1.79 ^a | 1.42 ^a | 0.414 ^d |
| | 0.10 | 3.10 ^b | 44.8 ^b | 2.82 ^b | 1.03 ^b | 69.6 ^b | 222 ^d | 307 ^b | 1.67 ^b | 1.33 ^b | 0.373 ^e |

Mean values within the same column with the same lower case letter are not significantly different according to Tukey's honestly significant difference (HSD) test at $P \leq 0.05$. FW – fresh weight; DW – dry weight

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Table 5. Effect of melatonin (MLT) on compatible solute accumulation in soybean plants grown under salt stress

| Treatment | MLT (mmol/L) | Proline (mmol/g FW) | Glycine betaine (mmol/g DW) |
|----------------------|--------------|---------------------|-----------------------------|
| NaCl (mmol/L) | | | |
| | 0 | 33.0 ^b | 2.05 ^b |
| | 100 | 57.6 ^a | 4.72 ^a |
| MLT (mmol/L) | | | |
| | 0.00 | 39.5 ^c | 3.00 ^b |
| | 0.01 | 41.4 ^b | 3.16 ^b |
| | 0.05 | 50.4 ^a | 3.80 ^a |
| | 0.10 | 50.2 ^a | 3.58 ^a |
| NaCl (mmol/L) | | | |
| | 0.00 | 31.1 ^e | 1.89 ^c |
| | 0.01 | 30.9 ^e | 1.91 ^c |
| | 0.05 | 36.7 ^d | 2.30 ^c |
| | 0.10 | 33.5 ^e | 2.10 ^c |
| | 0.00 | 47.8 ^c | 4.10 ^b |
| | 0.00 | 51.8 ^b | 4.40 ^b |
| | 0.05 | 64.1 ^a | 5.30 ^a |
| | 0.10 | 66.8 ^a | 5.07 ^a |

Mean values within the same column with the same lower case letter are not significantly different according to Tukey's honestly significant difference (HSD) test at $P \leq 0.05$. FW – fresh weight; DW – dry weight

Changes in compatible solute accumulation.

The effects of MLT on Pro and GB are shown in Table 5. Salinity stress significantly increased Pro and GB. Exogenous MLT significantly increased the accumulation of Pro and GB further over non-salt stressed plants. MLT (0.05 mmol/L) gave the highest values compared to all other MLT rates and 0.05 mmol/L MLT increased Pro and GB by 27.7% and 26.9%, respectively over MLT control. The interaction effects were significant in Pro and GB. Application of MLT (0.05 mmol/L) compared to control MLT under salinity stress further enhanced Pro and GB by 34.1% and 29.3%, respectively (Table 5). Sufficient accumulation of Pro protects the carboxylase activity of Rubisco (Khan et al. 2017, Hasan et al. 2021). In addition, GB improves the oxygen-evolving activity of PSII (Mohanty et al. 1993) and also prevents any possible damage to photosynthetic apparatus (Sakamoto and Murata 2002). Enhanced Pro and GB contents due to MLT may have contributed to the maintenance of water content, thereby avoiding the osmotic effects of salinity to a considerable extent.

Changes in ionic content and growth. Effects of MLT on N, K, Ca, Na, Cl, K:Na and Ca:Na ratio are shown in Table 6. Salinity stress significantly reduced N, K, and Ca while increased Na and Cl and raised K:Na and Ca:Na ratios. The exogenous

Table 6. Effect of melatonin (MLT) on nitrogen (N), potassium (K), calcium (Ca), sodium (Na), chlorine (Cl), K:Na ratio, and Ca:Na ratio in soybean plants grown under salt stress

| Treatment | MLT (mmol/L) | N | K | Ca | Na | Cl | K:Na | Ca:Na | |
|---------------|--------------|-------------------|-------------------|-------------------|--------------------|-------------------|-------------------|--------------------|-------------------|
| | | (mg/g DW) | | | | | ratio | | |
| NaCl (mmol/L) | 0 | 16.0 ^a | 13.4 ^a | 8.42 ^a | 1.65 ^b | 1.43 ^b | 8.48 ^a | 5.35 ^a | |
| | 100 | 10.6 ^b | 7.8 ^b | 5.61 ^b | 7.72 ^a | 3.29 ^a | 1.04 ^b | 0.76 ^b | |
| MLT (mmol/L) | 0.00 | 11.7 ^c | 9.3 ^c | 5.72 ^d | 5.34 ^a | 3.12 ^a | 3.46 ^d | 2.15 ^d | |
| | 0.01 | 12.1 ^c | 9.6 ^c | 6.42 ^c | 4.96 ^b | 2.80 ^b | 3.77 ^c | 2.37 ^c | |
| | 0.05 | 15.6 ^a | 12.9 ^a | 8.77 ^a | 3.71 ^d | 1.55 ^d | 7.16 ^a | 4.67 ^a | |
| | 0.10 | 13.8 ^b | 10.6 ^b | 7.16 ^b | 4.73 ^c | 1.96 ^c | 4.66 ^b | 3.01 ^b | |
| | 0.00 | 14.4 ^c | 11.6 ^c | 7.24 ^c | 1.89 ^e | 1.61 ^e | 6.14 ^d | 3.83 ^d | |
| NaCl (mmol/L) | 0 | 0.01 | 14.5 ^c | 12.0 ^c | 7.37 ^c | 1.81 ^e | 1.69 ^e | 6.64 ^c | 4.07 ^c |
| | | 0.05 | 18.8 ^a | 16.7 ^a | 10.69 ^a | 1.30 ^g | 1.02 ^g | 12.85 ^a | 8.23 ^a |
| | | 0.10 | 16.3 ^b | 13.2 ^b | 8.36 ^b | 1.59 ^f | 1.39 ^f | 8.30 ^b | 5.26 ^b |
| | | 0.00 | 9.0 ^e | 6.9 ^f | 4.19 ^e | 8.79 ^a | 4.63 ^a | 0.79 ^g | 0.48 ^g |
| NaCl (mmol/L) | 100 | 0.01 | 9.8 ^e | 7.3 ^{ef} | 5.46 ^d | 8.10 ^b | 3.91 ^b | 0.90 ^{fg} | 0.67 ^f |
| | | 0.05 | 12.4 ^d | 9.0 ^d | 6.85 ^c | 6.11 ^d | 2.08 ^d | 1.47 ^e | 1.12 ^e |
| | | 0.10 | 11.3 ^d | 8.0 ^e | 5.95 ^d | 7.87 ^c | 2.52 ^c | 1.02 ^f | 0.76 ^f |

Mean values within the same column with the same lower-case letter are not significantly different according to Tukey's honestly significant difference (HSD) test at $P \leq 0.05$. DW – dry weight

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Table 7. Effect of melatonin (MLT) on shoot length and shoot dry weight in soybean plants grown under salt stress

| Treatment | MLT (mmol/L) | Shoot length (cm) | Shoot dry weight (g/plant) |
|----------------------|--------------|-------------------|----------------------------|
| NaCl (mmol/L) | | | |
| 0 | | 20.4 ^a | 1.60 ^a |
| 100 | | 14.1 ^b | 0.92 ^b |
| MLT (mmol/L) | | | |
| 0.00 | | 15.3 ^c | 1.10 ^c |
| 0.01 | | 15.6 ^c | 1.12 ^c |
| 0.05 | | 19.7 ^a | 1.54 ^a |
| 0.10 | | 18.4 ^b | 1.28 ^b |
| NaCl (mmol/L) | | | |
| 0 | 0.00 | 18.2 ^b | 1.42 ^c |
| | 0.01 | 18.4 ^b | 1.46 ^c |
| | 0.05 | 23.2 ^a | 1.89 ^a |
| | 0.10 | 21.9 ^a | 1.63 ^b |
| 100 | 0.00 | 12.4 ^d | 0.78 ^f |
| | 0.01 | 12.8 ^d | 0.79 ^f |
| | 0.05 | 16.2 ^c | 1.19 ^d |
| | 0.10 | 14.8 ^c | 0.93 ^e |

Mean values within the same column with the same lower case letter are not significantly different according to Tukey's honestly significant difference (HSD) test at $P \leq 0.05$

application of MLT significantly increased N, K and Ca, while reduced Na and Cl. The increase in those traits was gradual till 0.05 mmol/L, then the increase was less in 0.01 mmol/L compared to 0.05 mmol/L. MLT (0.05 mmol/L) resulted in a further increase over MLT control by 33.4, 38.8 and 53.5% in N, K, and Ca, respectively, while caused more reduction by 30.6% and 50.3% in Na and Cl, respectively. Therefore, K:Na and Ca:Na ratios have risen by 106.7% and 117.0% due to MLT at 0.05 mmol/L (Table 6). Interaction effects were significant in all mentioned traits, and MLT application at 0.05 mmol/L under control salinity gave the highest values of N, K, Ca, K:Na and Ca:Na ratio. However, 0.05 mmol/L MLT under salinity stress resulted in an increase of 37.8, 30.4 and 63.5% in N, K, and Ca, respectively, while a reduction by 30.5% and 55.1% in Na and Cl, respectively. Thus, K:Na and Ca:Na ratios increased by 87.5% and 135.1%, respectively (Table 6). Relative to control, a significant decline in SL and SDW was observed, and MLT increased these traits gradually till 0.05 mmol/L, an increase was less in 0.01 mmol/L. MLT at 0.05 mmol/L increased SL and SDW by 28.8% and 40.0% respectively compared to control. Interaction effects were significant in both traits, and it was noticed that MLT at 0.05 mmol/L under control salinity gave the highest values (Table 7).

Table 8. Pearson's correlation coefficients among dry shoot weight and all measured traits of soybean plants sprayed with melatonin and grown under salt stress

| Trait | TC | Car | ALA | P _n | F _v /F _m | H ₂ O ₂ | O ₂ ⁻ | MDA | SDW |
|---------|-------|--------|--------|----------------|--------------------------------|-------------------------------|-----------------------------|--------|-------|
| SDW | 0.989 | 0.989 | 0.993 | 0.995 | 0.990 | -0.960 | -0.971 | -0.969 | 1.000 |
| P-value | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| Trait | MSI | LOX | MGO | Glo-I | SOD | CAT | APX | GR | SDW |
| SDW | 0.979 | -0.954 | -0.963 | -0.786 | -0.707 | -0.729 | -0.622 | -0.637 | 1.000 |
| P-value | 0.000 | 0.000 | 0.000 | 0.021 | 0.050 | 0.040 | 0.100 | 0.089 | 0.000 |
| Trait | AsA | GSH | TPh | Flav | DPPH* | Pro | GB | N | SDW |
| SDW | 0.932 | -0.591 | -0.660 | -0.652 | -0.592 | -0.664 | -0.725 | 0.992 | 1.000 |
| P-value | 0.001 | 0.123 | 0.075 | 0.080 | 0.122 | 0.073 | 0.042 | 0.000 | 0.000 |
| Trait | K | Ca | Na | Cl | K/Na | Ca/Na | SL | SDW | |
| SDW | 0.984 | 0.957 | -0.948 | -0.907 | 0.959 | 0.960 | 0.987 | 1.000 | |
| P-value | 0.000 | 0.000 | 0.000 | 0.002 | 0.000 | 0.000 | 0.000 | 0.000 | |

P-value – probability value (P -value ≤ 0.05 is statistically significant); TC – total chlorophylls; Car – carotenoids; ALA – δ -aminolevulinic; P_n – net photosynthesis; F_v/F_m – maximal PSII efficiency; H₂O₂ – hydrogen peroxide; O₂⁻ – superoxide anion radical; MDA – lipid peroxidation; MSI – membrane stability index; LOX – lipoxygenases; MGO – methylglyoxal; Glo-I – glyoxalase I; SOD – superoxide dismutase; CAT – catalase; APX – ascorbate peroxidase; GR – glutathione reductase; AsA – ascorbate; GSH – reduced glutathione; TPh – total phenolic contents; Flav – flavonoids; DPPH* – radical scavenging assay; Pro – proline; GB – glycine betaine; N – nitrogen; K – potassium; Ca – calcium; Na – sodium; Cl – chlorine; K:Na – potassium-to-sodium ratio; Ca:Na – calcium-to-sodium ratio; SL – shoot length; SDW – shoot dry weight

Correlation matrix. There was a highly significant positive association between SDW and each TC, Car, ALA, P_n , F_v/F_m , MSI, AsA, N, K, Ca, K:Na, Ca:Na and SL ($P \leq 0.01$) while significant with GB ($P \leq 0.05$). There were a negative association between SDW and each of H_2O_2 , $O_2^{\cdot-}$, MDA, LOX, MG, GSH, TPh and Na ($P \leq 0.01$) and significant with Glo-I, SOD, CAT ($P \leq 0.05$), while insignificant negative association in a linear way with APX, GR, Flav, DPPH, and Pro (Table 8).

Cluster analysis. In the present work, the tested traits were discriminated into three major clusters, namely, A, B and C. The first main cluster were divided into two sub-clusters. The first subcluster consisted of 13 traits, namely TC, Car, SL, ALA, P_n , F_v/F_m , N, SDW, MSI, K, K:Na, Ca:Na, and Ca, and, while the second subcluster included one traits, i.e., AsA. The second cluster (B) could be divided into two subclusters. The first subcluster consisted of H_2O_2 , MG, $O_2^{\cdot-}$, MDA, Na and LOX, while the second subcluster consisted of one trait, i.e., Cl. The

third main cluster consisted of Glo-I, CAT, GB, SOD, TPh, Flav, Pro, APX, GSH, DPPH and GR (Figure 1).

The response curve of shoot dry weight to different application levels of melatonin. Linear and quadratic responses of soybean SDW to different MLT levels grown under control salt (no salt stress) (A), under 0.01 mmol/L NaCl salt stress (B) and at an average combined salt plus salt-free conditions (C) are shown in Figure 2. Under the salt-free condition, with an MLT level increase of 0.01 mmol/L, the SDW was expected to increase by 2.59 mg/plant. The R^2 value is the regression sum of squares divided by the total sum of squares. This has increased from 30.3% (linear) to 94.4% (quadratic). This shows that 94.4% of the variation in SDW yields is explained by the quadratic regression model. In other words, the quadratic model is a significant better fit than the linear model. As seen in Figure 2A, for the quadratic curve, $SDW = 1.878$ g/plant was the maximum when $X = 0.0591$, so if MLT is applied at a level of 0.0591 mmol/L, the SDW is

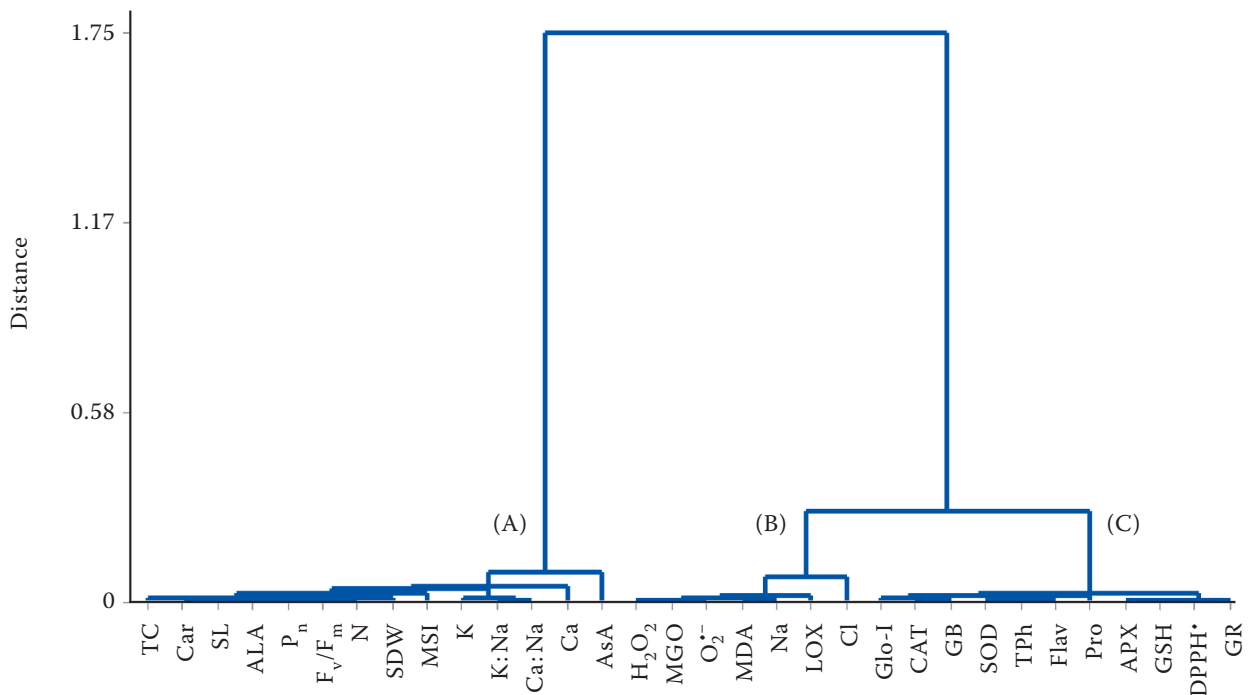


Figure 1. Dendrogram showing the distance among 32 soybean traits based on 8 treatments on soybean plants sprayed with melatonin and grown under salt stress. TC – total chlorophylls; Car – carotenoids; ALA – δ -aminolevulinic; P_n – net photosynthesis; F_v/F_m – maximum quantum efficiency of photosystem II; H_2O_2 – hydrogen peroxide; $O_2^{\cdot-}$ – superoxide anion radical; MDA – lipid peroxidation; MSI – membrane stability index; LOX – lipoxygenases; MGO – methylglyoxal; Glo-I – glyoxalase I; SOD – superoxide dismutase; CAT – catalase; APX – ascorbate peroxidase; GR – glutathione reductase; AsA – ascorbate; GSH – reduced glutathione; TPh – total phenolic contents; Flav – flavonoids; DPPH \cdot – radical scavenging assay; Pro – proline; GB – glycine betaine; N – nitrogen; K – potassium; Ca – calcium; Na – sodium; Cl – chlorine; K:Na – potassium-to-sodium ratio; Ca:Na – calcium-to-sodium ratio; SL – shoot length; SDW – shoot dry weight

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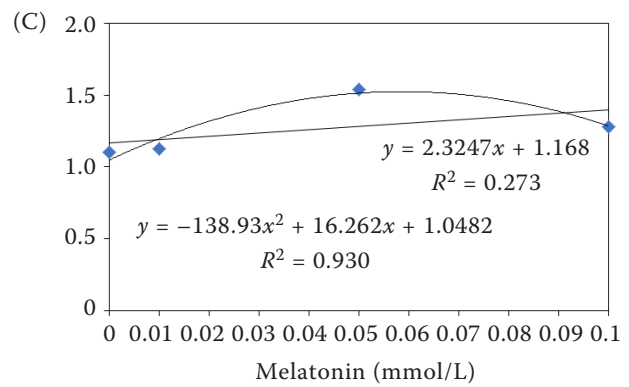
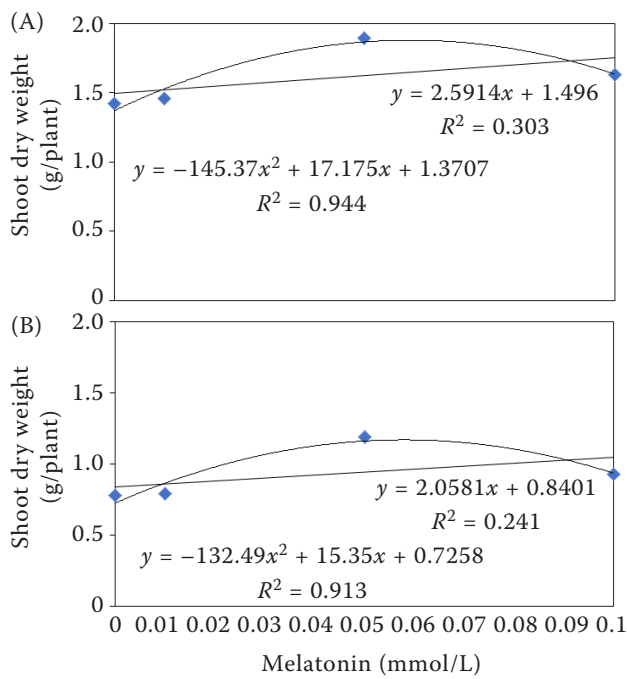


Figure 2. The response curve of shoot dry weight (g/plant) to melatonin in soybean plants grown under (A) control salt (no salt stress), (B) 100 mmol/L NaCl salt stress and (C) at an average combined salt plus salt-free conditions

expected to be about 1.878 g/plant. Under salt level with 100 mmol/L NaCl, with an MLT level increase of 0.01 mmol/L, the SDW was expected to increase by 2.06 mg/plant. This has increased from 24.1% (linear) to 91.3% (quadratic). In other words, the quadratic

model is a significant better fit than the linear model. As seen in Figure 2B, if MLT is applied at a level of 0.0579 mmol/L, the SDW is expected to be 1.170 g/plant. Under average combined salt plus salt-free conditions (C), with an MLT level increase of 0.01 mmol/L, the

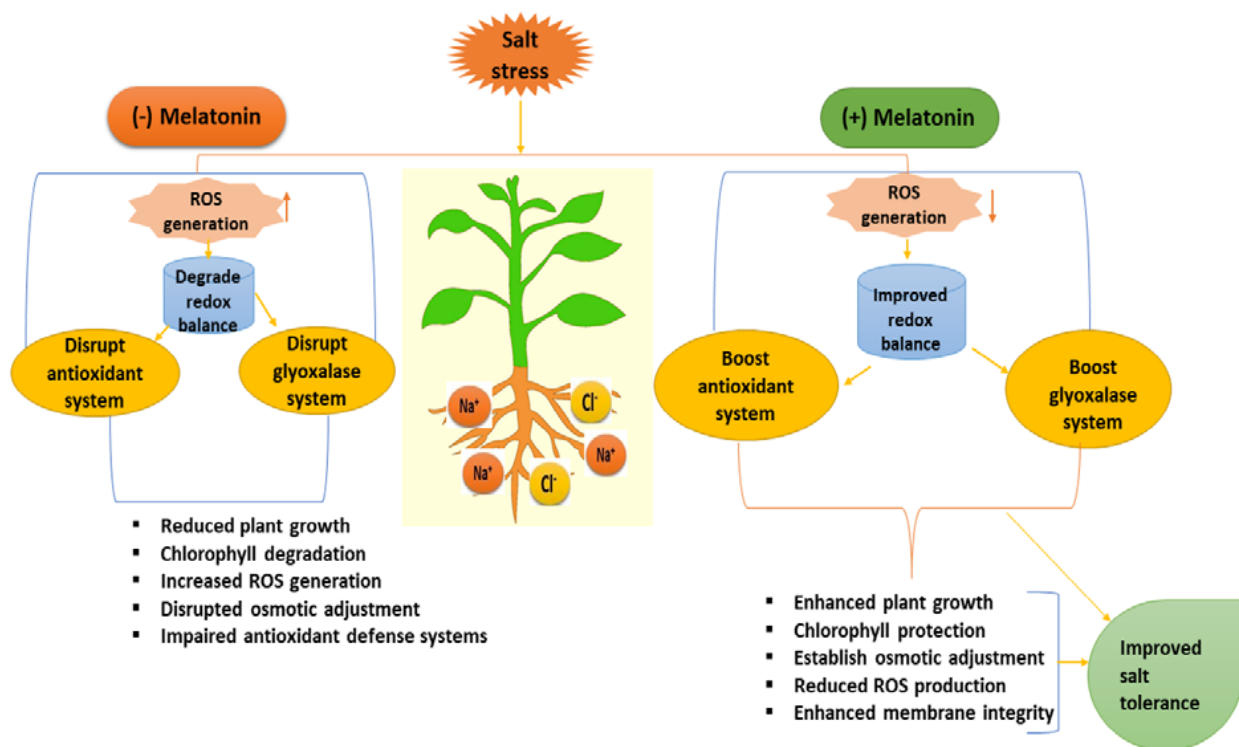


Figure 3. A schematic model figure is showing how melatonin confers salt stress in the soybean plant. ROS – reactive oxygen species

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SDW was expected to increase by 2.32 mg/plant. The R^2 value is the regression sum of squares divided by the total sum of squares. This has increased from 27.3% (linear) to 93.0% (quadratic). This shows that 93.0% of the variation in SDW yields is explained by the quadratic regression model. In the quadratic curve, if MLT is applied at a level of 0.0585 mmol/L, the SDW is expected to be 1.524 g/plant (Figure 2C). Results of the quadratic response of SDW yield to MLT levels of soybean plants grown under control salt (no salt stress) (A) or 100 mmol/L NaCl stress indicate that SDW yield appear to increase up to a maximum and then decrease as more MLT is added (Figure 2).

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