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Melatonin improves tolerance to salt stress and boron toxicity in soybean and pepper plants

Ali Sarioğlu, Cengiz Kaya

Harran University, Department of Soil Science and Plant Nutrition, Sanliurfa, Turkey

Abstract
The role of melatonin (MT) in promoting tolerance to salt stress and boron toxicity in soybean and pepper plants was investigated. The treatments comprised: Control (C), salt stress (SS; 100 mM NaCl), boron toxicity (BT; 2 mM B) and SS + BT. Harmful effects of combined stresses resulted in a greater decrease in plant biomass and chlorophyll contents in both plants compared to those of a single stress. However, SS and BT increased electron leakage, and contents of H_2O_2, proline and MDA, while an increase was observed in the activities of POD, SOD and CAT. Foliar applied MT (100 µM) caused an increase in fresh and dry mass and photosynthetic pigments of plants of both crops under both stresses imposed singly or in combination. However, melatonin reduced electron leakage, and the contents of H_2O_2 and MDA. Proline contents in the pepper plants increased with MT application, while they decreased in the soybean plants. The activities of vital antioxidant enzymes, POD, SOD and CAT were found to be increased with MT application in both plants.

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Introduction

Several agricultural practices are being carried out to increase agricultural production. Therefore, they result in decrease and deterioration of fertile soils due to excessive and irrational use of both chemicals and water. This situation also causes a decrease in product quality. The intensification of these agricultural practices leads to some new problems such as salt stress and boron toxicity (Dogan and Sarıoglu, 2020).

Effects of salts (mono-salts) on plants regarding water and ion uptake, rate of photosynthesis, osmotic effect, and oxidative metabolism, etc. have been extensively investigated (Liu et al., 2014), but the combined effects of salt with other toxic metals/nutrients on plants have not been deeply investigated. Such combined stresses may cause severe effects on plants than caused by either of the stresses applied singly. Salt stress significantly restricts water and nutrient intake by directly affecting both osmotic and ion uptake (Ahmed et al., 2015). Sodium (Na^+) accumulation in excessive amounts in the cytosols of salt stressed plants causes cell membrane injury, thereby causing electrolyte leakage; this leads to reduced CO_2 assimilation and other key metabolic processes (Pandol et al., 2012; Ahmad et al., 2014).

Boron (B) element, one of the nutrients essentially required for plant growth, occurs in excessive amount in dry saline areas (Pardossi et al., 2015). In arid regions, boron available in the root zone and soil profile is limited due to low rainfall (McDonald et al., 2010). As there is a narrow difference between the levels of B deficiency and B toxicity, so a slightly higher B level becomes toxic for most plants (Herrera-Rodríguez et al., 2010). Boron toxicity is known to impede several physio-biochemical processes in plants such as reduced photosynthetic rate and photosynthetic pigments, deterioration of bio-membranes, and triggering of oxidative stress (Li et al., 2017; Liu et al., 2017). It has been widely reported that high levels of B and salt may impede the growth and yield of different crops, e.g., tomato and cucumber (Alpaslan

***CONTACT:** CENGIZ KAYA; c_kaya70@yahoo.com; Harran University, Department of Soil Science and Plant Nutrition, Sanliurfa, Turkey

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and Güneş, 2001), wheat (Grieve and Poss, 2000), and spinach (Güneş et al., 2007a). Moreover, growth of tomato and cucumber was severely affected by salt stress and B high regimes applied in combination compared with that produced under the stresses when applied separately (Alpaslan and Güneş, 2001).

Melatonin (MT) is known to be a naturally synthesized molecule in plants (Martinez et al., 2018). This chemical substance has recently gained a ground, because its application to plants can enable them to thrive well under multiple stresses. For example, Huang et al. (2017) showed that MT application to kiwi fruit assuaged the injurious effects of Cd by upregulating the antioxidant defense system as well as in peach fruit placed in a cold storage (Cao et al., 2016). MT is also believed to play a promising role in the signal mechanisms of abiotically stressed plants (Martinez et al., 2018) as well as it acts as a powerful ROS scavenger (Allegra et al., 2003).

In the current research, the effects of salt stress and boron toxicity were examined singly or jointly on pepper and soybean plants as well as to assess that how far exogenous MT application regulates key physiological processes leading to improved growth in both types of plants under salt and boron stresses.

Materials and Methods

This research experimentation was performed as a pot trial in the glasshouses at the Harran University Soil Science and Plant Nutrition Department. In the study, different applications/treatments in 3 repetitions and their combinations were devised. The applications/treatments included salt treatment (100 mM NaCl), boron as 2 mM, and melatonin as 100 µM. Melatonin application was started after germination and applied 3 times a week, once every two days, and this treatment was completed before the stress applications. The plants in pots were irrigated with Hoagland's nutrient solution, salt and boron. The soybean seed used in the experiment was of cv. GAPSOY 16, obtained from the GAP Agricultural Research Institute. However, the seed of red sweet pepper (*Capsicum annuum* L.) was of cv. Samarkand.

The seeds of both crops were disinfected with NaOCl solution (1%) and planted five seeds in each pot. After germination, the number was reduced to three in each pot. Depending on the size of plants, water containing 100-1000 ml of nutrient solution was applied after a two-day interval to each pot during the experimentation, by maintaining the pH at 5.5 as stated in Kaya and Ashraf (2015).

After the stress effects appeared distinctively on the experimental plants, chlorophyll measurements were recorded with a SPAD device on three leaves with a similar physiological development in each pot before harvesting. Before terminating the experiment, root and shoot samples harvested were brought to the laboratory. After recording fresh mass of all samples, they were subjected to 70 °C in an oven until they reached constant mass and then their dry mass was determined. The key physiological analyses were made in the laboratory.

SPAD values

Chlorophyll measurements of soybean and pepper plants were made with a SPAD device, which enabled us to obtain information about the chlorophyll contents.

Electrolyte leakage (EL)

The protocol specified by Dionisio-Sese and Tobita (1998) was applied to appraise this attribute. A proportion (0.2 g) of fresh leaf was weighed and inserted in a test tube containing distilled water (10 mL). After two h of incubation, electrical conductivity (EC1) was measured. Then the test tubes with samples were autoclaved at 121 ^{o}C for 20 min and EC2 was read when the temperature dropped to 25 ^{o}C. Electrolyte leakage (% EL) was determined as a percentage of the values derived from the formula: (EC1 / EC2x100).

Hydrogen peroxide (H_2O_2)

Determination of H_2O_2 in leaf samples was determined as described in Loreto and Velikova (2001). A proportion (0.5 g) of the fresh leaf sample was weighed, pulverized in a solution containing 3 ml TCA (1%). The resulting homogenate was then properly subjected to centrifugation, and to an aliquot (0.75 ml) of the filtrate, 0.75 ml of 10 mM K buffer and 1.5 ml of 1 M KI were added. Absorbance of all treated samples was recorded at 390 nm.

Leaf malondialdehyde (MDA)

The method of Weisany et al. (2012) was adopted to appraise the levels of MDA in leaf tissues. Each leaf sample (100 mg) was macerated in 0.5 ml of 0.1% trichloroacetic acid. After properly centrifuging the samples, the supernatants were derived by paper filtration. A proportion of the filtrate was treated with 20% TCA, 0.5 ml of 1.5% thiobarbituric acid, and subjected to 95 ^{o}C for 25 min. Thereafter, the OD of the cooled sample mixtures was noted at 532 nm.

Leaf free proline

The method determined by Bates et al. (1973) was used to appraise the proline content in leaf tissues. A fraction (0.5 g) of leaf was weighed, 3% sulfosalicylic acid was added and then homogenized properly. To an aliquot of 2 ml of the filtrate, 2 ml each of ninhydrin solution and glacial acetic acid, were added. After placing the samples for 1 hour at 80 ^{o}C in a hot water bath, they were cooled down and then 4 ml of toluene were added to each sample mixture and read at 520 nm.

Antioxidant enzymes

Each (0.5 g) of fresh leaf samples was extracted in Na-P buffer (50 mM) with 1% soluble polyvinyl pyrrolidone. The mixtures were centrifuged at 20,000x*g* at 4 °C for 15 minutes. The activity of SOD was estimated as depicted by Beauchamp and Fridovich (1971), that of CAT following Kraus and Fletcher (1994), and of POD following Chance and Maehly (1955).

Analysis of data using statistical tools

The data obtained for each variable was subjected to the SPSS 21 software package to work-out ANOVAs. To assess if significant differences exist between treatment means, the Duncan's Multiple Range test at *P* < was employed.

Results

The stress treatments significantly ($P \leq 0.05$) reduced the plant fresh and dry weights of both crops. The application of two stresses had a drastic effect on the biomass of both plants, being more pronounced on the soybean plants (**Figures 1 & 2**). Melatonin (MT) application provided improvement in both plants against stress effects in terms of tissue biomass.

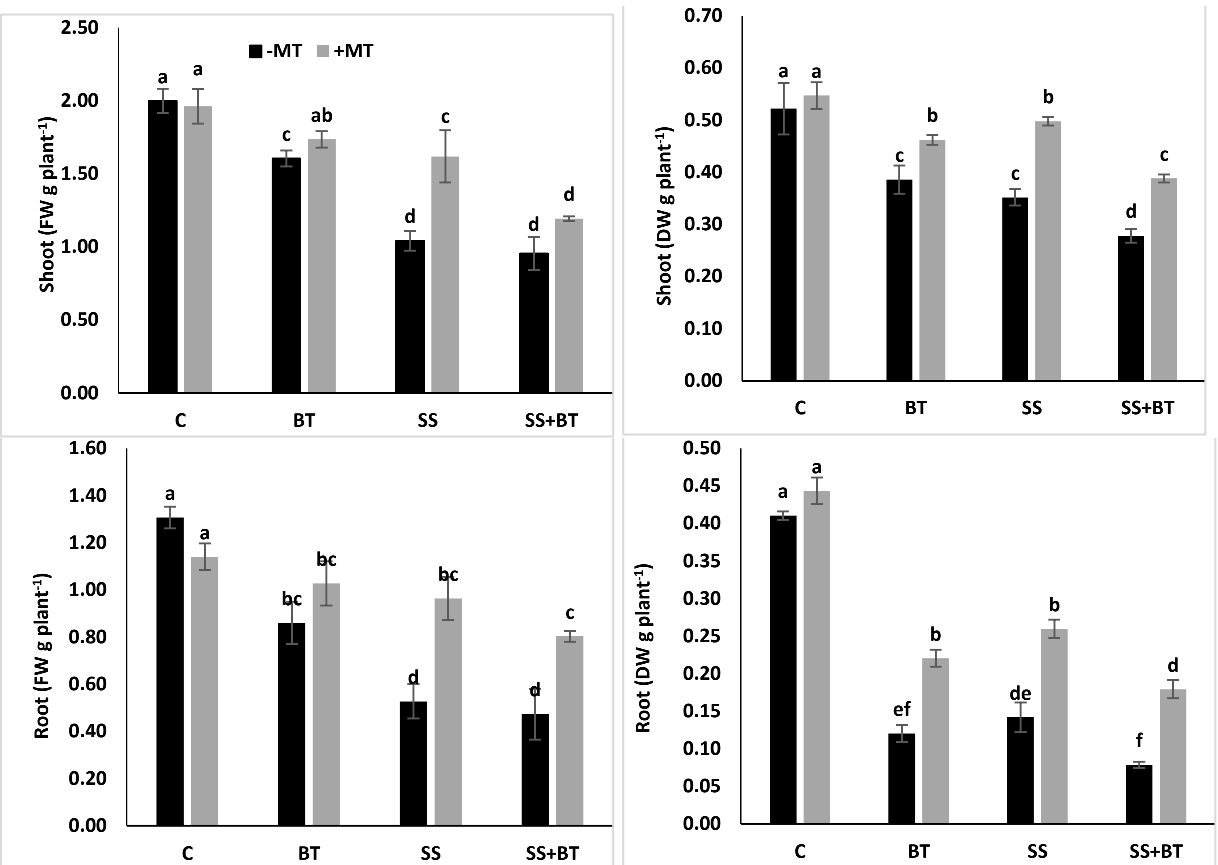

Figure 1: Shoot and root fresh and dry weights of pepper plants sprayed with melatonin (MT) under salt stress (SS), boron toxicity (BT) or a blend of both (SS + BT) (Mean ± S.E.)
Different alphabets on bars exhibit significant difference between means within each variable. C: treatment with nutrient solution alone; MT: foliar application of 0.1 mM melatonin; BT: 2 mM boric acid; SS: 100 mM NaCl

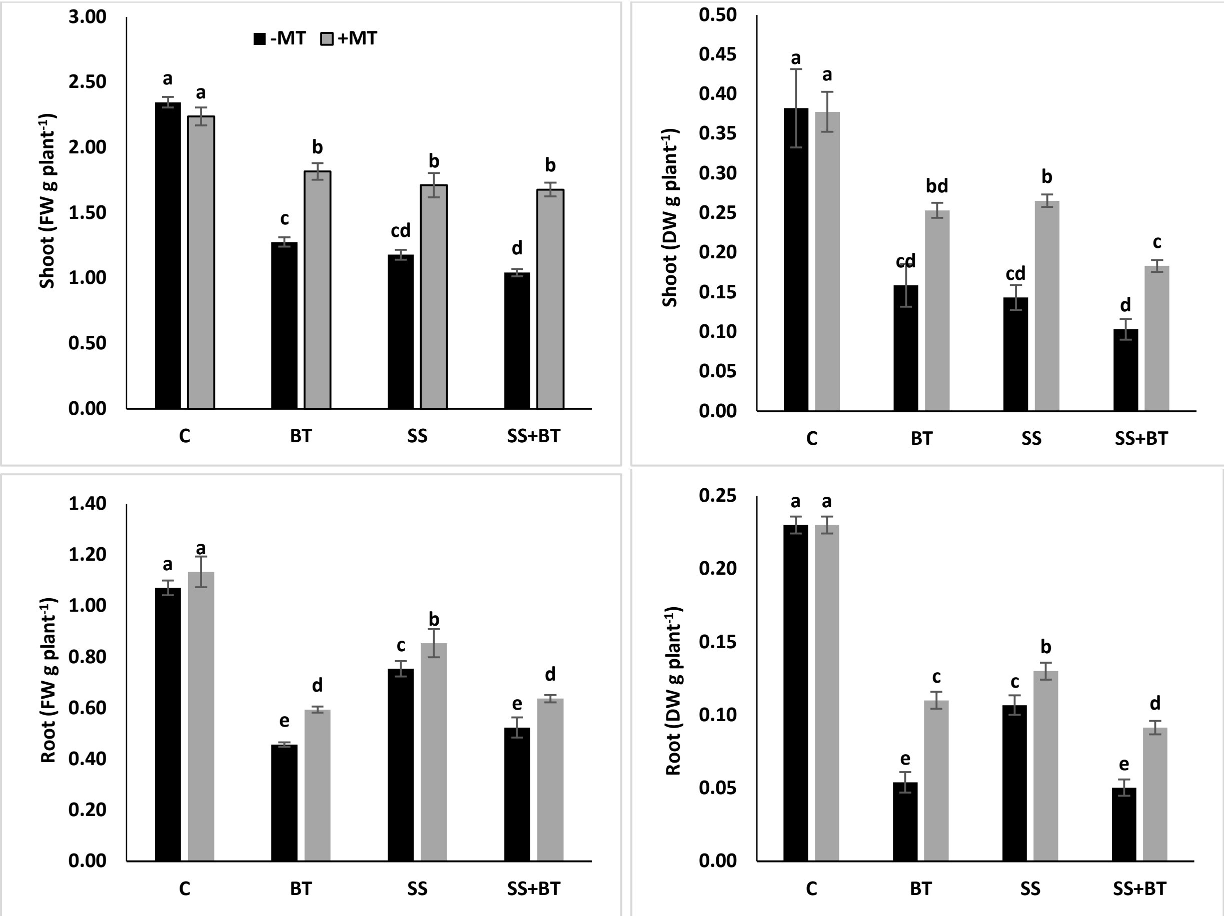

Figure 2: Shoot and root fresh and dry weight of soybean sprayed with melatonin (MT) under salt stress (SS), boron toxicity (BT) or a blend of both (SS + BT) (Mean ± S.E.)

Different alphabets on bars exhibit significant difference between means within each variable.C: treatment with nutrient solution alone; MT: foliar application of 0.1mM melatonin; BT: 2 mM boric acid; SS: 100 mM NaCl

The effects of boron toxicity (BT), salt stress (SS) and a blend of both stresses (SS + BT) on SPAD values of both crops were found to be significant (**Figure 3 A & B**) with respect to controls. As compared to those in pepper plants, the adverse effects of B and salt stresses were more marked in soybean plants in terms of SPAD values. The severe adverse effects on SPAD values were recorded to be due to the combined effect of both stresses. However, MT application mitigated the adverse effects of both stresses applied singly or jointly on the SPAD values of both plants.

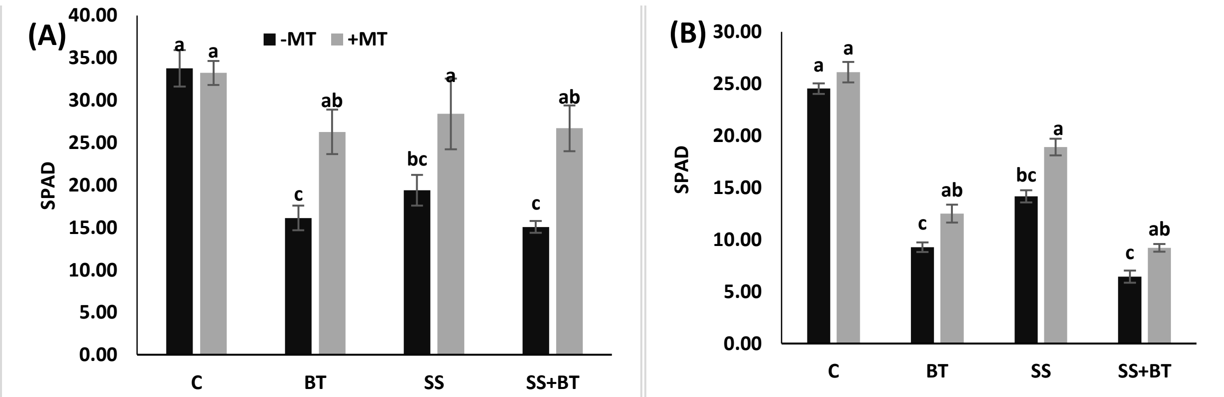

Figure 3: SPAD values of pepper (A) and soybean (B) sprayed with melatonin (MT) under salt stress (SS), boron toxicity (BT) or a blend of both (SS + BT) (Mean ± S.E.)

Different alphabets marked on bars exhibit significant difference between means within each variable. C: treatment with nutrient solution only; MT: foliar application of 0.1 mM melatonin; BT: 2 mM boric acid; SS: 100 mM NaCl

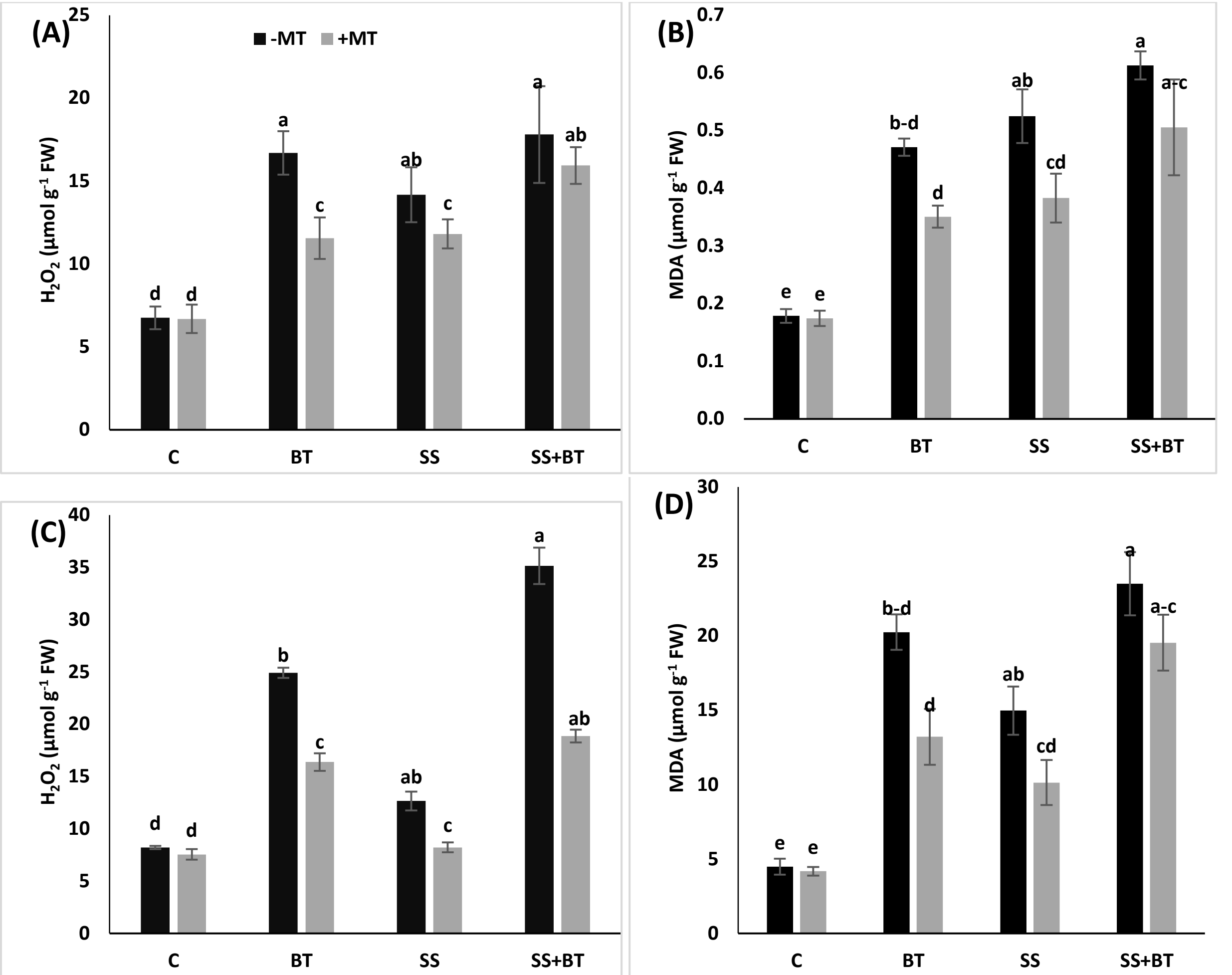

Figure 4: Hydrogen peroxide (H2O2) and malondialdehyde (MDA) of pepper and soybean plants sprayed with melatonin (MT) under salt stress (SS), boron toxicity (BT) or a blend of both (SS + BT) (Mean ± S.E.)

Different alphabets on bars exhibit significant difference between means within each variable. C: treatment with nutrient solution alone; MT: foliar application of 0.1 mM melatonin; BT: 2 mM boric acid; SS: 100 mM NaCl

BT application to pepper plants caused an increase in H_2O_2 levels by 147.3%, SS application by 110.1%, and joint (SS + BT) application by 163.7% compared to the controls. MT application applied to BT-stressed pepper plants decreased H_2O_2 level by 30.8%, MT applied to SS-plants by 16.7% and MT applied to SS + BT applied plants by 10.5%. In soybean plants, H_2O_2 contents (**Figure 4 C**) increased markedly (203.3%) by BT application, and SS application caused an increment of 54.1%. The highest increase was 327.4% by the joint application of both stresses (SS + BT). MT application to BT, SS and SS + BT stressed soybean plants decreased H_2O_2 contents by 34.3%, 35.1% and 46.3%, respectively.

The malondialdehyde (MDA) contents of pepper and soybean plants are presented in **Figure 4 B-D**. Application of BT, SS and two stresses together on pepper plant MDA content increased by 163.8%, 193.9% and 243.4%, respectively. MT application decreased these values in pepper plants by 25.6%, 24.1% and 17.5%, respectively. In soybean, on the other hand, the MDA contents increased by 352.4%, 234.6% and 425%, respectively, under BT, SS, and BT+SS. MT application decreased these values by 35%, 32.3% and 16.9%, respectively, under BT, SS, and BT+SS.

BT application increased the EL value by164.5% in the pepper plants and 117.9% in the soybean plants compared to the respective controls (**Figure 5 A & C**). SS application increased the percent EL value in pepper plants by 202.3% (**Figure 5 A**) and in soybean by 110.4%. Under the combined effect of the two stresses (SS + BT), the EL values increased by 204.7% in the pepper plants and 141.1% in the soybean plants (**Figure 5 C**) compared to the controls, and these values were recorded to be the highest. Melatonin application as BT + MT, TS + MT and TS + BT + MT to pepper plants reduced EL 42.1%, 15.1% and 8.3%, respectively. In soybean plants, these values were found to be reduced to 39.6%, 35.5% and 11.2%, respectively.

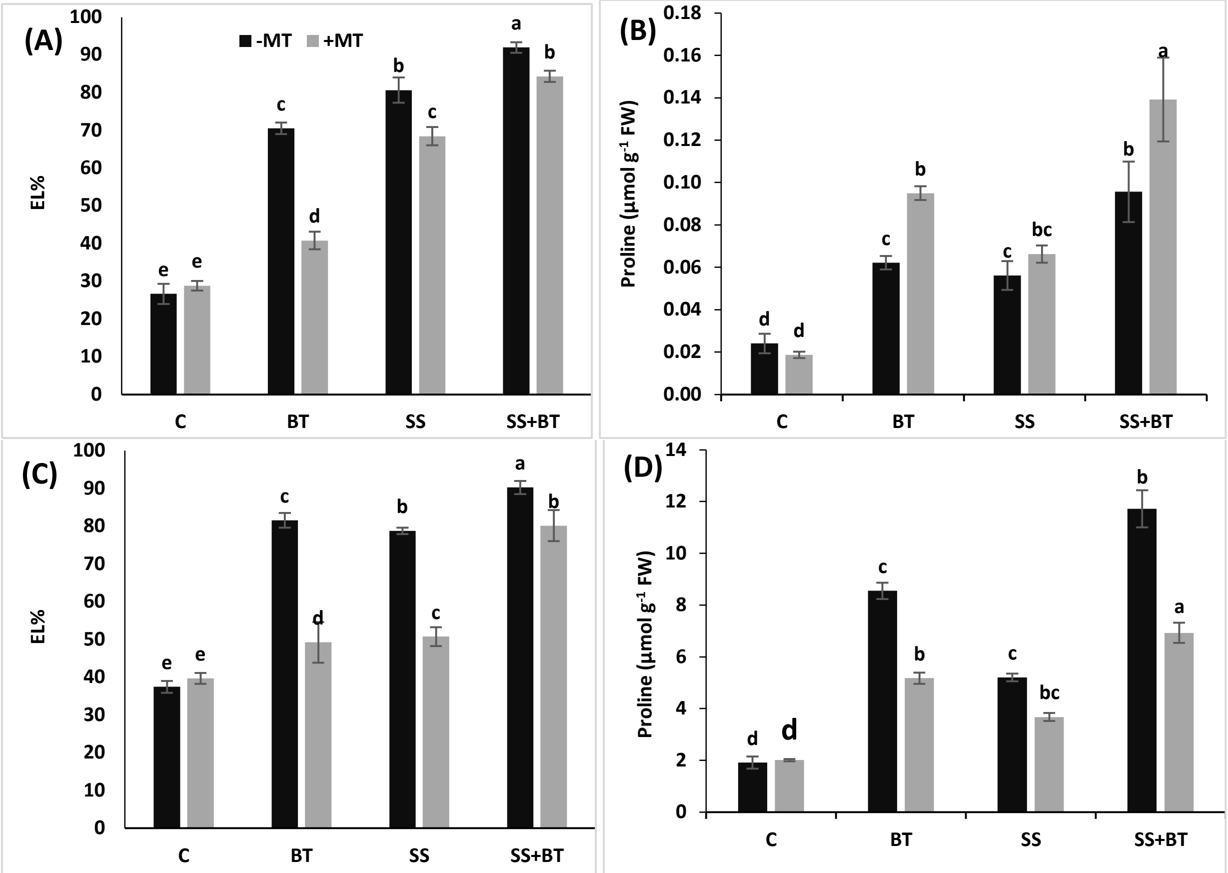

Figure 5: Electrolyte leakage (EL) and proline content of pepper (A, B) and soybean (C, D) plants sprayed with melatonin (MT) under salt stress (SS), boron toxicity (BT) or a blend of both (SS + BT) (Mean ± S.E.)
Different alphabets on bars exhibit significant difference between means within each variable. C: treatment with nutrient solution alone; MT: foliar application of 0.1 mM melatonin; BT: 2 mM boric acid; SS: 100 mM NaCl

In pepper plants, BT, SS and SS+BT increased the plant proline content by 158.3%, 133.4% and 297.7%, respectively (**Figure 5B**). MT application further increased the proline content in the pepper plants by approximately 60%. In soybean plants, BT, SS and SS+BT increased plant proline content by 346.7%, 171.8% and 512.5%, respectively (**Figure 5D**), while MT application reduced the proline content of plants by 55% under the effects of stresses compared with that in the pepper plants.

The BT application increased SOD, POD and CAT activities of pepper plants by 77.35%, 241.8% and 392.5%, respectively, over the controls (**Figure 6 A-C**). SS application increased SOD, POD and CAT activities by 78.8%, 312.2% and 320.3%, respectively. The joint (SS + BT) application significantly increased SOD, POD and CAT activities by 153.7%, by 1330.4% and 1208.34%, respectively. MT application led to further increases in SOD, POD and CAT activities by 15%, 20% and 32%, respectively.

BT application increased SOD, POD and CAT activities in the soybean plants by 262.1%, 254.9% and 85.9%, respectively (**Figure 7 A-C**). SS application increased SOD, POD and CAT activities by 243.8%, 334.4% and 176.5%, respectively. The combined stress (SS + BT) increased SOD, POD and CAT activities by 764.2%, 1627.2% and 752.5%, respectively. MT application led to further increases in SOD, POD and CAT activities by 34%, 62% and 72%, respectively.

Discussion

The premier purpose of this investigation was to find out how melatonin (MT) application to different plants would affect the key plant physiological parameters, despite the harmful effects that may occur in pepper and soybean plants grown under conditions of salt stress (SS), boron toxicity (BT) and a blend of the two stresses. High levels of salt and boron affected plant growth parameters and antioxidant enzymes significantly in the current experiment. However, the joint application of both stresses caused more harmful effects on plant biomass attributes in both crops. On the other hand, MT application on both crops under different stresses has been shown to mitigate the adverse effects (**Figure 1 & 2**). Different studies related to salt stress have shown (Tao et al., 2015, Coskun et al., 2016, Martinez et al., 2018) that externally applied MT to salt stressed tomato plants improved plant biomass. Another study with cucumber showed that MT application under salt stress increased plant biomass (Li et al., 2016).

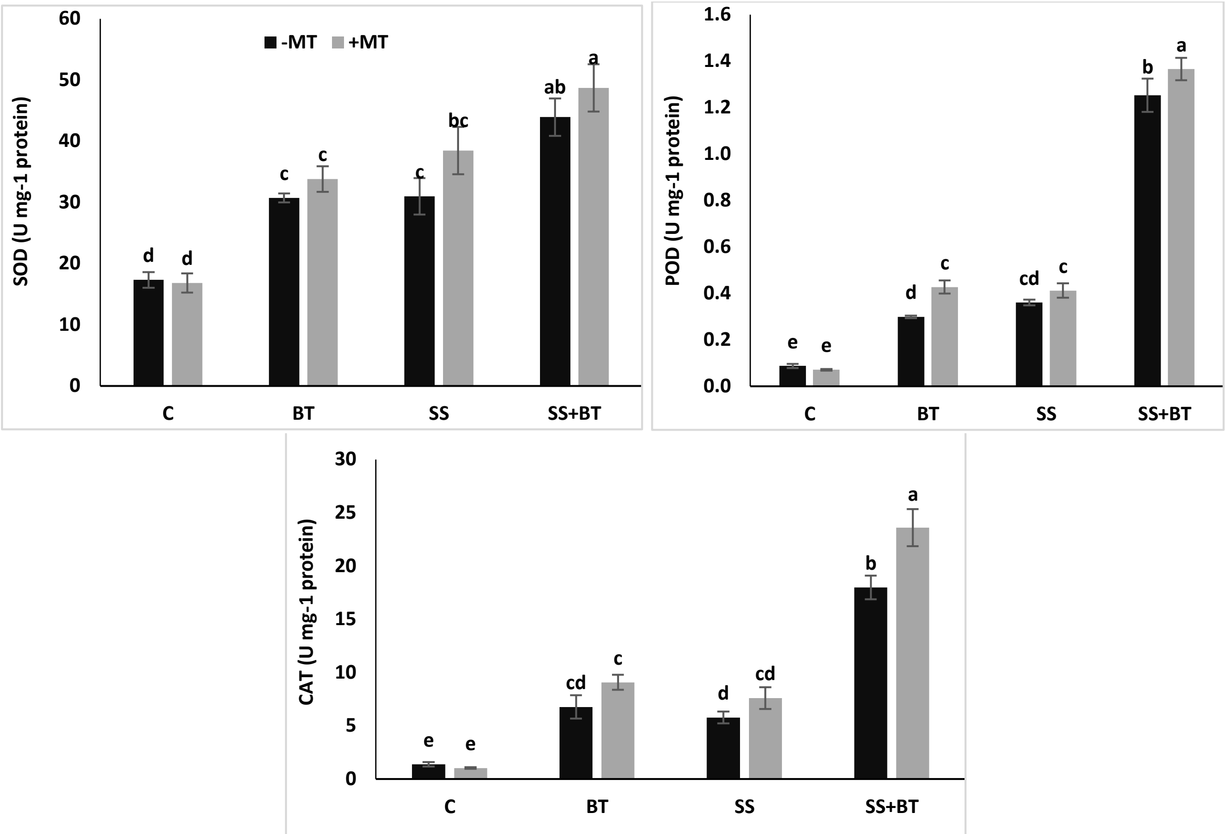

Figure 6: Activities of superoxide dismutase (SOD; A), peroxidase (POD) and catalase (CAT) of pepper plants sprayed with melatonin (MT) under salt stress (SS), boron toxicity (BT) or a blend of both (SS + BT) (Mean ± S.E.)
Different alphabets on bars exhibit significant difference between means within each variable. C: treatment with nutrient solution alone; MT: foliar application of 0.1 mM melatonin; BT: 2 mM boric acid; SS: 100 mM NaCl

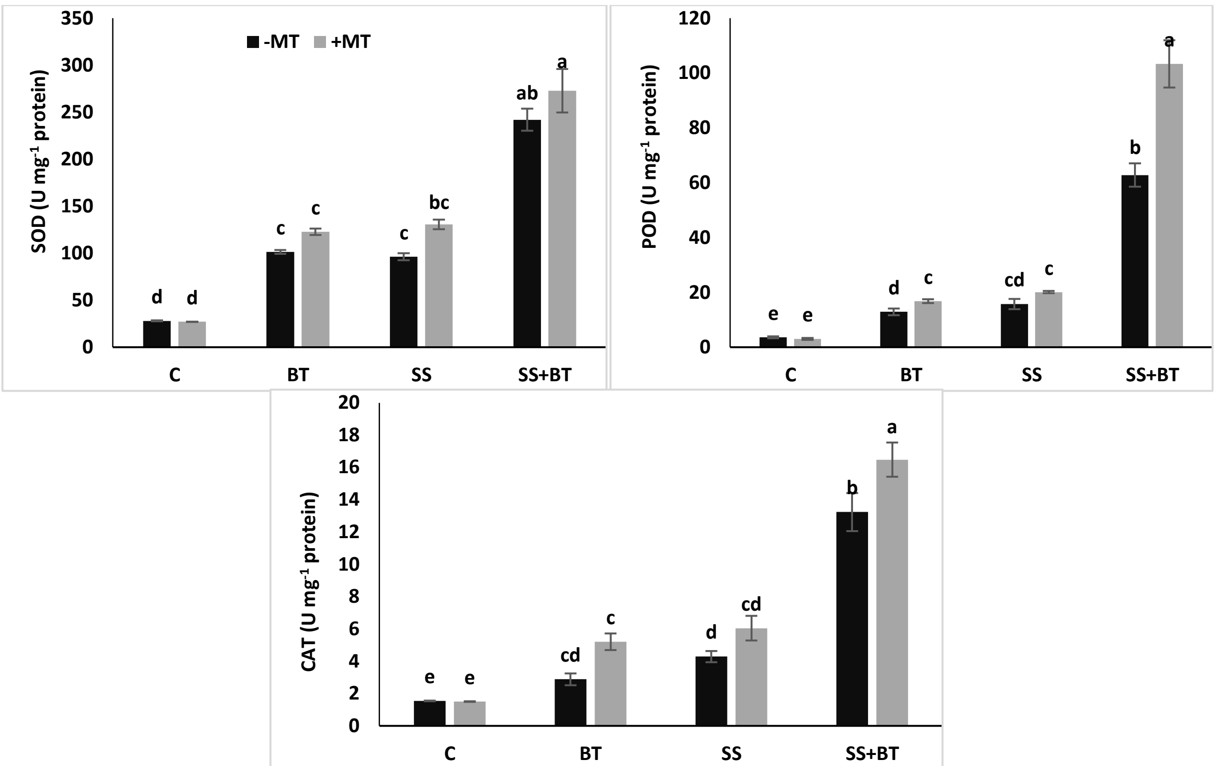

Figure 7: Activities of superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) of soybean plants sprayed with melatonin (MT) under salt stress (SS), boron toxicity (BT) or a blend of both (SS + BT)
(Mean ± S.E.); Different alphabets on bars exhibit significant difference between means within each variable. C: treatment with nutrient solution alone; MT: foliar application of 0.1 mM melatonin; BT: 2 mM boric acid; SS: 100 mM NaCl

Undoubtedly, B is an important element for plants, but it can become injurious to many plants when its level in the growth environment is above the optimum level. It is now widely reported that when plants are grown under conditions where boron is toxic, their growth and yield reduce to a considerable extent (Choi et al., 2007). Kaya et al. (2018) determined in their study that high boron in the growth medium negatively affected dry matter production in corn plants. However, exogenous melatonin application caused an improvement in plant dry matter.

In the current investigation, joint application of B and salt had a drastic effect on the growth of both pepper and soybean plants. It is likely that joint application of the two stresses might have perturbed water uptake in plants as it has been reported that the interaction between salt stress and boron toxicity could influence aquaporin functionality (Bastias et al., 2010; Martinez-Ballesta et al., 2008). However, studies in the literature are not many to explain this interaction.

Externally applied MT is believed to reduce the adverse effects of different individual stresses on plants, e.g., salt stress on wheat (Ke et al., 2018), cucumber (Zhang et al., 2020), grapevines (Xu et al., 2019), soybean (Alharbi et al., 2021), and boron toxicity in wheat (Al-Huqail et al., 2020), pepper (Sarafi et al., 2017), and spinach (Moussa and Algamal, 2017). However, in the literature, there is no study could be searched on the MT effect on plants under conditions where these two stresses coexist.

The SPAD values recorded in the present investigation enabled us to obtain information about chlorophyll content in the pepper and soybean plants (**Figure 3 A-B**). BT and SS stresses significantly decreased the plant chlorophyll content, but the combined effect of the two stresses was found to be the most devastating for chlorophyll content. Externally supplied MT has been reported to improve the chlorophyll content in different plants, so was observed in the current study wherein MT improved chlorophyll content in both crops but the effect of MT was more pronounced on pepper plants. Some earlier reports have exhibited that MT application under the effects of abiotic stresses cause improvement in the levels of key photosynthetic pigments (Wang et al., 2016; Martinez et al., 2018).

Significant increases in ROS levels are believed to occur in plants under stress conditions (Ahmad et al. 2017). In our study, it was determined that boron toxicity and salt stress significantly increased the H_2O_2 and MDA contents in the pepper and soybean plants, but externally applied MT reduced the levels of these ROS (**Figure 4 A-B-C-D**). Eser and Aydemir (2016) have reported similar results of increased levels of H_2O_2 and MDA in wheat plants under B toxicity. The combined application of the two stresses increased maximally the levels of both H_2O_2 and MDA, but MT application had a less effect on reducing the levels of these ROS in plants subjected to the blend of the two stresses than those in plants experiencing these stresses singly. These results cannot be explained as no similar information exists in the literature wherein the effect of MT application on plants exposed to combined application of B and salt has been reported.

Boron toxicity and salt stress induced electrolyte leakage in both plants grown under B, Salt, and B+Salt (**Figure 5 A-C**). This could be due to ROS accumulation caused by boron toxicity and salt stress, which may have caused membrane damage. In our study, combined stresses caused a severe damage to cell membranes as the values of EL were significantly higher than those recorded in both plants under individual stresses. Externally applied MT reduced this damage to a considerable extent. It is believed that MT has an important role as an effective antioxidant in reducing the negative effects of a stress by eliminating ROS formed in plants (Li et al., 2016). In contrast, some studies have shown that MT does not eliminate ROS formed under stress conditions, but it acts as a signal molecule in the synthesis of enzymatic and non-enzymatic antioxidants (Bonnefont-Rousselot et al., 2011, Li et al., 2016). However, this needs to be yet ascertained if MT functions directly as a scavenger of ROS or is involved in the synthesis of antioxidants as a signal molecule.

The plants of pepper and soybean under boron toxicity and salt stress applied singly or jointly showed increased activities of peroxidase (POD), superoxide dismutase (SOD), and catalase (CAT) to overcome the negative effects of stress-induced ROS. However, externally supplied MT further promoted the activities of all these enzymes under stress conditions. For example, Kaya et al. (2018) reported that boron applied at a level of 2 mM significantly increased the activities of enzymatic antioxidants (SOD, POD and CAT) in corn plants. Melatonin is believed to promote the activities of several antioxidant enzymes under stress conditions (Arnao and Hernandez-Ruiz, 2015). For example, Zhao et al. (2011) reported that MDA content was low in the tissues of *Rhamnus crenulata* callus, where MT was applied, as well as the activities of CAT and POD were high. In another similar study, application of 0.1 µM MT to the roots of tomato seedlings grown under drought stress increased the activities of SOD, POD and CAT which resulted in improved drought tolerance, mainly ascribed to reduced membrane damage (Liu et al., 2015).

Both pepper and soybean plants produced enhanced levels of proline under varying stresses (**Figure 5 B-D**). It has been previously reported that proline content generally increases in plants subjected to a stress (Hoque et al., 2007; Heidari and Karami, 2014). In the current study, it was observed that although proline content increased under B and salt stresses applied individually, its levels were maximum in both crop plants under the joint application of both stresses. However, externally applied MT increased proline levels in the pepper plants under stress conditions, while the stress-induced increase in proline content particularly in the soybean plants was decreased with MT application. Nonetheless, increased proline accumulation in plant tissues is a general phenomenon associated with tolerance to stress environments (Ashraf and Foolad, 2007; Bano et al., 2012).

Conclusion

Salt stress, boron toxicity, and especially their combined application significantly reduced plant biomass and photosynthetic pigments in pepper and soybean plants. On the contrary, these stresses increased the electrolyte leakage, and levels of proline, MDA and H_2O_2 as well as the activities of antioxidant enzymes, peroxidase, superoxide dismutase and catalase in both plant types. The effect of joint application of B and salt was found to be more pronounced compared with that of the either stress applied singly on different growth physiological attributes appraised in this study. Foliar-applied melatonin caused a significant increase in plant biomass and chlorophyll content in plants of both crops. In contrast, it reduced electrolyte leakage and the levels of MDA and H_2O_2 in both plants, but it promoted the activities of the afore-mentioned key antioxidant enzymes thereby leading to improved stress tolerance in pepper and soybean plants.

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