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Research article

Alleviation of drought stress damages by melatonin and *Bacillus thuringiensis* associated with adjusting photosynthetic efficiency, antioxidative system, and anatomical structure of *Glycine max* (L.)

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ABSTRACT

These experiments were performed to study the effect of exogenous treatment with melatonin at 100 μM and seed treatment with *Bacillus thuringiensis* (10^{6-8} CFU/cm^3) on growth, physio-biochemical characters, antioxidant enzymes, and anatomical features of soybean plants cv. Giza 111 under drought conditions. The findings showed that leaves number, nodules number, branches number, relative water content (RWC), chlorophyll content, and maximum quantum efficiency of PSII (Fv/Fm) were significantly reduced in soybean under drought stress. In addition, anatomical structure of stems and leaves were negatively affected in stressed plants. Moreover, proline, electrolyte leakage (EL%) lipid peroxidation (MDA), superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and antioxidant enzymes, such as catalase (CAT), superoxide dismutase (SOD), and peroxidase (POD), were significantly increased under drought stress. However, application of melatonin or *Bacillus* caused an improvement in growth characters, such as branches number, and increased chlorophyll *a* and *b* content, RWC as well as Fv/Fm in drought stressed soybean plants. Furthermore, melatonin and *Bacillus* treatments showed a significant decrease in EL%, MDA, $O_2^{\cdot-}$ and H_2O_2, besides regulating the activity of antioxidant enzymes under drought stress. The stems and leaves anatomical structure, such as lamina thickness, lower and upper epidermis thickness, number of xylem vessels/bundle, stem diameter, xylem vessels diameter, and phloem thickness, were improved under drought conditions with melatonin and *Bacillus* treatments. Therefore, the outcomes of this investigation recommended the use of melatonin as foliar spray and *Bacillus thuringiensis* as seed treatment, which could regulate a number of stress-responsive mechanisms to protect the stressed soybean plants, improve their growth under drought stress.

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1. Introduction

Soybean is one of the most significant oilseed crops with a great economic importance. It is a main source of proteins for human food and animal feeding. Soybean plants require a large amount of water for growth and yield production [1]. So, drought is one of the critical abiotic factors that affect soybean and many economic plants [2–4]. Drought is one of the most harmful stresses due to its effects on morphological characters [5,6], chlorophyll concentrations [7], and the activities of antioxidant enzymes [8,9] in many plants. Moreover, drought causes a negative effect on seed germination, development, and yield production of different plants. Under drought stress, the production of ROS, such as O_2^-, hydrogen peroxide, and hydroxyl radicals (OH), was significantly increased [10–12]. This increase in ROS levels can be controlled with the regulation of antioxidant defense system, such as catalase (CAT), peroxidase (POX), ascorbate peroxidase (APOX), proline, and ascorbic acid [13–15]. Malondialdehyde (MDA) and electrolyte leakage (EL%) were dramatically increased in stressed plants, this increase could be attributed to the damages of the organelles under various stresses [16–19]. The harmful effects of drought were recorded on stem height, chlorophyll content, relative water content, hydrogen peroxide, superoxide, electrolyte leakage, and yield in many plants [20–23]. Chlorophyll fluorescence parameters are important and fast indicators to evaluate the efficacy of photosynthetic organs and chlorophyll contents in the plants. They indicate the plant health under various stress factors [24,25]. These parameters, like nonphotochemical quenching (NPQ), electron transport rate (ETR), the optimal/maximal quantum yield (Fv/Fm), and actual photochemical quantum yield (FPSII), are used to identify the harmful effects of drought [26,27]. Moreover, drought stress leads to negative effects on anatomical characters, such as stem diameter, lamina thickness, xylem, phloem tissues, and stomata numbers in several plants [2,28]. Many studies have been conducted to find the sustainable strategies to manage drought stress. The application of PGPR is one of the most effective strategies to improve plant growth under drought conditions [7]. The PGPRs play important roles in improving plant growth and development; increase nutrient availability and uptake, consequently increase the yield under normal and environmental stress conditions [29–31]. Pervious study of Abdelaal et al. [32] showed that application of PGPRs provided the greatest values in stripped stalks, juice, and syrup yields of sweet sorghum plants. The positive effects of PGPRs may be due to their role in enhancing the nodulation, nodule size, nitrogen fixation, and grain yield [33–35]. This role may be associated with the production of phytohormones, antioxidant compounds, and vitamins that improve relative water content and plant growth characters [36]. *Bacillus* is an important plant promoting bacteria. It can promote nutrient cycle, and improve nitrogen fixation, phosphorus solubilization, and phytohormone synthesis. This promoting effect can be enhanced by the activation of enzymatic system, such as catalase, polyphenol oxidase, and superoxide dismutase [5].

Phytohormones play important role in protecting plant cells from negative effects of environmental stresses [37,38]. Melatonin is an important hormone and biological regulator of circadian rhythms, immunological systems, and antioxidative mechanisms in animals [39,40]. Moreover, it is an important phytohormone that modulates the biochemical and physiological processes which regulate plant growth and development [41]. The previous studies found that melatonin plays a significant role in plant protection from many environmental stresses [42]. It has a significant function as antioxidant, so it can alleviate the adverse effects of stress conditions in many plants, such as wheat and maize [43,44]. Under drought, melatonin improves photosynthesis, inhibits chlorophyll deprivation, and improves plant biomass [45,46]. In addition, application of melatonin reduces oxidative damage caused by drought stress in many plants, such as maize [47], alfalfa [48], apple [49], and tobacco plants [50]. Earlier studies have focused on the useful effects of PGPRs and melatonin on plant stress tolerance in many crops, whereas few studies have examined the effect of PGPRs and melatonin on photo-synthetic efficiency, antioxidative system, and anatomical structure of soybean plants under drought conditions. Therefore, the objective of the current study was to explain the tolerance mechanism of soybean plants to drought conditions using *Bacillus thuringiensis* and melatonin to improve the growth characters of soybean associated with morphological, physiological, and anatomical features. This study not only provided valued information for exploring the mechanism of *Bacillus thuringiensis* and melatonin in alleviating the oxidative and osmotic stress, but also laid the foundation for cultivating high yield of stressed soybean plants under drought conditions besides achieving sustainable agriculture.

2. Materials and methods

2.1. Conditions of experiment and treatments

The experiments were performed in the two seasons 2022 and 2023 in the private farm Gharbia governorate, Egypt, to study the

Table 1
The weather conditions of the experimental site.

Months	Temp (°F)		Humidity (%)	Wind	Ligh period (h)	Dark period (h)
	Max	Low				
May	43.29	13.60	51.25	3.16	13.7h	10.3h
June	46.08	18.83	50.81	3.11	14.1h	9.9h
July	41.73	20.55	51.19	2.87	13.9h	10.1h
August	42.75	21.24	54.00	2.77	13.2h	10.8h
September	40.62	19.32	54.62	2.71	13.3h	10.7h
October	40.80	16.40	59.88	2.55	11.4h	12.6h
November	29.33	10.47	60.56	2.20	10.6h	13.4h

effect of melatonin at 100 μM and *Bacillus thuringiensis* (10^{6-8} CFU/cm^3) on morphological, physio-biochemical, and anatomical characters of soybean plants cv. Giza 111. The experiment was arranged in a completely randomized design with four replicates. The seeds of soybean (*Glycine* max L.) Giza 111 were obtained from Agricultural Research station, Sakha, Egypt, and sown on 14th May in the both seasons. Each plot unit was 3.5 m length x 6 m width, and contained 5 ridges. The seeds were sterilized and washed with distilled water. Soybean seeds were treated with *Bacillus thuringiensis* (10^{6-8} CFU/cm^3), and kept at room temperature for 6 h. The seeds were then planted within the row in hills 15 cm apart. The recommended amounts of nitrogen, phosphorus, and potassium (NPK) fertilizers were added as following: phosphorus was added at 55 kg ha^{-1} as superphosphate during soil preparation, and potassium was added at 72 kg ha^{-1} as potassium sulfate after 35 days from sowing, while ammonium nitrate (33.5 % N) was used as a source of nitrogen at the rate of 50 kg N ha^{-1} and added in equal doses. The first dose was applied after 20 days of sowing before the first irrigation. The weather conditions have been presented in Table 1. The drought stressed plants were exogenously sprayed twice with melatonin every 15 days after 30 days from sowing. The experiment includes 4 treatments as follow.

- Irrigation with 100 % from water requirements (Control).
- Irrigation with 75 % from water requirements [Drought (D)].
- Irrigation with 75 % from water requirements + *Bacillus thuringiensis* (Drought + Ba-cillus).
- Irrigation with 75 % from water requirements + melatonin (Drought + melatonin).

2.2. *Morphological characters*

After 60 days from sowing, morphological characters, such as leaves number, branches number, and nodules number per plant, were determined.

2.3. *Physiological characters*

2.3.1. *Determination of chlorophyll a and b*

The extraction from fresh soybean leaves (0.5 g) was done using *N*–N Dimethyl formamide. Afterwards, chlorophyll *a* and *b* concentrations were measured using spectrophotometer at 647 nm and 664 nm [51].

2.3.2. *Relative water content (RWC%)*

The samples of soybean fresh leaves were prepared. Ten leaf discs were taken to determine RWC by using the formula RWC% = (FW − DW)/(TW − DW) × 100 [52], where FW: fresh weight, DW: dry weight, and TW: turgid weight.

2.3.3. *Determination of total phenolics compounds*

Soybean fresh leaves (0.5 g) were homogenized with 80 % acetone according to the method of Julkunen-Tiitto [53]. The total phenols were spectrophotometrically monitored at 765 nm.

2.3.4. *Proline determination*

Leaves sample (0.5 g) was homogenized in 3 % sulfosalicylic acid (10 mL) at 4 °C. The extract was filtered. In a test tube, 2 mL of each filtrate, acid-ninhydrin and glacial acetic, was mixed and incubated at 100 °C for 1 h. The mixture was placed on ice. After this, 4 mL of toluene was used to extract the reaction mixture. Using spectrophotometer, proline was determined at 520 nm in soybean fresh leaves as μg g^{-1} FW [54].

2.3.5. *Electrolyte leakage determination (EL%)*

Ten discs (1 cm^2) of soybean fresh leaves were taken, and EL% was determined by using initial conductivity/final conductivity × 100 [55].

2.3.6. *Determination of lipid peroxidation*

Fresh leaf (0.2 g) was homogenized in 2 mL trichloroacetic acid, and centrifuged for 15 min at 14,000×*g*. 2 mL aliquot was added to 3 mL thiobarbituric acid (0.5 %) and 5 % trichloroacetic acid, and heated in water path for 30 min. The mixture was placed on ice, and centrifuged at 5000×*g* for 15 min. Lipid peroxidation was assayed as malondialdehyde (MDA) at 450 nm, 532 nm, and 600 nm using a spectrophotometer as μmol g^{-1}FW [56].

2.3.7. *Chlorophyll fluorescence parameters*

Chlorophyll fluorescence parameters (Fo, Fm, and Fv) were measured at 60 days from the sowing in the middle-aged soybean leaves in accordance with Schreiber [57], using a chlorophyll fluorometer (PEA, Hansatech Instrument Ltd., version 1.21, Norfolk, UK). The middle-aged soybean leaves (four replicates) were taken to measure fluorescence parameters between 10:00 h and 12:00 h in sunny conditions. The variable fluorescence (Fv) can be measured by using formula (Fv)=(Fm−Fo), where (Fo) indicates minimum chlorophyll fluorescence, and (Fm) indicates maximum fluorescence. The maximum efficiency of PSII was calculated as follow: Fv/Fm = (Fm − Fo)/Fm.

2.3.8. *Enzymes activity*

Soybean leaves were used for enzymes activity. The activity of catalase (CAT) was measured using spectrophotometer at 240 nm based on the rate of H_2O_2 consumption as μmol min^{-1} mg protein^{-1} in accordance with Havier and McHale [58]. Activity of superoxide dismutase (SOD) was measured at 560 nm as μmol min^{-1} mg protein^{-1} using method of Giannopo-litis and Ries [59]. Peroxidase (POD) activity was measured according to the method of Chance and Maehly [60], using a spectrophotometer at 470 nm for 2 min at an interval of 20s. Peroxidase (POD) activity was expressed according to the following equation as μmol min^{-1} mg protein^{-1}. The activity $= (\textit{first reading} - \textit{last reading}\,/\textit{time})$X 5.19.

2.3.9. *Detection of reactive oxygen species ($O_2\cdot^-$ and H_2O_2)*

Soybean fresh leaves were vacuum infiltrated with 10 mM potassium phosphate buffer (pH 7.8) containing 0.1 w/v % NBT according to Ádám et al. [61] or 0.1 w/v % DAB (Fluka, Buchs, Switzerland). NBT- and DAB-treated samples were incubated under daylight for 20 min and 2 h respectively, and subsequently cleared in 0.15 w/v % trichloroacetic acid in ethanol: chloroform 4:1 v/v for 1 day [62]. The discoloration of leaf discs was quantified and presented as μmol g^{-1} FW using a ChemiImager 4000 digital imaging system (GE Healthcare Bio-Sciences AB Björkgatan,751 84 Uppsala, Sweden).

2.4. *Anatomical characters*

For anatomical investigation, the samples (0.5 cm) of third internode and leaf (terminal leaflet) from soybean plants were taken in the second season, and saved in Formalin Acetic Alcohol (FAA) for killing and fixation. The samples were impeded in paraffin wax. The cross sections were done with rotary microtome at 10–15 μm and fixed on glass slides through Haupt's adhesive. They were saved in the refrigerator for 12 h, and kept to dry for 12h. The slides were dipped in xylene to remove the wax, and the double staining with Safranin- Fast green was done. The sections were cleared by xylene, and were mounted in Canada balsam. Digital images of leaves and stems were recorded using a photomicroscope with a digital camera, and the following characters were recorded: stem diameter, xylem vessel diameter, phloem thickness, leaf lamina thickness, and number of vessels [63–65].

2.5. *Statistical analysis*

Statistical analysis was done using one-way Anova (ANOVA) procedures [66], using the MSTAT-C statistical software package, and the means between treatments were compared by Duncan [67] when the difference was significant ($p \leq 0.05$).

3. Results

3.1. *Morphological characters*

Based on the results in Fig. 1, drought stress (D) (70 % of water requirements) significantly decreased leaves number (34.3 % and

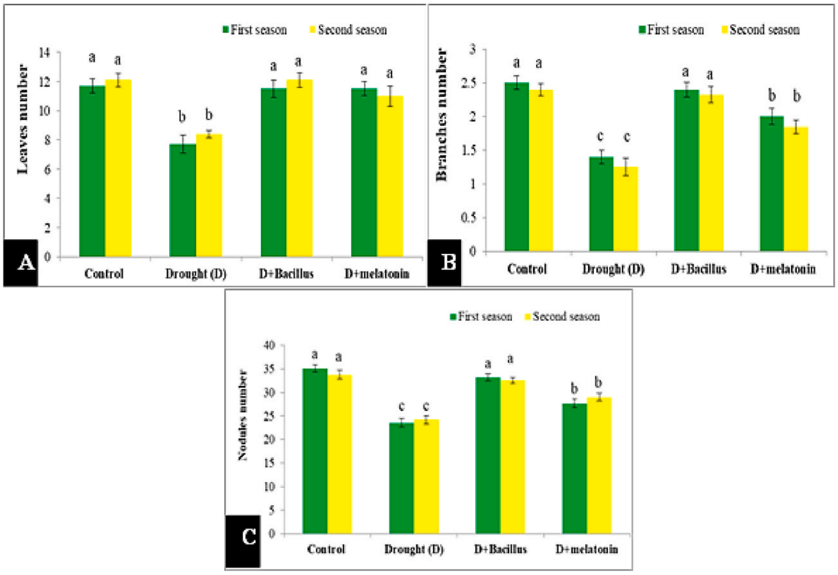

Fig. 1. Effect of melatonin and *Bacillus* thuringiensis on number of leaves (A), branches number (B), and nodules number (C) in soybean plants under drought stress during 2022 and 2023 seasons. The value within the same column with the different letter is significantly different at 5 % probability level by DMRT.

31.7 %), branches number (44.1 % and 48.2 %), and nodules number (37.4 % and 31.6 %) per soybean plant in both seasons respectively. However, leaves number, branches number, and nodules number in treated soybean plants with melatonin and *Bacillus thuringiensis* under drought conditions displayed a significant increase as compared to the untreated soybean plants (D). Overall, treatments of 100 μM melatonin (exogenously) and *Bacillus* (seed treatment) significantly increased leaves number (Fig. 1A), branches number (Fig. 1B), and nodules number (Fig. 1C) per plant in the stressed soybean plants as compared to stressed plants without treatments. The best results in leaves number (51 % and 48.4 %), branches number (74.5 % and 81.3 %), and nodules number (62 % and 53.6 %) were recorded with *Bacillus* treatment as compared to control treatments in the two seasons respectively.

3.2. Physiological characters

3.2.1. Chlorophyll contents and chlorophyll fluorescence parameters

The results in Fig. 2 exhibited a significant decrease in chlorophyll *a* and chlorophyll *b* concentrations in stressed soybean plants (D) in both seasons as compared to control treatment. However, exogenous application of melatonin and *Bacillus* as seed treatment significantly increased chlorophyll *a* concentration (43.47 % and 37.5 %) and chlorophyll *b* concentration (31.03 % and 31.85 %) in droughted soybean plants as compared to stressed plants without treatments (D) during the two seasons respectively (Fig. 2A and B). Under drought conditions, chlorophyll fluorescence parameters (Fig. 2C–E) were negatively affected. The minimum fluorescence (Fo) was increased significantly, while the maximam fluorescence (Fm) was decreased significantly in the stressed soybean plants as a negative indicator to drought stress. Moreover, the maximum quantum efficiency of PSII (Fv/Fm) significantly decreased in the stressed soybean as compared to the control treatment (6 % and 7.3 %) in the both seasons respectively. On the other hand, the stressed soybean plants treated with melatonin and *Bacillus* exhibited a significant increase in Fv/Fm ratio (5.1 % and 6.5 % with melatonin treatment and 2.5 % and 3.9 % with *Bacillus* treatment) during the two seasons as compared to the stressed untreated plants. Furthermore, *Bacillus* treatment was the superior, and provided the best results of chl a, chl b, Fo, Fm, and Fv/Fm in soybean plants under drought conditions as compared to the stressed plants without treatments.

3.2.2. Total soluble sugars, total phenolic compounds, relative water content (RWC%), and proline content

The results in Fig. 3 exhibited that total soluble sugars (Fig. 3A) and proline content (Fig. 3D) significantly augmented in the stressed plants under drought conditions as compared to control treatment in both seasons. However, drought stress led to significant decreases in total phenolic compounds and RWC% (44.8 % and 21.43 %) in the first season and (37.25 % and 21 %) in the second

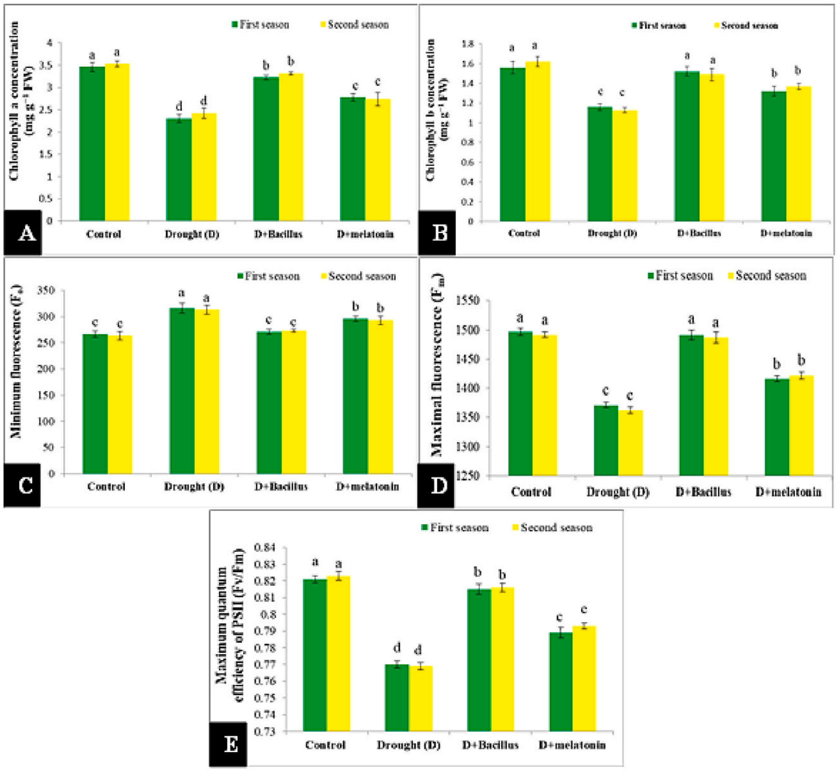

Fig. 2. Effect of melatonin and *Bacillus thuringiensis* on chlorophyll *a* (A), chlorophyll *b* (B), minimum fluorescence (Fo) (C), maximam fluorescence (Fm) (D), and maximum quantum efficiency of PSII (Fv/Fm) (E) in soybean plants under drought stress during 2022 and 2023 seasons. The value within the same column with the different letter is significantly different at 5 % probability level by DMRT.

season in the stressed soybean plants. Application of melatonin and *Bacillus* caused a significant decrease in total soluble sugars and proline in the stressed plants during both seasons. Additionally, total phenolic compounds and RWC% were increased significantly (69.4 % and 28.9 %) in the first season and (32.8 % and 30.9 %) in the second season in the stressed plants treated with *Bacillus* in both seasons as compared to untreated stressed plants (Fig. 3B–C). However, application of melatonin caused a significant increase in total phenolic compounds (26.27 % and 20.3 %) and RWC% (29.8 % and 21 %) in the stressed plants during the two seasons.

3.2.3. Electrolyte leakage (EL%), lipid peroxidation (MDA), hydrogen peroxide (H_2O_2), and superoxide ($O_2{\cdot}^-$) levels

Drought stress induced a significant increase in EL% (57.14 % and 53.96 %) and MDA (54.39 % and 60 %) as an indicator to oxidative damage in the stressed soybean plants compared with control treatment during the both seasons (Fig. 4A–B). In contrast, the treatment with melatonin significantly reduced EL% (17.5 % and 23.15 %) and MDA content (23.07 % and 27 %) in the stressed plants as compared to the stressed plants without treatments under drought conditions in the two seasons. Moreover, application with *Bacillus* significantly decreased EL% (34.34 % and 33.11 %) and MDA content (24.67 % and 28.9 %) in the stressed plants. Furthermore, reactive oxygen species, mainly hydrogen peroxide (H_2O_2) and superoxide ($O_2{\cdot}^-$), were significantly increased in the stressed plants under drought conditions (78.2 % and 76.83 %) in the first season and (64.8 % and 48.79 %) in the second season (Fig. 4C–D).

Contrariwise, exogenous application of melatonin and *Bacillus* as seed treatment revealed positive effects on H_2O_2 and $O_2{\cdot}^-$ and significantly reduced their levels in soybean plants under drought conditions compared with the stressed untreated plants. In this context, the best treatment which caused significant decreases in EL% (34.3 % and 33.11 %), MDA (24.67 % and 27.34), H_2O_2 (26.67 % and 26.86 %), and $O_2{\cdot}^-$ (40.12 % and 38.14 %) in the stressed plants in both seasons was *Bacillus* followed by melatonin treatment.

3.2.4. Activity of catalase (CAT), superoxide dismutase (SOD), and peroxidase (POD)

The activity of antioxidant enzymes, particularly catalase (CAT), superoxide dismutase (SOD), and peroxidase (POD) as main important enzymes, was significantly increased under drought stress in the stressed plants compared with control treatment in the two seasons (Fig. 5A–C). The increase in CAT was 40.57 % and 52.02 %, however the increase in SOD was 71.5 % and 72.4 %, and the increase in POD was 46.24 % and 50.9 %). Conversely, the application of melatonin and *Bacillus* significantly reduced the activity of catalase, superoxide dismutase, and peroxidase in the stressed plants as compared to the untreated plants under drought stress during two seasons.

3.3. Anatomical characters

The results in Figs. 6 and 7 showed that the anatomical characters of soybean leaves and stems were adversely affected under drought compared with control treatment. A significant reduction was recorded in the anatomical stem characters such as stem diameter, xylem vessels diameter, epidermis thickness, phloem thickness, and number of xylem vessels/vascular bundle in the stressed soybean plants (Fig. 6B) compared with control plants (Fig. 6A). Nevertheless, the applications of melatonin and *Bacillus thuringiensis* had a positive impact, and led to improvement in the anatomical features of stem such as stem diameter, xylem vessels diameter, and number of vessels in stressed soybean plants under drought conditions (Fig. 6C–D). Regarding the anatomical charters of soybean leaf (Fig. 7), the significant decrease in lamina thickness, epidermis thickness, and number of xylem vessels/bundle was observed in

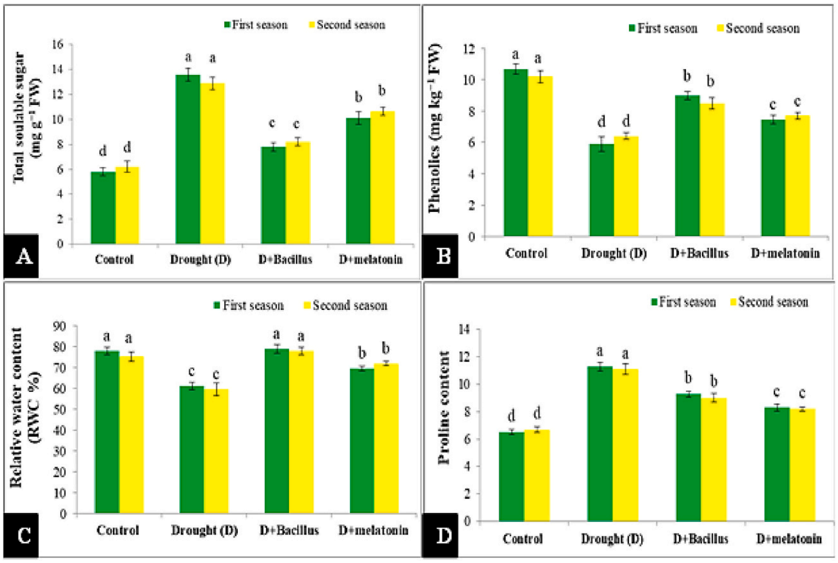

Fig. 3. Effect of melatonin and *Bacillus thuringiensis* on total soluble sugars (A), total phenolics compound (B), relative water content (C), and proline content (D) in soybean plants under drought stress during 2022 and 2023 seasons. The value within the same column with the different letter is significantly different at 5 % probability level by DMRT.

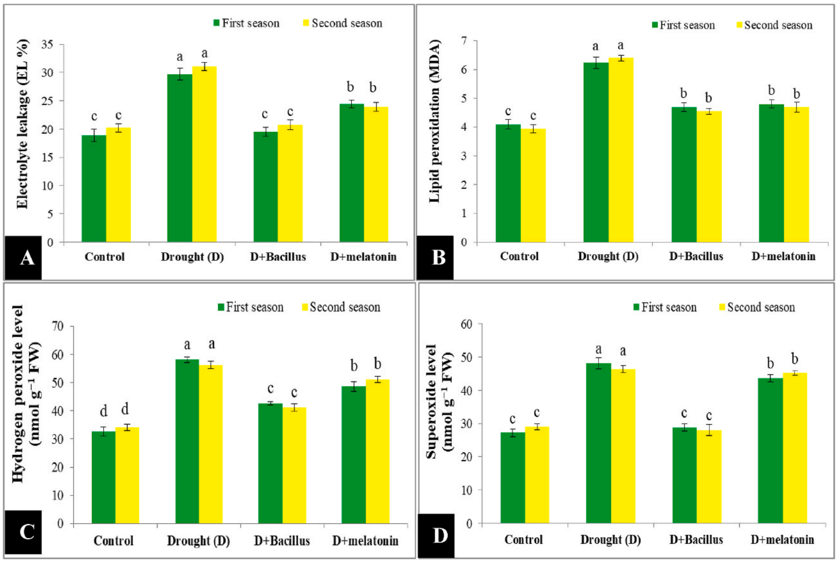

Fig. 4. Effect of melatonin and *Bacillus thuringiensis* on electrolyte leakage (A), lipid peroxidation (MDA) (B), hydrogen peroxide (C), and superoxide level (D) in soybean plants under drought stress during 2022 and 2023 seasons. The value within the same column with the different letter is significantly different at 5 % probability level by DMRT.

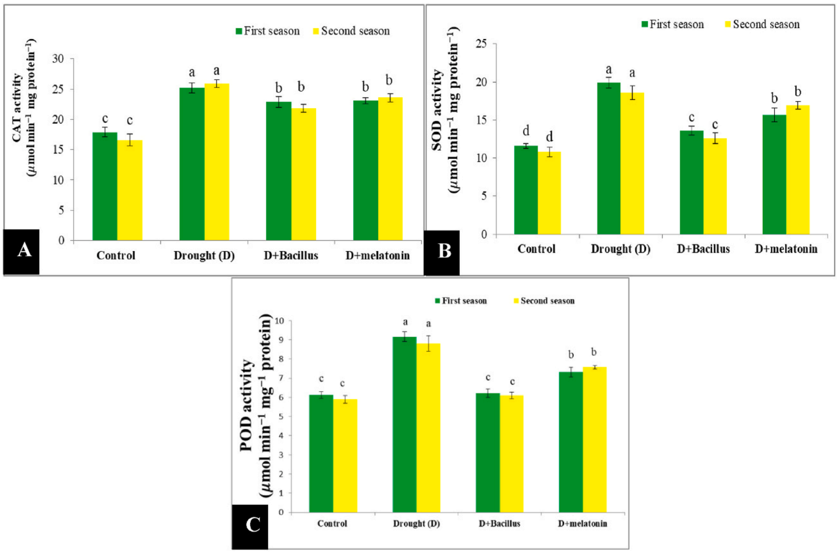

Fig. 5. Effect of melatonin and *Bacillus thuringiensis* on CAT (A), SOD (B), and POD activities (C) in soybean plants under drought stress during 2022 and 2023 seasons. The value within the same column with the different letter is significantly different at 5 % probability level by DMRT.

stressed plants (Fig. 7B) compared with control treatment (Fig. 7A). On the other hand, the anatomical structure of aforementioned characters was improved with application of melatonin and *Bacillus* in the drought stressed soybean plants (Fig. 7C–D).

E: Epidermis Col: Collenchyma VB: Vascular bundle PhT: Phloem tissue XT: Xylem tissue P: Pith.

UE: Upper epidermis PT: Palisade ST: Spongy tissue PhT: Phloem XT: Xylem tissue LE: Lower epidermis.

4. Discussion

Soybean is a drought sensitive crop. It can be seriously affected by drought stress. In this context, the results in Fig. 1 revealed that the decrease in morphological characters such as leaves number, branches number, and nodules number/plant in the stressed soybean plants was pronounced in the two seasons. This reduction may be due to the harmful effects of drought on water and nutrients uptake, and might reduce the cells division and expansion [68], resulting in decrease in the morphological characters like leaves number,

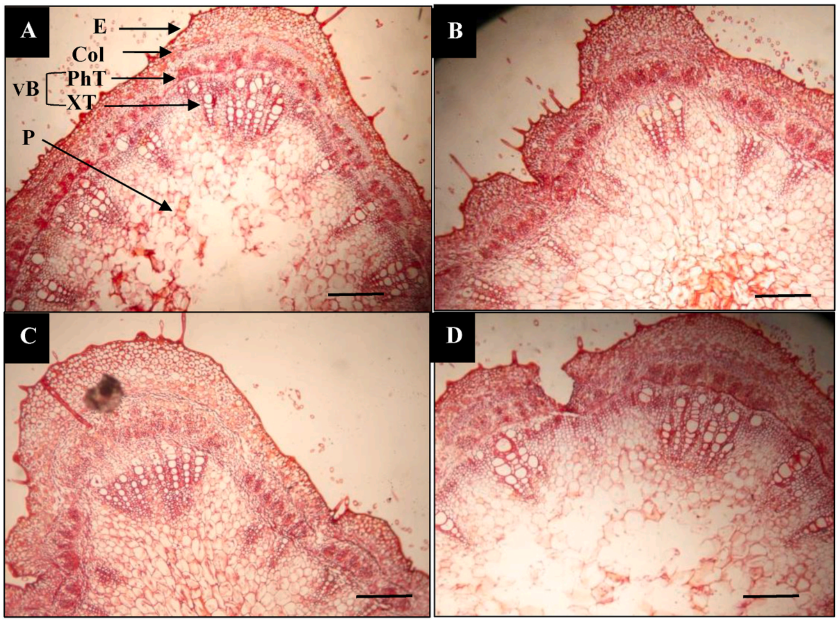

Fig. 6. Effect of melatonin and *Bacillus thuringiensis* on anatomical characters of soybean stems under drought stress conditions. A. Control B. Drought (D) C. D + *Bacillus* D. D + Melatonin.

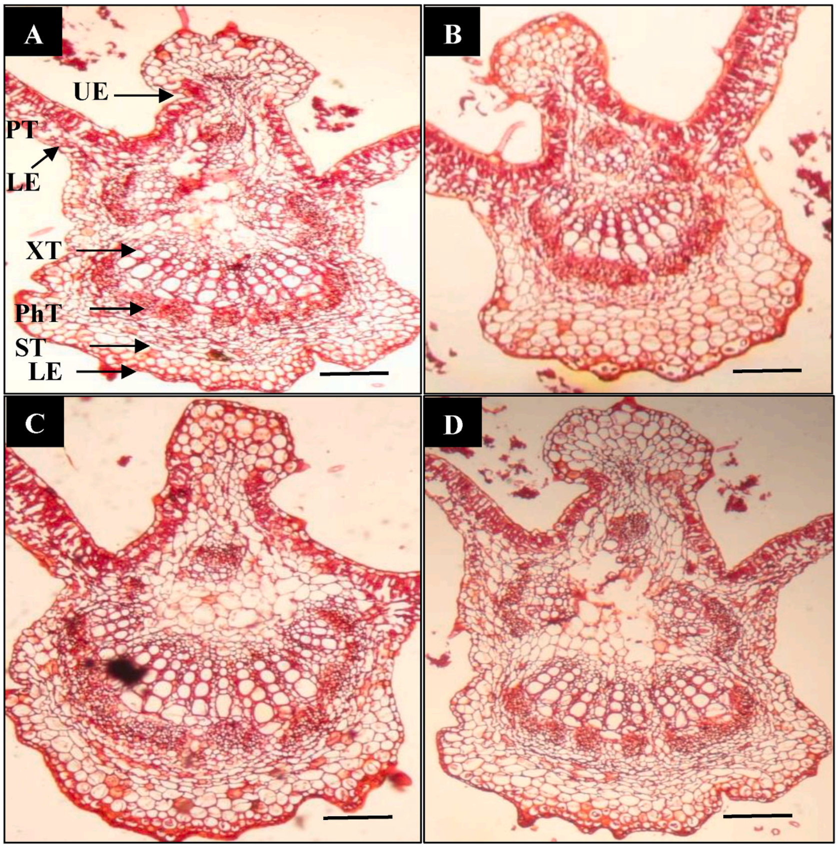

Fig. 7. Effect of melatonin and *Bacillus thuringiensis* on anatomical characters of soybean leaves under drought stress conditions. A. Control B. Drought (D) C. D + *Bacillus* D. D + Melatonin.

branches number, and nodules number. Under drought conditions, low availability of soil water directly hampers plant morphology. The negative effects of drought on morphological characters were recorded in several crops such as maize [3], soybean plants [8], barley [28], faba bean [32], and wheat [69]. The previous studies displayed that melatonin can improve morphological characters of several crops [70–72]. In addition, application of *Bacillus* as seed treatment led to a significant increase in leaves number, branches number, and nodules number/plant in stressed plants. This useful impact of *Bacillus* may be due to its role in improving soil hydrological parameters, such as soil structure, water holding capacity, and infiltration, which help roots to penetrate the soil, obtain more water, and increase the availability of water and nutrients, consequently increasing the morphological parameters. The mode of action of Bacillus in improving drought tolerance could be attributed to the increase in nitrogen content, which acts as an important nutrient. It also plays an important role in regulating hydraulic under drought stress [5,73,74]. Moreover, *Bacillus* is able to solubilize P and K+ under stress conditions, resulting in improving the nutritional status of plants. Additionally, PGPB plays a significant role in improving drought tolerance by releasing some phytohormones, like auxins and gibberellins, and enzymes like ACC-deaminase, which are important for increasing plant growth during many growth processes. This pivotal role of *Bacillus* would translate into improved nodules formation and plant nutrition of the stressed soybean plants [75].

In the current research, the findings in Fig. 2 exhibited that chl a and chl b concentrations significantly decreased in drought stressed soybean plants compared with control treatment. In addition, minimum fluorescence (Fo), maximam fluorescence (Fm), and maximum quantum efficiency of PSII (Fv/Fm) were negatively affected due to drought stress. This adverse effect on chl a, chl b, Fo, Fm, and Fv/Fm could be due to the oxidative stress and excessive formation of ROS, which increase chlorophyllase enzyme activity damaging the thylakoid membranes, consequently increasing the chlorophyll degradation. Moreover, drought may cause inhibition to electron transport chin at the PSII sites, consequently reducing chl a and chl b content as well as the maximum quantum efficiency of PSII (Fv/Fm). The negative effect on chlorophyll content and chlorophyll fluorescence parameters was recorded in some crops under various stresses [24,76–78]. Chlorophyll content and chlorophyll fluorescence parameters in stressed soybean plants were improved significantly with melatonin and *Bacillus* treatments under drought conditions. These results could be attributed to the important role of melatonin as growth regulator and *Bacillus* as a source for promoting substances, which increased chlorophyll concentration and the maximum quantum efficiency of PSII (Fv/Fm). The results of this study are in agreement with the results of some researchers, who found that melatonin significantly improved the Fv/Fm and enhanced photosynthetic efficiency under stress conditions [79,80].

In response to drought stress, total soluble sugars and proline content were significantly increased in stressed soybean plants, however total phenolic compounds and relative water content were significantly decreased (Fig. 3). The increase in proline and total soluble sugars may be due to the fact that drought stress induces proline production and total soluble sugars formation. Some researchers showed that proline content was higher in numerous stressed plants than in control plants [4,7,18]. In this context, Mohammad-khani and Heidari [81] found that proline in drought stressed corn plants was 1.56–3.13 times higher than that in control plants. These results may be due to the fact that proline and total soluble sugars are important osmoregulators and essential metabolites, which play pivotal roles in protecting cell membrane, and keep plant metabolism active under various stress conditions [82,83]. This study recorded a significant decrease in the total phenolics compounds and relative water content in the stressed plants. This reduction may be due to the negative effect of drought on cell membrane stability, leading to additional cell wall damage and decrease in RWC% [84]. In summary, melatonin and *Bacillus* treatments help stressed soybean plants to overcome the damaging effect of drought, improve RWC% and total phenolics compounds, and regulate total soluble sugars as well as proline content.

It is well established that EL%, lipid peroxidation (MDA), H_2O_2, and $O_2\cdot^-$ levels as stress signals were significantly augmented in stressed plants. The results of this study in Fig. 4 showed a significant increase in EL%, MDA, H_2O_2, and $O_2\cdot^-$ levels in the stressed soybean plants under drought stress. This result might be due to the fact that drought stress causes oxidative stress and lipid peroxidation for plant organelles, mainly mitochondria, plasma membrane, and chloroplast, resulting in an increase in EL%, MDA, H_2O_2, and $O_2\cdot^-$ levels. Numerous studies indicated that EL%, MDA, H_2O_2, and $O_2\cdot^-$ levels were significantly elevated under several stresses [85–89]. Conversely, application of *Bacillus* and melatonin led to decrease in EL%, MDA, H_2O_2, and $O_2\cdot^-$ levels in the stressed plants. This result may be due to the fact that melatonin and *Bacillus* led to accumulate amino acids, increase chlorophylls pigments such as chlorophyll *a* and *b*, and improve the production of metabolites, causing oxidative stress reduction and increased photosynthesis, consequently decreasing EL%, MDA, H_2O_2, and $O_2\cdot^-$ levels under drought conditions. Several studies have confirmed that inoculation with *Bacillus* is an important approach for ROS scavenging in the drought stressed plants [90]. Furthermore, melatonin treatment improved photosynthetic pigments, regulated phytohormones under drought stress conditions, and played several roles in phenotypic, molecular, and physiological processes [91]. Antioxidant enzymes play an important role in stress tolerance, CAT, SOD, and POD as main antioxidant enzymes were significantly increased in stressed plants compared with control treatment in the two seasons (Fig. 5). This result may be due to the over production of antioxidant enzymes to scavenge reactive oxygen species and mitigate the oxidative damage in soybean plants under drought stress [92,93]. This result was in harmony with the results of Khaffagy et al. [8], El-Yazied et al. [70], and Alshammari et al. [94]. Application of melatonin and *Bacillus* alleviated the oxidative stress and regulated the activity of CAT, SOD, and POD in stressed soybean plants. This positive effect could be due to the protective role of melatonin and *Bacillus* in maintaining membrane stability, stimulating plant development and osmoregulation, and mitigating the oxidative stress by regulating the enzymes activity. The findings of this study are in agreement with the result of Gusain et al. [95], Kumar et al. [96], and Alhashimi et al. [97]. Furthermore, melatonin may improve stress tolerance by regulating the metabolism of carbohydrates and amino acids [98,99].

Anatomical features are very important indicators under various stresses. The anatomical features of soybean leaves and stems were adversely affected under drought stress compared with control treatment. A significant reduction was recorded in the anatomical characters of stem and leaf. This negative effect on xylem vessels diameter, stem diameter, epidermis, phloem thickness, and number of xylem vessels in the stressed soybean plants may be due to the harmful impact of drought on plant growth and differentiation.

However, applications of melatonin and *Bacillus* improved the anatomical characters of stem and leaf of stressed soybean under drought stress. The effect of melatonin and *Bacillus* may be due to the synthesis of secondary metabolites, hormones, and antioxidants, which enhance nitrogen fixation and produce siderophores that increase plant tolerance [100], and consequently improve the anatomical characters of plant. Moreover, the helpful effect of melatonin was recorded in many plants such as maize. In maize, melatonin improves ultra-structure of the leaves under drought conditions [101,102]. Generally, this study showed that application of melatonin or *Bacillus* can mitigate the damaging effect of drought on physiological, biochemical processes, and anatomical characters of soybean plants.

5. Conclusions

The findings of this study confirmed that drought stress harmfully affects the morphophysiological, biochemical, and yield characteristics under drought stress. However, application of melatonin or *Bacillus* improved morphological characters such as leaves number, and increased chlorophyll and relative water content as well as the maximum quantum efficiency of PSII (Fv/Fm) in the stressed soybean plants. In addition, EL%, MDA, and ROS were decreased with melatonin and *Bacillus* treatments under drought stress. Furthermore, the anatomical structure of soybean leaves and stems was improved with application of melatonin or *Bacillus* treatments. Thus, the application of melatonin or *Bacillus thuringiensis* provides the protection to the stressed soybean plants confirmed by the morphological, physio-biochemical, and anatomical structure under drought stress.

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Data availability

Data included in article/supp. Material/referenced in article.

CRediT authorship contribution statement

Wasimah B. Al-Shammari: Writing – review & editing, Writing – original draft, Resources, Methodology, Funding acquisition, Data curation, Conceptualization. **Arwa Abdulkreem AL-Huquil:** Writing – review & editing, Writing – original draft, Software, Methodology, Data curation, Conceptualization. **Kholoud Alshammery:** Writing – review & editing, Resources, Formal analysis. **Salwa Lotfi:** Writing – original draft, Software, Resources, Funding acquisition, Data curation. **Haya Altamimi:** Writing – original draft, Resources, Funding acquisition. **Abeer Alshammari:** Writing – original draft, Software, Resources, Funding acquisition. **Nadi Awad Al-Harbi:** Writing – review & editing, Writing – original draft, Data curation. **Afaf Abdullah Rashed:** Writing – review & editing, Validation, Formal analysis. **Khaled Abdelaal:** Writing – review & editing, Writing – original draft, Supervision, Software, Investigation, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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