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Melatonin confers drought stress tolerance in soybean (*Glycine max* L.) by modulating photosynthesis, osmolytes, and reactive oxygen metabolism

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Abstract

In order to investigate the role of melatonin in the drought tolerance, we examined pigments, gas exchange, osmolytes, and reactive oxygen radical metabolism in soybean plants. Drought declined photosynthetic pigments and caused irreversible reduction in net photosynthesis, which was followed by stomatal limitation for 5 and 10 d and nonstomatal limitation for 15 d. Soluble sugar, soluble proteins, and proline concentrations were higher in drought-stressed seedlings compared with the control. The contents of superoxide anion, hydrogen peroxide, and malondialdehyde increased during drought stress indicating oxidative stress. Drought stress also increased superoxide dismutase, peroxidase, and catalase activities. Melatonin treatment improved the tolerance of drought-treated plants, which was possibly due to the enhanced content of osmolytes and higher antioxidant enzyme activities that retard dehydration and lipid peroxidation.

Additional key words: carotenoid; chlorophyll; gas-exchange parameters; plant growth regulator; reactive oxygen species.

Introduction

Drought is an abiotic stress that has drastic effect on the growth and development of plants, especially at the seedling stage, which affects the global grain production (Du *et al*. 2004, Basu *et al*. 2010, Deng *et al*. 2012, Hu *et al*. 2013, Kaczmarek *et al*. 2017). In spring, drought can kill seedlings through their root systems, which are not fully developed (Kaczmarek *et al*. 2017). With the rising global temperature, the timing and magnitude of drought stress has been increasing yearly, especially, in arid and semiarid regions (Wang *et al*. 2013, Harrison *et al*. 2014).

Many studies have reported that when plants encounter a water deficit, there is a disturbance in water balance, and a decline in photosynthesis (Fu and Huang 2001, Hu *et al*. 2013, Cui *et al*. 2017). Kaczmarek *et al*. (2017) proposed that osmolyte accumulation was the first line of defense against drought. The most common compatible osmolytes, such as soluble sugars, soluble proteins, and proline, can reduce membrane permeability, and play an important role in maintaining water balance in plants under mild water stress (Du *et al*. 2004). Through stomatal and nonstomatal limitations, drought stress inhibits photosynthesis and is usually accompanied by a decline in light energy absorption. Stomatal closure leads to a leakage of electrons towards O_2, resulting in increased reactive oxygen species (ROS) generation in water-deficient plants (Basu *et al*. 2010). Superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2), which are the major ROS, accumulate in cells and cause oxidative stress (Basu *et al*. 2010, Deng *et al*. 2012). These ROS can disrupt normal metabolism during stress, leading to membrane lipid peroxidation, chlorophyll (Chl) loss, and enzyme inactivation, which can accelerate plant senescence. Superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) are antioxidant enzymes that play key roles in eliminating excessive ROS in cell, and maintain ROS homeostasis and tolerance to stress, including drought stress (Basu *et al*. 2010, Deng *et al*. 2012).

Melatonin (*N*-acetyl-5-methoxytryptamine) is a steroidal tryptamine that is a well-known hormone in plants and animals. In 1995, melatonin was discovered in higher plants, and it is a plant growth regulator (PGR) that is widely used to regulate growth and enhance plant resistance (Dubbels *et al*. 1995, Hattori *et al*. 1995). In a normal growth environment, melatonin has been shown to be a ubiquitous modulator of developmental processes, such as the preservation of Chl, promotion of photosynthesis (Tan *et al*. 2012), and regeneration of root system architecture (Zhang *et al*. 2014). In addition, melatonin is an effective

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Abbreviations: Car – carotenoid; CAT – catalase; Chl – chlorophyll; C_i – intercellular CO_2 concentration; DAB – 3,3-diaminobenzidine; E – transpiration rate; FM – fresh mass; g_s – stomatal conductance; MDA – malondialdehyde; NBT – nitroblue tetrazolium chloride; PGR – plant growth regulator; P_N – net photosynthetic rate; POD – peroxidase; ROS – reactive oxygen species; RSWC – relative soil water content; SOD – superoxide dismutase; VC – cotyledon stage; V1 – the second trifoliate leaf stage.
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free radical scavenger and antioxidant in plants (Tan *et al*. 2012) that protects against multiple abiotic stresses, such as salt stress (Li *et al*. 2012), heavy metal stress (Posmyk *et al*. 2008), and UV radiation (Zhang *et al*. 2012). Previous studies have shown that melatonin can increase the germination rate and promote root growth in cucumber (Zhang *et al*. 2012), and delay drought-induced senescence in apple leaves (Wang *et al*. 2013). Although there are many studies on the effects of melatonin on drought stress, the results have significant limitations: (*1*) the research subjects are mainly horticultural crops (such as cucumbers, apples, *etc*.), and there are few studies on field crops; and (*2*) polyethylene glycol (PEG) was used to simulate drought, which cannot truly reflect the natural process of drought (Basu *et al*. 2010, Deng *et al*. 2012). The mechanisms underlying the effect of melatonin on soybean drought tolerance are unknown. Therefore, in this study, using cv. Suinong 26 as the test material, we simulated natural drought and normal water treatment at the seedling stage. We administered melatonin by spraying in order to test the effects of melatonin on the growth, photosynthesis, and antioxidant system of soybean seedlings under drought stress, which could provide a theoretical basis for the cultivation of drought-resistant soybeans.

Materials and methods

Experimental site and design: The experimental site was chosen at Heilongjiang Bayi Agricultural University located in Northeast China (124°19'–125°12'E, 45°46'–46°55'N) in 2018. The soybean (*Glycine max* L.), variety Suinong 26, was sown at a rate of eight seeds per plastic pot (33 × 30 cm) in a mixture of vermiculite and perlite in a 1:1 (v/v) ratio and watered with full strength Hoagland solution once per day from sowing to emergence, and twice per day after emergence. In order to prevent excessive accumulation of salt in the mixed perlite and vermiculite, the pots were watered with distilled water once every 5 d. Three seedlings were retained at the cotyledon stage (VC).

The melatonin (*Sigma*, St. Louis, MO, USA) and drought treatments were applied at the second trifoliate leaf stage (V1). Before the treatments were applied, the pots were divided into two groups, drought-stressed and well-watered. Drought-stressed pots were kept at 45% relative soil water content (RSWC), and well-watered pots were watered up to 80% RSWC (Hu *et al*. 2013). Half of the pots were sprayed with 45 mL of a 100 mg L^{-1} melatonin solution (Wang *et al*. 2013) for 3 d before the drought treatment. The rest were sprayed with distilled water as a control treatment. Each pot was weighed daily for 15 d to maintain the soil moisture levels at 45 or 80% by adding lost water. There were four treatments: (*1*) control (CK): well-watered (80%) and sprayed with distilled water; (*2*) CK + M: well-watered (80%) and sprayed with melatonin; (*3*) drought (D): drought-stressed (45%) and sprayed with distilled water; (*4*) D + M: drought-stressed (45%) and sprayed with melatonin. The experimental design was a randomized complete design with four replications. Fully expanded leaves (*i.e*., second trifoliate leaves from the main apex) were sampled from each treatment at three time points (5, 10, and 15 d) after exposure to drought treatments, and frozen in liquid nitrogen, and stored at –80°C.

Photosynthetic pigments concentrations: Chl *a*, Chl *b*, Chl (*a*+*b*), and carotenoid (Car) concentrations were determined according to the method of Arnon (1949) with a minor modification. Fresh leaf tissue from fully expanded healthy leaves (100 mg) was soaked in 10 mL of ethanol absolute for 24 h until the pellets became colorless. The optical density (D) of the solution was measured at 470, 649, and 665 nm, using a UV-visible spectrophotometer (*Jenway 6850 UV-Vis*, *Cole-Parmer Ltd*., UK). Contents were calculated as follows:

$$\text{Chl } a = 13.95\ D_{665} - 6.88\ D_{649}$$

$$\text{Chl } b = 24.96\ D_{649} - 7.32\ D_{665}$$

$$\text{Car} = (1000\ D_{470} - 2.05\ \text{Chl } a - 111.48\ \text{Chl } b)/245$$

Gas-exchange parameters: The net CO_2 assimilation rate (P_N), stomatal conductance (g_s), transpiration rate (E), and intercellular CO_2 concentration (C_i) were measured in the youngest and fully expanded leaves using a portable photosynthesis system (*Li-Cor 6400*, *Li-Cor Inc*., Nebraska, USA) under the following conditions: light intensity of 1,000 μmol(photon) m^{-2} s^{-1}, CO_2 concentration of 380 μmol mol^{-1}, flow rate of 500 μmol s^{-1}, leaf temperature of 27 ± 2°C, and relative humidity of 65 ± 5%. Measurements were taken from 9:30 to 11:30 h.

Osmolyte concentrations: Soluble sugars were extracted according to the method of Hendrix (1993) with slight modifications. Frozen leaf samples (about 1.0 g of fresh mass) were grounded in liquid nitrogen, extracted in 7.0 mL of 80% (v/v) ethanol three times, immersed in a 80°C water bath for 15 min, and centrifuged at 6,000 × *g* for 5 min. After centrifugation, the supernatant was recovered and brought to a volume of 25 mL. Then 0.5 mL of the supernatant was mixed with 2.0 mL of distilled water and 6.5 mL of anthrone solution. The absorbance of the supernatant was read at 620 nm using a UV-Vis spectrophotometer (*Jenway 6850 UV-Vis*, *Cole-Parmer Ltd*., UK).

The soluble protein concentration was estimated spectrophotometrically according to the method of Smith *et al*. (1985). Freshly harvested leaf samples (1.0 g fresh mass) were homogenized with 0.1 M phosphate buffer (pH 6.75). Then, the homogenates were centrifuged at 15,000 × *g* for 15 min. Next, 5 μL of supernatant was transferred to tubes and mixed with 1.5 mL of BCA reagent (bicionchonic acid + $FeCl_3$). The samples were incubated in boiling water for 5 min and then cooled to room temperature. The absorbance at 562 nm was measured with a spectrophotometer (*Jenway 6850 UV-Vis*, *Cole-Parmer Ltd*., UK) and the concentration of soluble protein was expressed as mg g^{-1}.

The proline concentration was assayed according to the method of Bates *et al*. (1973) with slight modifications. Approximately 0.5 g of leaf sample was boiled in 3% 5-sulfosalicylic acid and then centrifuged at 11,500 × *g*

for 12 min. Then, 2.0 mL of the supernatant was mixed with 2.0 mL of glacial acetic acid and 2.0 mL of acid-ninhydrin, boiled for 30 min, and then cooled to room temperature. The developed color was extracted with 5 mL of toluene, and the absorbance was read at 520 nm using a UV-Vis spectrophotometer (*Jenway 6850 UV-Vis*, *Cole-Parmer Ltd*., UK).

Histochemical localization of hydrogen peroxide and superoxide anion: Leaves were collected from the same position in the plants and were then stained with nitroblue tetrazolium chloride (NBT) and 3,3-diaminobenzidine (DAB) solution according to the method of Chen *et al*. (2010) and Wei *et al*. (2015), respectively. The reaction of DAB with H_2O_2 produced brown spots, and the reaction of NBT with $O_2^{\bullet-}$ produced dark blue spots. All the leaves from the different treatments were incubated for 6 h and were immersed in a boiling mixed solution (ethanol:glacial acetic acid, 3:1, v/v) to visualize the spots.

Measurement of superoxide anion, hydrogen peroxide, and lipid peroxidation: The superoxide anion ($O_2^{\bullet-}$) production rate was determined as previously described (Wang and Luo 1990). Briefly, leaf samples (0.2 g) were homogenized in 5 mL of ice cold 50 mM phosphate buffer (pH 7.8) and then centrifuged at 16,000 × *g* for 20 min at 4°C. The supernatant was mixed with 1.0 mL of hydroxylamine hydrochloride and 2.0 mL of extraction buffer, and incubated for 20 min. The production rate of $O_2^{\bullet-}$ was measured spectrophotometrically at 530 nm (*UV-3600 Plus*, *Shimadzu*, Japan) and expressed as mmol min^{-1} g^{-1}(protein).

The H_2O_2 concentration was determined by the method of Patterson *et al*. (1984). Firstly, 0.1 g of leaf tissue was homogenized with 5 mL of ice-cold acetone and centrifuged at 3,000 × *g* for 10 min. Then, 1.0 mL of the supernatant was added to 0.1 mL of 5% titanium sulfate, and 0.2 mL of concentrated ammonia and centrifuged at 3,000 × *g* for 10 min again. The residual tissue was washed with 5 mL of sulfuric acid. Absorbance was measured spectrophotometrically at 415 nm. The H_2O_2 concentration was calculated using H_2O_2 as a standard and expressed as μmol g^{-1}(FM).

Lipid peroxidation was determined based on the malondialdehyde (MDA) concentration according to the method of Guidi *et al*. (2000). Approximately 1.0 g of fresh leaves was homogenized in 5 mL of 0.05 M phosphate buffer (pH 7.8) and was centrifuged at 12,000 × *g* for 20 min. The mixture contained 2 mL of the supernatant and 2 mL of 0.6% thiobarbituric acid in 10% trichloroacetic acid containing 0.25% thiobarbituric acid, which was incubated in water bath at 95°C for 30 min, quickly cooled in an ice bath, and then centrifuged at 10,000 × *g* for 10 min. The absorbance of the supernatant was determined at 450, 532, and 600 nm (*Jenway 6850 UV-Vis*, *Cole-Parmer Ltd*., UK). Lipid peroxidation was expressed as μmol g^{-1}(FM).

Antioxidant enzyme assays: Using a precooled mortar and pestle, soybean leaves (0.5 g) were homogenized in 10 mL of 50 mM ice cold PBS buffer (pH 7.0) and then centrifuged at 11,500 × *g* for 15 min at 4°C. The supernatant was used for the enzyme activity assay.

Superoxide dismutase (SOD, EC 1.15.1.1.) was determined by measuring the ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT), according to the method of Alonso *et al*. (2001) with some modifications. The reaction mixture, which contained 2.4 mL of 50 mM phosphate buffer (pH 7.8), 0.2 mL of 75 mM NBT, 0.2 mL of 13 mM methionine, 0.1 mL of 100 mM EDTA, and 0.2 mL of 2 mM riboflavin (added last) was added to tubes containing 0.2 mL of enzyme extract. These tubes were exposed to fluorescent tubes emitting a photon flux density of around 600 μmol m^{-2} s^{-1} for 10 min and then covered with a black cloth. The change in the absorbance at 560 nm was read with a UV-Vis spectrophotometer (*Jenway 6850 UV-Vis*, *Cole-Parmer Ltd*., UK). The unit of SOD activity was defined based on the standard curve and was expressed as μmol min^{-1} mg^{-1}(protein).

Peroxidase (POD, EC 1.11.1.7.) activity in the leaves was measured according to the method of Maehly (1954) based on the increase in absorbance at 470 nm. Peroxidase was assayed using guaiacol as the substrate. The assay solution for POD activity (3.0 mL) contained 0.3% H_2O_2, 50 mM phosphate buffer (pH 7.0), and 0.2% guaiacol. The reaction was initiated by adding 0.2 mL of crude enzyme extract. The activity of POD was expressed as μmol min^{-1} mg^{-1}(protein).

Catalase (CAT, EC 1.11.1.6.) activity was assayed according to the method of Fu and Huang (2001) by monitoring the disappearance of H_2O_2 as the decrease in the absorbance at 240 nm for 1 min (*Jenway 6850 UV-Vis*, *Cole-Parmer Ltd*., UK). The reaction mixture contained 1.5 mL of 50 mM sodium phosphate buffer (pH 7.8), 0.1 mL of enzyme extract, and 0.3 mL of 1.5 M H_2O_2. The activity was calculated using the extinction coefficient of 39.4 M^{-1} cm^{-1} and expressed as μmol min^{-1} mg^{-1}(protein).

Statistical analysis: The data are presented as the means of four replicates. All data were subjected to analysis of variance (*ANOVA*) and *Duncan*'s multiple range test. Differences between the treatments and control were considered significant at $P<0.05$, calculated by using *SPSS* (*21.0*) software. Figures were drawn with *OriginPro 9.1* software (*OriginLab*, Northampton, MA, USA).

Results

Photosynthetic pigments: Melatonin treatment increased the contents of Chl and Car under both well-watered and drought-stress conditions (Fig. 1). Drought stress significantly decreased the concentration of Chl *a*, Chl *b*, Chl (*a*+*b*), and Car, from day 5 to day 15, these indicators decreased by 18.4–42.2, 39.7–82.7, 29.0–45.5, and 49.1–60.9%, respectively, when compared to well-watered plants. However, exogenous melatonin effectively relieved these decreases in drought-stressed plants. From day 5 to day 15, the above indicators [Chl *a*, Chl *b*, Chl (*a*+*b*), and Car] decreased by 7.5–25.3, 18.1–42.2, 12.8–33.8, and 54.8–73.3%, respectively.

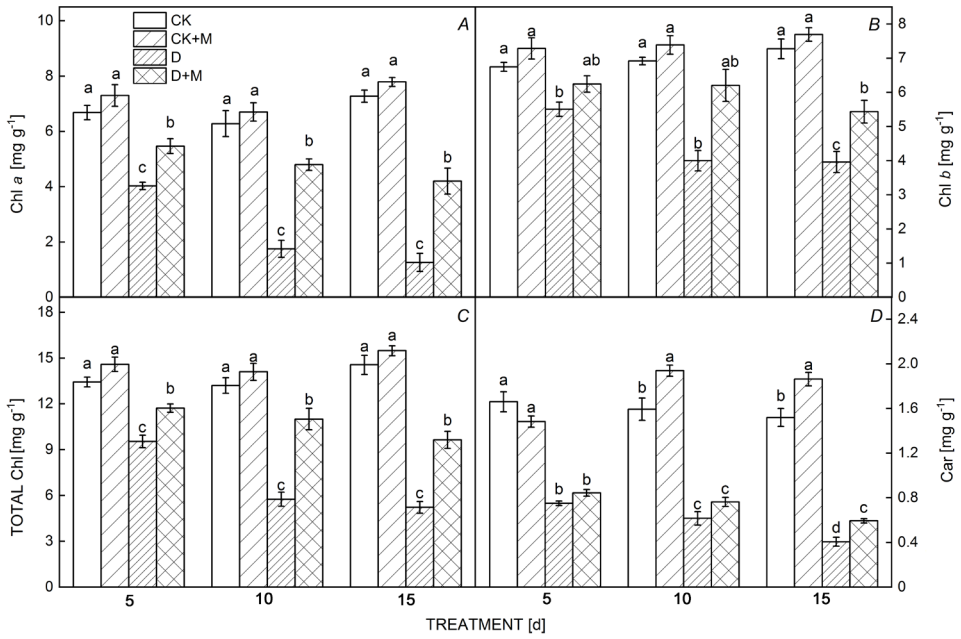

Fig. 1. Effect of melatonin on chlorophyll (Chl) *a* (*A*), Chl *b* (*B*), Chl (*a*+*b*) (*C*), and carotenoid (Car) concentration (*D*) in soybean leaves under well-watered and drought stress conditions. All data are means ± SE (n = 4). *Different letters* above horizontal lines indicate significant differences (P<0.05) between treatments. CK – well-watered con-ditions, CK + M – well-watered conditions + melatonin, D – drought stress, D + M – drought stress + melatonin.

Photosynthesis: As shown in Fig. 2, melatonin significantly increased P_N, g_s, and E under well-watered conditions. With prolonged treatment time, P_N increased from 5.9 to 13.4%. Drought stress sharply decreased P_N and g_s. However, melatonin markedly alleviated the drought-induced reductions in P_N and g_s. Compared to well-watered plants, P_N was reduced by 31.5–67.2% in drought-stressed plants, and by 13.4–43.6% in melatonin-treated drought-stressed plants.

Osmolyte concentration: Drought stress increased the concentrations of various osmolytes, such as soluble sugar, soluble protein (Fig. 3), and proline (Table 1), and compared to well-watered plants, the concentrations of soluble sugar after 5, 10, and 15 d of drought stress increased by 16.8, 49.9, and 89.1%, respectively. In contrast, melatonin-pretreated drought-stressed soybean plants showed increases of 4.8, 13.4, and 15.3% after 5, 10, and 15 d of drought stress, respectively (Fig. 3*A*). Compared with well-watered plants, the increases in soluble protein were 30.0, 53.6, and 74.6% in drought-stressed plants, respectively. Compared to drought-stressed plants without melatonin, drought-stressed plants pretreated with melatonin showed 8.6, 15.0, and 19.2% higher concentrations of soluble protein after 5, 10, and 15 d of drought stress, respectively (Fig. 3*B*). Without melatonin, a significant increase in the proline concentration was observed in drought-stressed plants over time as compared with the contents in well-watered plants (Table 1). Soybean seedlings showed higher endogenous proline concentrations, which increased by

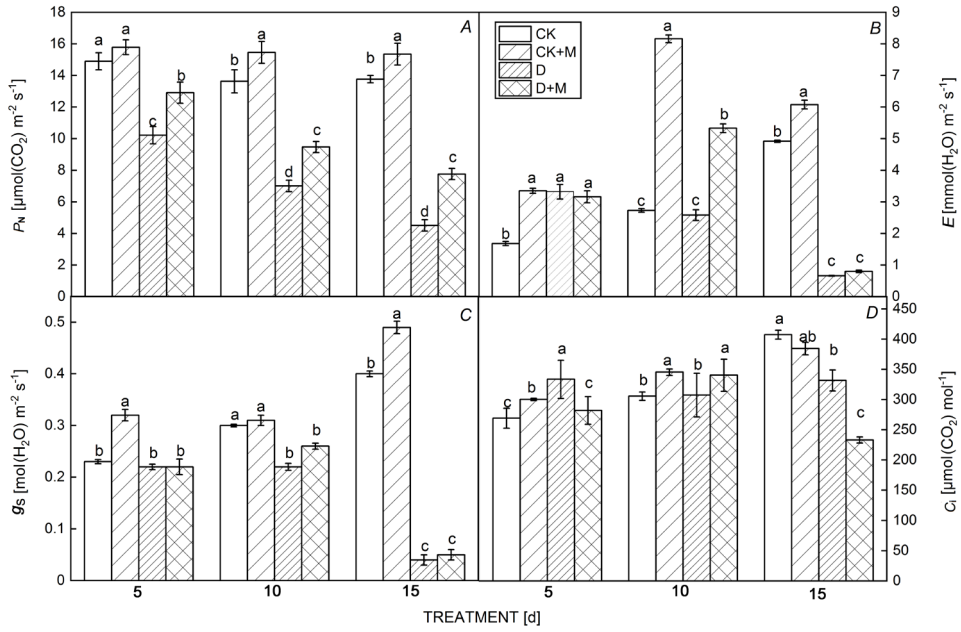

Fig. 2. Effect of melatonin on net photosynthetic rate (P_N) (*A*), transpiration rate (*E*) (*B*), stomatal conductance (g_s) (*C*), and intercellular CO_2 concentration (C_i) (*D*) in soybean leaves under well-watered and drought stress conditions. All data are means ± SE (n = 4). *Different letters* above horizontal lines indicate significant differences (P<0.05) between treatments. CK – well-watered conditions, CK + M – well-watered conditions + melatonin, D – drought stress, D + M – drought stress + melatonin.

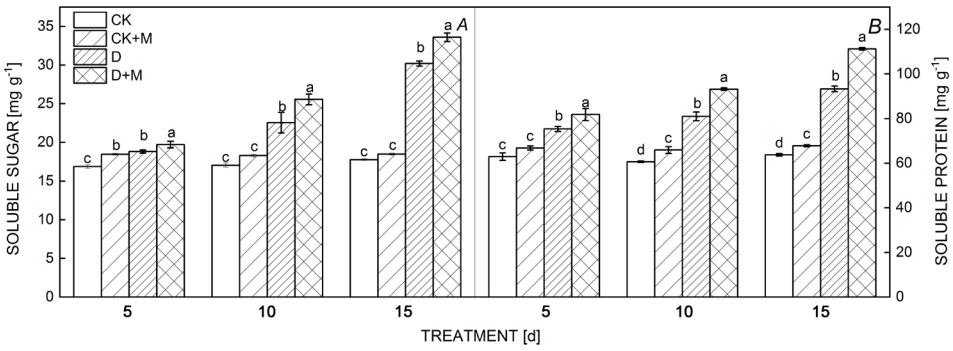

Fig. 3. Effect of melatonin on soluble sugar (*A*) and soluble protein (*B*) concentration in soybean leaves under well-watered and drought stress conditions. All data are means ± SE (n = 4). *Different letters* above horizontal lines indicate significant differences (P<0.05) between treatments. CK – well-watered conditions, CK + M – well-watered condi-tions + melatonin, D – drought stress, D + M – drought stress + melatonin.

Table 1. Effects of melatonin on proline content in soybean under drought stress. All data are means ± SE (n = 4). *Different letters* within the same row represent significant differences (P<0.05). CK – well-watered conditions, CK + M – well-watered conditions + melatonin, D – drought stress, D + M – drought stress + melatonin.

Drought stress [d]	Proline [μg g^{-1}(FM)]			
	CK	CK + M	D	D + M
5	17.42 ± 0.29^{c}	17.10 ± 0.50^{c}	22.70 ± 0.72^{b}	24.96 ± 1.04^{a}
10	18.00 ± 0.46^{d}	16.23 ± 0.14^{c}	40.51 ± 0.27^{b}	54.44 ± 0.40^{a}
15	17.62 ± 0.53^{c}	16.69 ± 0.79^{c}	76.48 ± 0.27^{b}	83.84 ± 0.27^{a}

30.3, 125.1, and 334.0% after 5, 10, and 15 d of drought stress, respectively, compared with well-watered plants. These results showed that the application of melatonin effectively maintained the higher water potential and cell turgor in drought-stressed soybean seedlings.

Reactive oxygen species: Leaves were soaked in NBT and DAB solutions to visualize the spots of H_2O_2 and $O_2^{\bullet-}$ concentrations, respectively. Drought-stressed leaves showed a significant increase in deep blue spots of $O_2^{\bullet-}$ and dark brown patches of H_2O_2 (Fig. 4). However, compared with drought-stressed plants, the $O_2^{\bullet-}$ and H_2O_2 spots were somewhat reduced following exogenous melatonin pretreatment, which is indicative of a reduction in oxidative stress. However, the melatonin treatment did not reduce the H_2O_2 and $O_2^{\bullet-}$ spots in well-watered plants.

Our data confirmed that drought stress induced significant accumulation of H_2O_2, $O_2^{\bullet-}$, and MDA when compared to the corresponding levels in well-watered plants (Fig. 5, Table 2). Melatonin treatment decreased the contents of H_2O_2, $O_2^{\bullet-}$, and MDA under both well-watered and drought-stressed conditions. Compared with well-watered plants, drought stress induced significant accumulation of H_2O_2, $O_2^{\bullet-}$, and MDA. At 5, 10, and 15 d, the H_2O_2 concentration increased by 27.6, 48.6, and 81.9%, $O_2^{\bullet-}$ content was elevated by 34.8, 62.8, and 98.4%, and MDA concentration increased by 37.4, 72.3, and 96.0%, respectively. Exogenous application of melatonin significantly reduced H_2O_2, $O_2^{\bullet-}$, and MDA concentrations in drought-stressed soybean leaves. In short, melatonin treatment of water-deficient plants alleviated the toxic effects on cellular metabolism.

Antioxidant enzymes: SOD, POD, and CAT activities were analyzed in the soybean plants. As shown in Table 3, these enzymes were not altered by melatonin pretreatment in well-watered plants. Exposure to drought stress increased the activity of SOD, POD, and CAT, and melatonin pretreatment markedly improved the activities of these enzymes. After 5, 10, and 15 d of drought stress, SOD activity increased by 26.2, 49.4, and 63.1%; POD activity

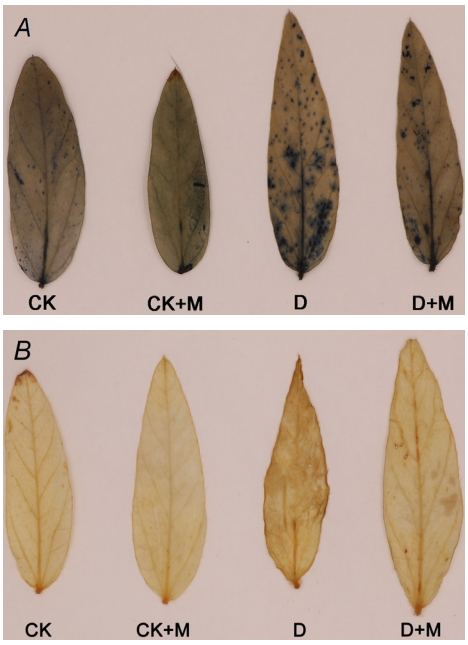

Fig. 4. Histochemical localization of superoxide anion ($O_2^{\bullet-}$) (*upper panel*) and hydrogen peroxide (H_2O_2) (*lower panel*) in soybean leaves under well-watered and drought stress conditions. CK – well-watered conditions, CK + M – well-watered conditions + melatonin, D – drought stress, D + M – drought stress + melatonin.

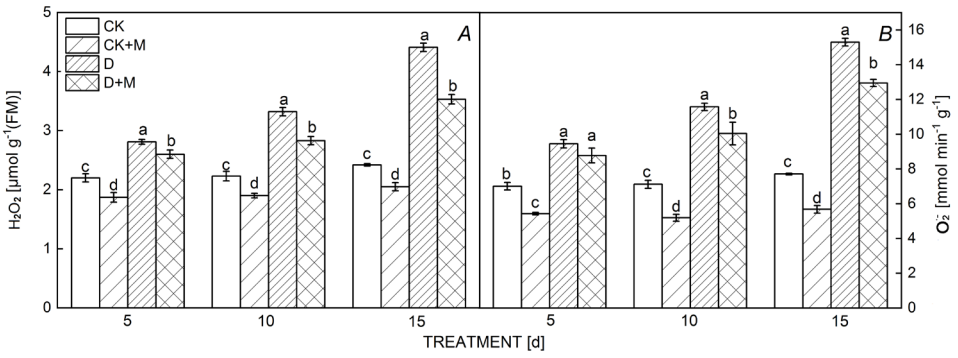

Fig. 5. Effect of melatonin on hydrogen peroxide (H_2O_2) (*A*) and superoxide anion ($O_2^{\bullet-}$) (*B*) in soybean leaves under well-watered and drought stress conditions. All data are means ± SE (n = 4). *Different letters* above horizontal lines indicate significant differences (P<0.05) between treatments. CK – well-watered conditions, CK + M – well-watered conditions + melatonin, D – drought stress, D + M – drought stress + melatonin.

Table 2. Effects of melatonin on malondialdehyde concentration in soybean leaves exposed to drought stress. All data are means ± SE (n = 4). *Different letters* within the same row represent significant differences (P<0.05). CK – well-watered conditions, CK + M – well-watered conditions + melatonin, D – drought stress, D + M – drought stress + melatonin.

Drought stress [d]	Malondialdehyde [nmol g^{-1}(FM)]			
	CK	CK + M	D	D + M
5	0.44 ± 0.03^{b}	0.38 ± 0.01^{b}	0.60 ± 0.02^{a}	0.57 ± 0.02^{a}
10	0.37 ± 0.01^{c}	0.32 ± 0.01^{c}	0.64 ± 0.02^{a}	0.55 ± 0.03^{b}
15	0.40 ± 0.02^{c}	0.36 ± 0.03^{c}	0.79 ± 0.02^{a}	0.67 ± 0.04^{b}

Table 3. Effects of melatonin on catalase (CAT), superoxide dismutase (SOD), and peroxidase (POD) activity in soybean leaves exposed to drought stress. All data are means ± SE (n = 4). *Different letters* within the same row represent significant differences (P<0.05). CK – well-watered conditions, CK + M – well-watered conditions + melatonin, D – drought stress, D + M – drought stress + melatonin.

Enzyme activity [μmol min^{-1} mg^{-1}(protein)]	Drought stress [d]	CK	CK + M	D	D + M
CAT	5	29.84 ± 0.22^{c}	30.28 ± 0.97^{c}	50.00 ± 0.73^{b}	71.44 ± 1.52^{a}
	10	28.84 ± 0.97^{c}	30.56 ± 0.61^{c}	78.38 ± 1.59^{b}	121.44 ± 0.74^{a}
	15	27.52 ± 1.71^{c}	28.56 ± 0.48^{c}	100.23 ± 2.86^{b}	127.58 ± 1.07^{a}
SOD	5	83.61 ± 3.21^{c}	84.54 ± 4.31^{c}	105.50 ± 2.87^{b}	127.16 ± 1.98^{a}
	10	81.03 ± 3.87^{c}	81.81 ± 4.06^{c}	121.07 ± 5.26^{b}	143.94 ± 2.76^{a}
	15	82.65 ± 3.61^{c}	82.68 ± 3.06^{c}	134.83 ± 4.29^{b}	152.00 ± 6.83^{a}
POD	5	119.33 ± 8.66^{c}	133.33 ± 9.54^{c}	221.17 ± 10.21^{b}	251.33 ± 9.05^{a}
	10	104.33 ± 8.02^{c}	112.63 ± 6.95^{c}	236.67 ± 10.31^{b}	262.50 ± 10.71^{a}
	15	147.67 ± 7.53^{c}	165.50 ± 5.80^{c}	392.33 ± 12.40^{b}	452.65 ± 15.81^{a}

increased by 85.3, 126.8, and 165.7%; and CAT activity increased by 139.4, 321.7, and 363.5%, respectively, as compared with the activities in well-watered plants.

Discussion

Chl, the major molecule responsible for photosynthesis, is fragile and easily damaged by ROS, which are generated by environmental stress (Tan *et al*. 2012). Because of the critical function of Chl, it must be preserved for the survival, growth, and production of plants. Melatonin, as an antioxidant, can prevent the degradation of Chl. Wang *et al*. (2013) uncovered that melatonin could preserve the integrity of Chl and increase the photosynthetic efficiency of Chl under both normal conditions and drought stress. The present work indicated that drought significantly decreased the concentration of Chl and Car, whereas melatonin pretreatment ameliorated this decrease (Fig. 1).

Photosynthesis is a key process in the primary metabolism of plants, and it plays an important role in plant performance under drought stress. Jia *et al*. (2008) and Hu *et al*. (2013) considered that drought stress inhibits photosynthesis through nonstomatal limitation. However, Yin *et al*. (2005) proposed that drought-mediated inhibition of photosynthesis may be due to stomatal closure. In the present study, we showed that P_N and g_s significantly decreased under drought stress (Fig. 2). After drought stress for 5 and 10 d, the reduced values of P_N were accompanied by a significant decrease in g_s and C_i, which indicated that the drought stress-induced P_N decrease was mainly due to the stomatal limitation. However, under the drought stress for 15 d, the reduced values of

P_N were accompanied by a significant decrease in g_s and an increase in C_i, which indicated that the drought stress-induced decline in P_N was mainly due to the nonstomatal limitation. This difference may be due to the destruction of the chloroplast structure in soybean leaves under drought stress, resulting in damage to the photosynthetic organs, decreasing photosynthetic activity and increasing the concentration of CO_2. The present results were consistent with the findings in other studies (Yin *et al*. 2005, Jia *et al*. 2008, Hu *et al*. 2013). In our study, melatonin treatment significantly increased P_N and E in soybean leaves when compared to the corresponding levels in drought-stressed leaves.

Soluble sugar, proline, and soluble protein, the most common compatible osmolytes, are actively accumulated to maintain a higher water potential and cell turgor under drought stress, which is the first line of defense against drought (Du *et al*. 2004, Kaczmarek *et al*. 2017). Osmolytes are synthesized in response to drought stress and do not interfere with the normal cellular biochemical reactions (Du *et al*. 2004). The present study showed that the concentrations of various osmolytes, such as soluble sugar, proline, and soluble protein, increased in drought-stressed plants (Fig. 3, Table 1). Compared to drought-stressed plants without melatonin, melatonin-pretreated plants showed significantly higher concentrations of osmolytes.

Earlier studies frequently reported that ROS actively accumulated in drought-stressed plants and damaged cell function (Basu *et al*. 2010, Deng *et al*. 2012, Wang *et al*. 2013). As an antioxidant, melatonin can directly interact with ROS and modulate the activity of antioxidant enzymes in response to excessive ROS (Shi *et al*. 2015). Wang *et al*. (2013) considered that melatonin directly scavenges ROS, and in particular, it controls the burst of H_2O_2, decreases the contents of MDA, and increases the activity of CAT and POD. Cui *et al*. (2017) reported that when wheat seedlings were exposed to a water deficit, the exogenous application of melatonin significantly reduced the concentrations of H_2O_2, $O_2^{\bullet-}$, and MDA, which was attributed to the increased antioxidant enzyme activity. In this present study, the H_2O_2 and $O_2^{\bullet-}$ localized in the leaf tissue of the soybean plants were visualized by histochemical staining, and drought stress induced excessive accumulation of H_2O_2 and $O_2^{\bullet-}$ (Fig. 4). H_2O_2, $O_2^{\bullet-}$, and MDA, the indicators of oxidative stress, were actively accumulated in the drought-stressed leaves (Fig. 5, Table 2). However, the application of exogenous melatonin reduced the H_2O_2 and $O_2^{\bullet-}$ spots in drought-stressed plants and the concentrations of H_2O_2, $O_2^{\bullet-}$, and MDA declined, which is indicative of a reduction in oxidative stress. These results were in agreement with several previous reports (Basu *et al*. 2010, Deng *et al*. 2012, Wang *et al*. 2013, Cui *et al*. 2017).

POD and CAT can effectively scavenge H_2O_2 (Wang *et al*. 2013). The decrease in the concentration of ROS was initially attributed to the antioxidant capacity of melatonin, as evidenced by the improved activity of antioxidant enzymes. This study showed that drought stress treatment increased the activities of SOD, POD, and CAT in the soybean leaves (Table 3). Exogenous melatonin induced upregulation of these enzymes compared to those observed under drought stress (Table 3). Thus, the protective effect of melatonin against drought stress could be ascribed to its antioxidant capacity and free radical scavenging.

Conclusion: The results of the present study demonstrated that drought stress decreased the concentrations of photosynthetic pigments and P_N, and increased $O_2^{\bullet-}$, H_2O_2, and MDA concentrations. The results also showed that, compared to drought-stressed plants, foliar application of melatonin (100 mg L^{-1}) at the seedling stage improved photosynthesis and maintained the balance in ROS metabolism (ROS production and removal). This suggests that exogenous melatonin is an effective protectant that improves drought tolerance in soybean seedlings by enhancing antioxidant enzymes and reducing oxidative damages.

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