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ORIGINAL ARTICLE

Exogenous application of melatonin improves salt tolerance of sugar beet (*Beta vulgaris* L.) seedlings

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Abstract

Salt stress usually results in severe physiological damage to plants. Melatonin (*N*-acetyl-5-methoxytryptamine) is an important growth regulator that adapts plants to abiotic stress. The present study evaluated the role of melatonin application on inducing salt tolerance in sugar beet (*Beta vulgaris* L.). The protective role of melatonin (0, 30, 60, and 90 μM) was examined by measuring leaf photosynthetic characteristics, antioxidant system, and osmotic adjustment substances of sugar beet seedlings under salt (300 mM Na^+) and non-salt stresses. The results showed that salt stress resulted in significantly reduced biomass, reduced photochemical activity of photosystem II (PSII), and evoked the production of reactive oxygen species (ROS). In contrast, the application of melatonin significantly increased antioxidant enzyme activities (SOD, POD, and CAT) under salt stress, reduced ROS accumulation (MDA and $O_2 \cdot^-$), and enhanced photosynthesis in seedlings. There was no significant difference in the above indicators of melatonin pretreatment under control condition (non-salinized). On day 1 of stress application, the concentration of sucrose decreased significantly, and the concentration of proline and H_2O_2 increased significantly under melatonin treatment. On day 7, soluble sugar and betaine concentrations increased significantly. Current research speculates that melatonin enhances cellular energy metabolism and may be involved in activating the antioxidant system to eliminate ROS. In conclusion, these results indicated that the application of 60 μM melatonin could act as a feasible way to alleviate the salt stress in sugar beet production.

Keywords Salt tolerance · Melatonin · Antioxidant enzymes · Hydrogen peroxide · Sugar beet

Abbreviations

Car	Carotenoids
CAT	Catalase
Chl	Chlorophyll
*C*i	Intercellular CO_2 concentration
E	Transpiration rate
F_v/F_m	Maximum quantum yield of PSII
LRWC	Leave relative water content
MDA	Malondialdehyde content
g_s	Stomatal conductance
*P*n	Net photosynthetic rate
q_L	Estimates the fraction of open PSII centers
POD	Peroxidase
q_P	Photochemical quenching
ROS	Reactive oxygen species
SOD	Superoxide dismutase
Y(II)	Effective quantum yield of PSII
Y(NO)	Quantum yield of nonregulated non-photochemical energy dissipation
Y(NPQ)	Quantum yield of regulated non-photochemical energy dissipation

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Introduction

Salinity accounts for a main abiotic stress inhibiting plant growth in the world (Zhu 2001). Currently, approximately 900 million hectares of land is salinized globally, including

around 20% of cultivated land and 50% of irrigated land (Cristiano et al. 2016; FAO 2009). Furthermore, the annual world farmland has been reduced by 2 million hectares owing to secondary salinization, resulting in reduced plant productivity (Ke et al. 2016).

Salinity is a complex abiotic stress, including physiological water deficiency, ion toxicity, oxidative damage, nutrient disturbance, metabolic disturbance, photoinhibition and altered main cell enzymatic activities (Chen et al. 2007; Cuin and Shabala 2010; Farouk and Al-Amri 2019c; Farouk and Arafa 2018; Helaly et al. 2018; Munns and Tester 2008). Plants can establish the antioxidant systems and accumulate osmotic adjustment substances in the presence of abiotic stresses (Farouk and Al-Amri 2019c). However, the in-build system of plants to adapt to salinity is insufficient to prevent damage to crops. Therefore, it is urgent to find effective ways to improve the salt tolerance of crops.

Melatonin is an endogenous growth regulator synthesized by animals and plants under stress (Kolár and Macháckova 2010; Reiter et al. 2014). As melatonin was first discovered in plants in 1993, considerable progress has been made in this field (Farouk and Al-Amri 2019a, b; Van Tassel et al. 2010). Until date, several studies have demonstrated that plant melatonin could be an essential regulator of redox homeostasis (Arnao and Hernández-Ruiz 2018). It is associated with several physiological functions such as growth (Farouk and Al-Amri 2019b), rooting (Arnao and Hernández-Ruiz 2017), seed germination (Zhang et al. 2017b), and photosynthesis (Li et al. 2017a), as well as protection against abiotic stress (Arnao and Hernández-Ruiz 2015, 2017; Farouk and Al-Amri 2019b; Wang et al. 2018). Melatonin plays a critical role in plants to scavenge reactive oxygen species (ROS), which is the first-line defense to resist environmental and endogenous oxidative stresses (OS) (Arnao and Hernández-Ruiz 2018; Farouk and Arafa 2018). Wang et al. (2016) investigated the potential role of melatonin in salt tolerance and found that 50 to 150 μM exogenous melatonin promoted cucumber seedling growth and photosynthesis under salt stress (200 mM NaCl). Similarly, Li et al. (2017a) found that melatonin treatments at diverse concentrations (50, 150, and 500 μM) mitigated OS and the reduced photosynthetic rate of watermelon leaf samples under 300 mM NaCl stress. According to the results from Siddiqui et al. (2019), applying melatonin promoted tomato plant development in the meantime of reducing ROS contents through increasing the non-enzymatic antioxidant and antioxidant (including catalase and superoxide dismutase). However, information on how melatonin is involved in the salt tolerance of crops is still scarce (Chao et al. 2012; Wei et al. 2015).

Sugar beet (*Beta vulgaris* L.) represents the main sugar crop in the world, which shows high resistance to abiotic stresses (Bor et al. 2003). However, there is at present no literature related to melatonin involvement in regulating sugar beet response to abiotic stress is available. This work determined the roles of exogenous melatonin in sugar beet growth, antioxidant system, osmotic regulation and photosynthesis in the presence of salt stress; analyzed the regulatory mode; and laid the foundation for further elucidating the physiological and molecular mechanisms of its regulation of salt stress.

Materials and methods

Plant materials and growth conditions

Our experiments were carried out in the Northeast Agricultural University (126°63′ E, 45°44′ N, Harbin, P.R. China). We grew the sugar beet seeds (KWS0143, *KWS*, Germany) into pots that contained vermiculite within a plant growth room under the following conditions, 23 °C at day, 18 °C at night and 14 h/10 h light/dark cycle.

In our experiments, the relative humidity (RH) and light intensity were set at 60 ± 5% and 450 μmol m^{-2} s^{-1} PAR, respectively. Following seedling emergence, they were irrigated once a day with a 1/2 concentration of Hoagland's solution for 10 days. The uniform seedlings were later transferred to the above nutrient solution. The pH was maintained at 7.0 during the entire plant development period, while nutrient solution was replaced at intervals of 3 days.

Experimental design

Melatonin pretreatment (a culture with 1/2 Hoagland's solution containing 0, 30, 60 or 90 μM melatonin for 3 days) was performed upon the unfolding of the initial true leaf pair. Salt treatment (S; NaCl and Na_2SO_4 in a 2:1 molar ratio) was started after the pretreatment (after 3 days), following which the Na^+ concentration elevated to 100, 200 and 300 mM in sequence within 3 days (Hossain et al. 2017). A randomized block design comprised 8 treatments: (1) 1/2 Hoagland's solution (M0); (2) 1/2 Hoagland's solution + 30 μM melatonin (M30); (3) 1/2 Hoagland's solution + 60 μM melatonin (M60); (4) 1/2 Hoagland's solution + 90 μM melatonin (M90); (5) 1/2 Hoagland's solution + 300 mM Na^+ (M0 + S); (6) 1/2 Hoagland's solution + 30 μM melatonin + 300 mM Na^+ (M30 + S); (7) 1/2 Hoagland's solution + 60 μM melatonin + 300 mM Na^+ (M60 + S); (8) 1/2 Hoagland's solution + 90 μM melatonin + 300 mM Na^+ (M90 + S). Sugar beet seedlings were sampled at 1 day and 7 days after salt (300 mM Na^+) treatment, and physiological parameters were measured. Each collected sample was subjected to liquid nitrogen freezing at once, followed by preservation under − 80 °C for subsequent biochemical analysis. All

experiments were performed thrice and each treatment included three biological replicates.

Endogenous melatonin, Na^+, and K^+, concentration measurements

Melatonin was extracted from the leaves of sugar beet in line with Pape and Lüning's description (2006). Afterwards, we used the melatonin ELISA kit (EK-DSM; Buhlmann Laboratories AG, Schonenbuch, Switzerland) to quantify endogenous melatonin in line with specific protocols. Melatonin concentrations were expressed as pg g^{-1} (fresh mass, FM).

The concentrations (mg g^{-1} DW) of Na^+ and K^+ were determined using the method described by Chen et al. (2018). Leaf samples were subjected to 48 h of oven-drying under 75 °C. Later, HNO_3:$HClO_4$ (5:1 v/v) was added to digest 0.1 g dry sample till the solution became clear. Thereafter, we measured K^+ and Na^+ contents through inductively coupled plasma mass spectrometry (Optimal 2100DV; PerkinElmer Instruments, Waltham, MA).

Leaf relative water content and photosynthetic pigments' concentration

We determined relative water content (RWC) by the formula as follows: RWC = (fresh weight − dry weight)/(turgid weight − dry weight) × 100% (Smart and Bingham 1974).

We measured photosynthetic pigment contents by the acetone approach (Lichtenthaler and Wellburn 1983). To be specific, we isolated photosynthetic pigments from the freshly prepared samples contained within the 80% acetone. Then, the UV-754 spectrophotometer (Zealquest Scientific; Shanghai, China) was employed to measure supernatant absorbance (OD) values at 470, 645, and 663 nm. Later, the corrected extinction coefficients were utilized to calculate the Car, chlorophyll (Chl) a and Chl (a + b) contents, which were presented in the manner of mg g^{-1} FW.

Gas exchange and photosynthetic activity measurements

We adopted the photosynthesis system (GFS-3000; *WALZ*, Germany) to measure gas exchange in the leaf samples attached using the light source of 3040-L LED. Typically, we set the photosynthetic photon flux density (PPFD) and cuvette air flow rate as 400 µmol m^{-2} s^{-1} and 750 mL min^{-1}, respectively. At the same time, we determined the stomatal conductance (g_s), net photosynthetic rate (P_N), transpiration rate (E) and intercellular CO_2 contents (C_i). Each experiment was carried out for thrice, with 5 leaf samples from diverse plants being used under every replicate. In this experiment, we used the initial 2 completely folding leaf samples on the top (Zou et al. 2019).

Chlorophyll fluorescence measurements

After leaf samples were adapted to dark for 25 min, we used the Portable Chlorophyll Fluorometer (PAM-2500; *WALZ*, Germany) to measure Chl fluorescence parameters within leaf samples. In the meantime, we measured the maximal (F_v/F_m) and actual ([Y(II)]) photochemical efficiency of PSII, as well as the non-photochemical [Y(NPQ)] and photochemical (q_P) quenching, respectively (Pfündel et al. 2008).

Determination of RuBPcase activity

RuBPcase (ribulose-1, 5-bisphosphatecarboxylase) activity was determined using an ELISA kit according to the manufacturer's (CHUNDUBIO Ltd., China). 5 mL PBS (pH = 7.4) was used to extract 0.5 g freshly prepared leaf samples. Supernatant OD value was detected at 450 nm, while the eventual enzymatic activity was presented in the manner of µmol CO_2 g^{-1} FM^{-1} min^{-1}.

Determination of malondialdehyde (MDA), superoxide radical, and hydrogen peroxide (H_2O_2)

Thiobarbituric acid (TBA) was used to extract MDA, and later the supernatant OD values were measured at 450, 532 and 600 nm according to Shi et al.'s method (2013).

We applied Liu and Pang's approach (2010) to determine superoxide ($O_2\cdot^-$) contents. $O_2\cdot^-$ was extracted from plant materials using the potassium phosphate buffer (pH 7.8) supplemented with 7 mM α-naphthylamine and 17 mM sulfonamide, and incubated for 20 min under 25 °C. Finally, we determined the OD value at 530 nm and presented $O_2\cdot^-$ content in the manner of nmol g^{-1} FM min^{-1}.

H_2O_2 contents were measured according to Velikova et al.'s description (2000). First, root tissues were extracted with 0.1% (w/v) trichloroacetic acid, followed by the addition of PBS and the 1 M KI solution to react for 1 h in dark. Then, we determined the OD value at 390 nm. A standard curve was produced using solutions of known H_2O_2 concentration, with H_2O_2 content expressed in mM g FM^{-1}.

Analysis of antioxidant enzymes

Superoxide dismutase (SOD, EC 1.15.1.1) activity was measured using the NBT method of Stewart and Bewley (Stewart and Bewley 1980). Enzymatic activities were presented in the manner of unit g^{-1} (FM). To be specific, we monitored the guaiacol oxidation rate by Fu et al.'s method

(2014) to determine peroxidase (POD, EC 1.11.1.7) activity at 470 nm, then expressed in mmol (guaiacol) min^{-1} g^{-1} (FM). In addition, we determined catalase (CAT, EC 1.11.1.6) activity by Aebi's method (1984).

Flavonoids' concentration measurements

Contents of flavonoids were determined by Pekal and Pyrzynska's method (2014). Briefly, 0.3 mL $NaNO_2$ (5%, w/v) was blended with 1 mL of the 50% ethanolic extract, followed by the addition of 0.5 mL $AlCl_3$ (2%, w/v), as well as 0.5 mL NaOH solution (1 M) for neutralization. At 10 min later, we detected the OD value at 510 nm. Meanwhile, the standard flavonoid solution was utilized to prepare the standard curve.

Determination of soluble sugar and sucrose

We measured the content of soluble sugar through the anthrone approach according to Spiro's description (1966). In brief, we blended 100 µL root extract into the solution supplemented with 2.1 mM anthrone, 1.09 mM thiourea and 1.08 M H_2SO_4 till 3 ml. Thereafter, we heated the mixed solution for 10 min under 100 °C, and measured the OD value at 620 nm. In addition, we also constructed the calibration curve based on D-glucose for reference.

The sucrose content was determined by a spectrophotometric method following Alcázar et al. description (2005).

Determination of proline and betaine concentration

Proline concentration was determined as described by Bates et al. (1973). 3% (w/v) sulfosalicylic acid was utilized to extract 0.5 g samples. Thereafter, acid ninhydrin along with glacial acetic acid was added, and the obtained mixed solution was maintained for 1 h under 100 °C within the water bath, while the ice bath was used to terminate the reaction. We used toluene to extract proline and measured its OD value at 520 nm. Meanwhile, the proline level was determined based on the standard curve, which was presented in the manner of standard µg g^{-1} FM.

This study determined the glycine betaine (betaine) level according to Nishimura et al. description (2001). In brief, 0.1 g dried leaf powder was blended sufficiently with 5 ml distilled water. Then, we extracted the supernatants and filtered them using the 0.2-µm filter membrane. For the subsequent esterification, Gorham's approach (1984) after modification was applied. To be specific, 100 µL standard betaine solution or plant extract was added into the microtube and blended with 50 µL buffer solution consisting of 100 mM KH_2PO_4,100 mM $KHCO_3$ and acetonitrile at 1:1:4 (v/v), followed by the addition of 300 µL of the 20 mg/mL p-bromophenacyl bromide solution contained within acetonitrile. Later, we capped the tube and then heated it for 90 min under 80 °C. Afterwards, we adopted the centrifugal evaporator to evaporate the reaction mixture till dryness under 80 °C. Later, the electrolyte solution was run, and each sample was injected in hydrostatic mode (10 cm, 10 s), with the potential applied and peak being 15 kV and 254 nm, respectively. At last, we used the standard betaine solution calibration curve to obtain betaine levels in plant extracts.

Statistical analysis

Values were presented in the manner of mean ± SD. All experiments were totally randomized and three replicates were set for each experiment. GraphPad Prism 8.3 (San Diego, CA, USA) and Visio 2013 were used to create figures. SPSS22.0 (IBM, Chicago, IL) was employed for all statistical analyses, while Duncan's multiple range test was conducted to compare means.

Results

Effects of exogenous application of melatonin on the growth in sugar beet under salt stress

Relative water content and plant biomass of the leaves were measured to study how exogenous melatonin affected sugar beet development. As shown in Fig. 1a, the LRWC was significantly reduced under salt stress, whereas melatonin pretreatment significantly attenuated leaf water loss. On day 7, comparison with the non-salt control, the LRWC of M0 + S was significantly reduced by 43.66%, whereas the change of M60 + S treatment was insignificant. No significant was observed in the biomass of sugar beet seedlings between treatments under non-salt conditions (Fig. 1b). The application of salt stress inhibited plant development under salt stress in the presence or absence of melatonin (Fig. 2). For salt-exposed sugar beet seedlings subjected to melatonin pretreatment, their biomass elevated. Compared with M0 + S, the biomass of M30 + S, M60 + S, M90 + S leaves and petiole was increased significantly ($P < 0.05$), of which M60 increased 51.00% of leaf biomass and 43.79% of whole plant biomass.

Effects of exogenous application of melatonin on endogenous melatonin, Na^+ and K^+ concentration measurements under salt stress

As shown in Fig. 1c, the endogenous melatonin concentration of the leaves increased remarkably with exogenous treatment, and especially under salt stress. As shown in Fig. 1d, e, the application of exogenous melatonin significantly ($P < 0.05$) reduced the Na^+ concentration of the beet

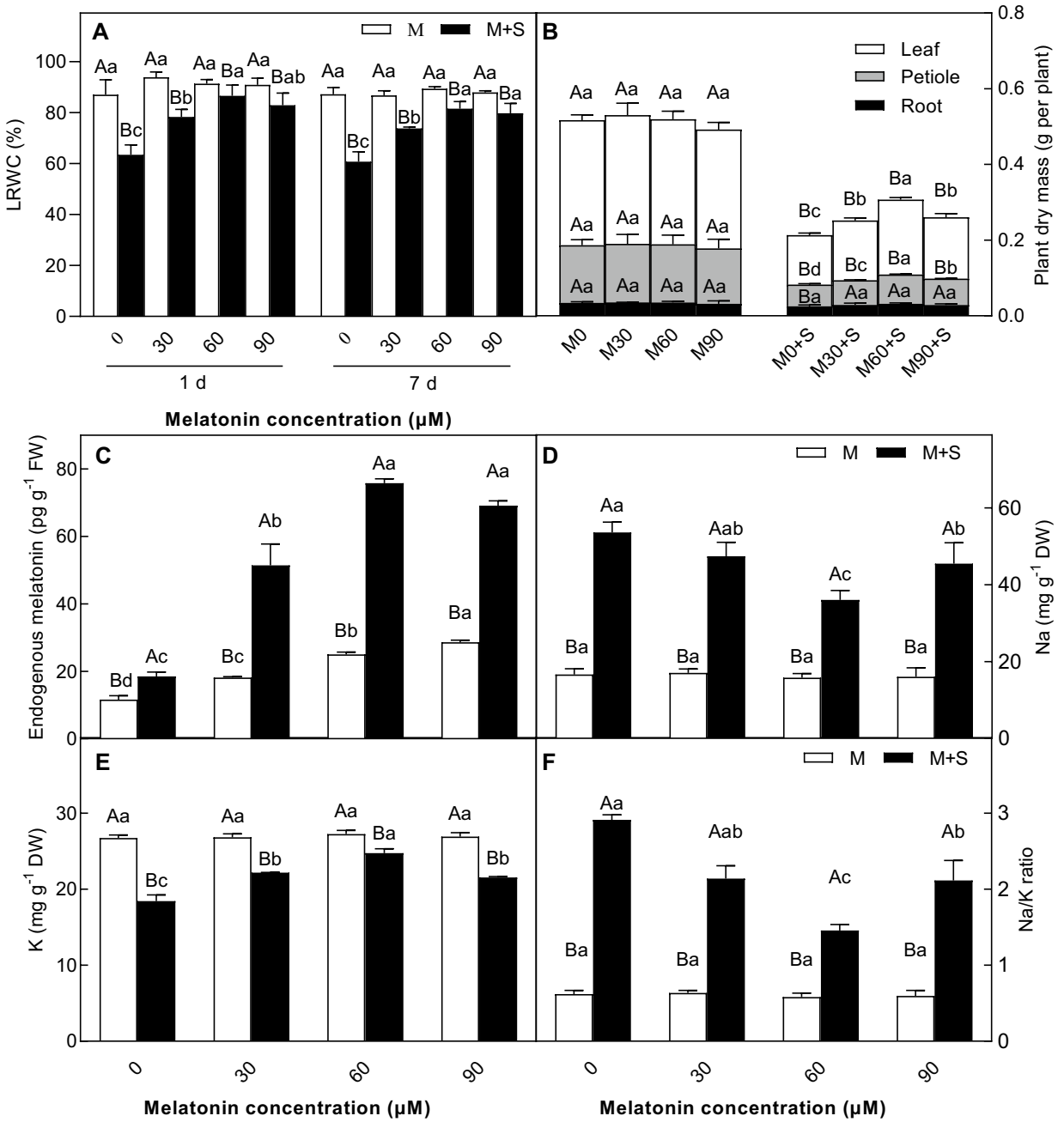

Fig. 1 Effects of different concentrations of melatonin on the growth of sugar beet seedlings. **a** Leaf relative water content of sugar beet seedlings under 0, 30, 60 and 90 μM melatonin and exposed to salt stress for 1 day. **b** Plant biomass in sugar beet seedlings with 0, 30, 60, and 90 μM melatonin treatments and exposure to salt stress for 7 days. **c** Melatonin concentration in sugar beet leaves following different treatments for 7 days. **d** Na^+ content **e** K^+ content, and **f** Na + /K + ratio in sugar beet leaves following different treatments for 7 days. M represents no salt stress after melatonin pretreatment, while M + S stands for melatonin treatment combined with salt treatment. In the figures, the diverse uppercase letters on the top of columns stand for statistically significant differences ($P < 0.05$; Duncan's range test) in salt treatment compared with control. The diverse lowercase letters on the top of columns stand for statistically significant differences ($P < 0.05$; Duncan's range test) among melatonin treatments. The same as below.

seedlings and increased the K^+ concentration, while reducing the Na^+/K^+ ratio. Compared with M0 + S, the Na^+/K^+ ratio of M30 + S, M60 + S, and M90 + S was decreased significantly ($P < 0.05$), of which 60 mM melatonin effect was the most obvious.

Fig. 2 Growth performance of sugar beet seedlings under salinity and melatonin conditions

Effects of exogenous application of melatonin on photosynthesis and chlorophyll fluorescence in sugar beet under salt stress

Photosynthesis shows tight correlation with plant development. Therefore, we analyzed the gas exchange chlorophyll concentration, carotenoids, together with Chl fluorescence parameters. As a result, melatonin made no obvious difference to the above parameters under non-salt stress conditions (Figs. 3 and 4). However, carotenoids and Chl markedly reduced after salt treatment ($P < 0.05$). On day 7, the concentration of chlorophyll and carotenoids of melatonin treatment M60 + S and M90 + S were higher compared with M0 + S. The chlorophyll content of M60 was increased by 51.08%, whereas carotenoid concentration was increased by 47.70%. As shown in Fig. 3d, RuBPcase activity showed no significant change between treatments on the first day of stress. On day 7, salt stress significantly decreased RuBPcase activity, which then increased and finally decreased with increasing melatonin concentration. Among them, M60 + S-treated treatment showed the greatest enzymatic activity, evidently increased compared with control.

Leaf F_v/F_m and q_P significantly decreased on the day 1 of salt stress, and were efficiently kept under melatonin exposure (Fig. 4). On day 7 of salt stress, the difference of F_v/F_m and q_P between all treatments was insignificant, whereas the Y(II) for M60 + S treatment was still significantly increased. Despite after a decrease on day 7, leaf Y(NPQ) increased substantially after salt treatment. Melatonin efficiently suppressed the elevation in Y(NPQ), whereas M60 treatment produced the lowest effect.

Salt exposure remarkably decreased photosynthesis (Fig. 5). On day 1 of stress, P_N, E and Gs of M0 + S decreased sharply, until these recovered on day 7. The melatonin treatment significantly alleviated the salt stress, and M60 + S-treated P_N, E and g_s higher than M0 + S by 818.25%, 605.95% and 705.11%, respectively.

Exogenous melatonin mitigated ROS damage while enhancing the antioxidant enzymatic activities within salt-exposed sugar beet

As shown in Fig. 6a–c, on day 1 and 7 day after salt stress, compared with M0, M0 + S resulted in increased in MDA concentrations of sugar beet leaves by 174.13% and 203.61% and $O_2 \cdot^-$ by 55.54% and 72.90%, respectively. However, melatonin treatment significantly reduced MDA and $O_2 \cdot^-$ under salt stress. Compared with M0 + S, M60 + S produced lower MDA (− 46.30% and − 12.89%) and $O_2 \cdot^-$ (− 31.24% and − 23.19%) on days 1 and 7 ($P < 0.05$). On day 1 of salt stress, with the increase in melatonin concentration, the concentration of H_2O_2 in leaves first increased first and then decreased. The trend on day 7 of stress was the opposite of that on day 1, in which M60 + S and M90 + S was significantly reduced.

The results showed that under non-salt stress conditions, melatonin did not change the activity of SOD, POD and CAT (Fig. 6). Under salt stress, the activity of SOD, POD, and CAT in melatonin-treated sugar beet seedlings was significantly higher than that under M0 + S. On day 1 of stress, the SOD, POD, and CAT activities of M60 + S treatment were 68.46%, 210.56%, and 79.13% higher than that under M0 + S, respectively. On day 7 of stress, SOD and CAT activities were maximal, and the CAT activity was approximately twice that on day 1. However, compared day 7, the POD activity was stronger on day 1 under salt stress.

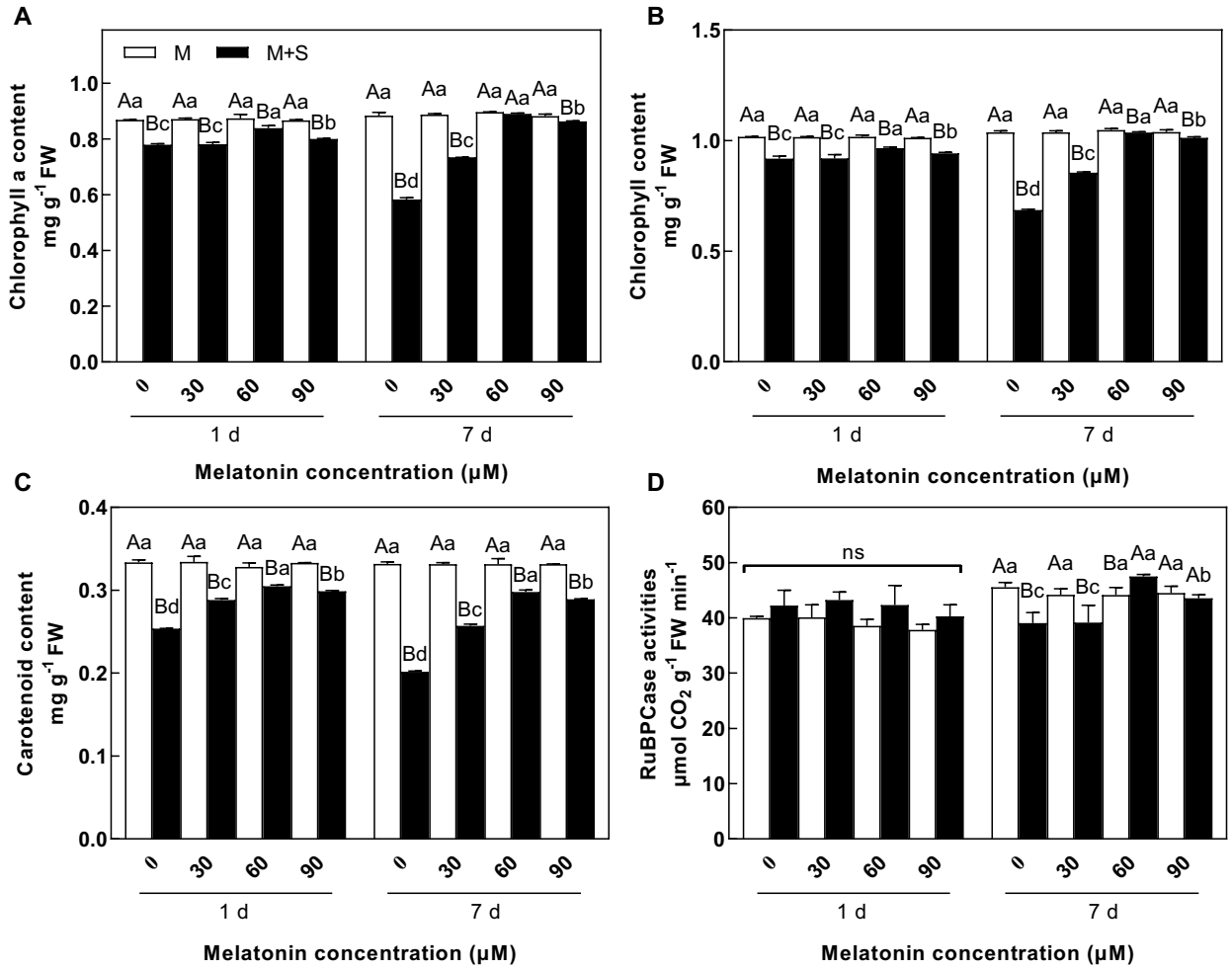

Fig. 3 Effects of different concentrations of melatonin on photosynthetic pigments and the RuBPcase activity. **a** Chlorophyll *a* concentration, **b** chlorophyll concentration, **c** carotenoid concentration and **d** RuBPcase activity of sugar beet seedlings with 0, 30, 60, and 90 μM melatonin treatments and exposure to salt stress for 1 and 7 days

Exogenous application of melatonin altered accumulation of osmolytes in sugar beet under salt stress

The accumulation of proline, betaine, and flavonoids in sugar beet seedlings was evaluated in this study. Figure 7 demonstrates that salt stress significantly enhanced the accumulation of compatible solutes in sugar beet seedlings. Salt stress reduced the sucrose concentration of leaves; however, it increased the soluble sugar concentration. In the absence of salt exposure, melatonin treatment did not significantly affect sucrose or reduce sugar level. Upon salt exposure, melatonin treatment remarkably decreased sucrose level within leaves of sugar beet while markedly increasing soluble sugar content ($P < 0.05$). Compared with day 1 of salt stress, the trend on day 7 was more pronounced.

Relative to controls, betaine and proline contents markedly elevated upon salt exposure ($P < 0.05$), whereas the concentration of flavonoids was significantly reduced considerably. On day 1 of salt stress, melatonin treatment significantly increased the concentration of proline and betaine in sugar beet leaves ($P < 0.05$) and inhibited the reduction in flavonoid concentration. The increase in proline concentration was the most pronounced, M30 + S, M60 + S and M90 + S increased by 91.22%, 216.95%, and 28.21%, respectively, compared with M0 + S (Fig. 7). Compared with day 1 day, proline concentration was significantly decreased on day 7 of salt stress, whereas the concentration of betaine increased significantly by 4.86%, 34.78%, and 29.89%, respectively.

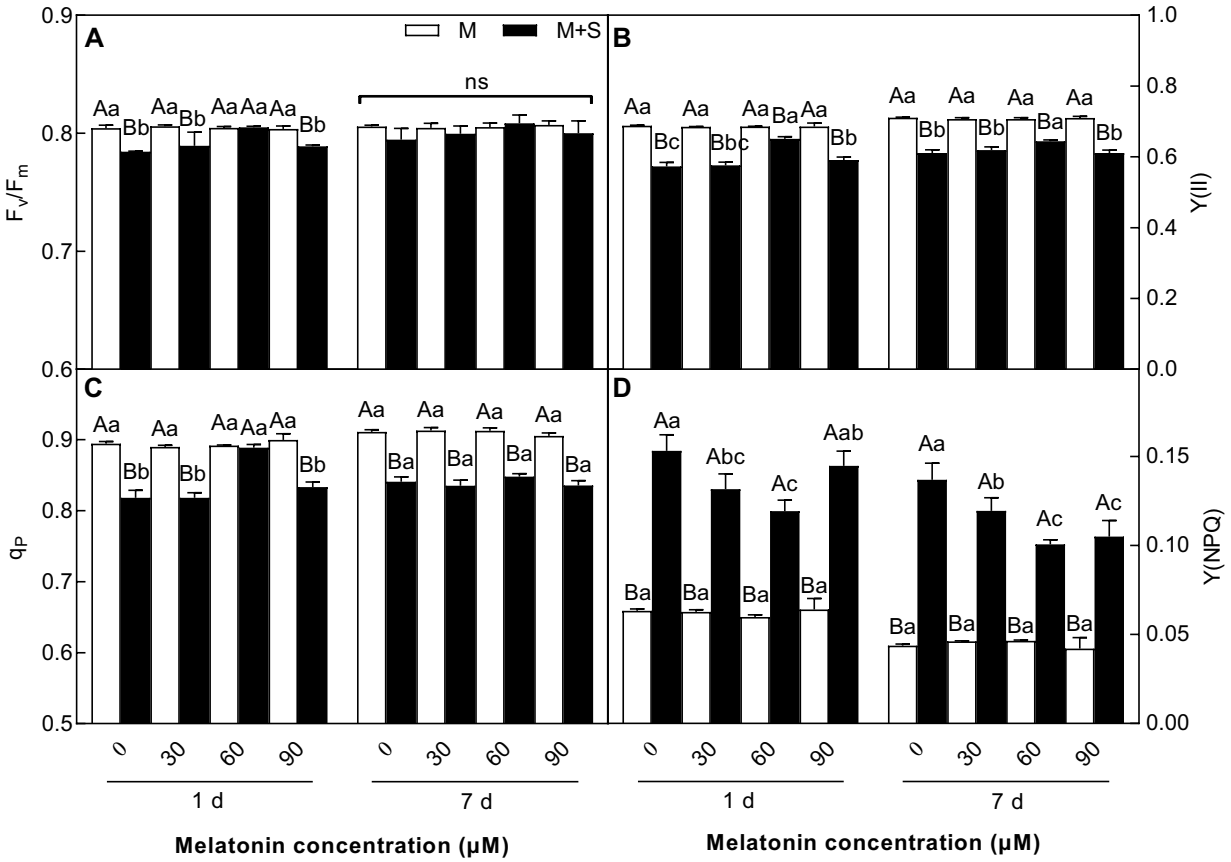

Fig. 4 Effects of different concentrations of melatonin on chlorophyll fluorescence parameters. **a** F_v/F_m, **b** Y(II), **c** q_P and **d** Y(NPQ) of sugar beet seedlings with 0, 30, 60 and 90 μM melatonin treatments and exposed to salt stress for 1 and 7 days

Discussion

Biomass is a reliable indicator of plant growth, whereas salt stress significantly suppresses plant growth (Egea et al. 2018; Sui et al. 2018). Melatonin exerts a vital part in the increase of biomass through elevating P_N in the exposure to salt (Li et al. 2017b). Overcoming osmotic stress accounts for a crucial strategy for the adaptation to salt exposure in plants. In this work, salt exposure markedly suppressed sugar beet seedling growth, reduced P_N, and promoted plant leaf dehydration, resulting in reduced plant biomass (Fig. 1). However, melatonin effectively alleviated the water loss of beet seedlings and significantly improved P_N, which in turn increased the biomass of the plants.

Different concentrations of exogenous melatonin could increase the endogenous melatonin concentration only to a certain extent, with no absolute effect. The leaf endogenous melatonin content was dose-dependent when sugar beet roots were pre-treated with exogenous melatonin (Fig. 1c). On the contrary, certain studies have suggested that relatively high exogenous melatonin concentration has a more effective promoting effect on tomato (Siddiqui et al. 2019) and corn seedlings (Chen et al. 2018). Compared with the results of these studies, multiple concentrations may be more representative. Consistent with the observations from Li et al. on watermelon seedlings (Li et al. 2017a), exogenous melatonin content had first increased and then weakened impacts on sugar beet seedling growth.

Salt exposure has certain impact on plant photosynthesis via non- and stomatal limitations (Zhou et al. 2016). The stomatal limitation is attributed to the partial closure of the stomata, resulting in a reduced concentration of CO_2 entering the mesophyll cells of the plant, which in turn blocks photosynthesis (Sharkey et al. 2007). In this work, the decrease in E and g_s and the high level of C_i indicated that reduced P_N could be a result of physiological dryness which caused by the high osmotic stress of salinity, leading to almost complete closure of stomata (Fig. 4). The carbon dioxide generated simultaneously during plant respiration increases C_i. In the late stage of melatonin application, P_N, g_s, and C_i increased indicating melatonin maintenance of stomatal opening under salt stress as the primary reason for elevated P_N in the leaves of sugar beet seedlings.

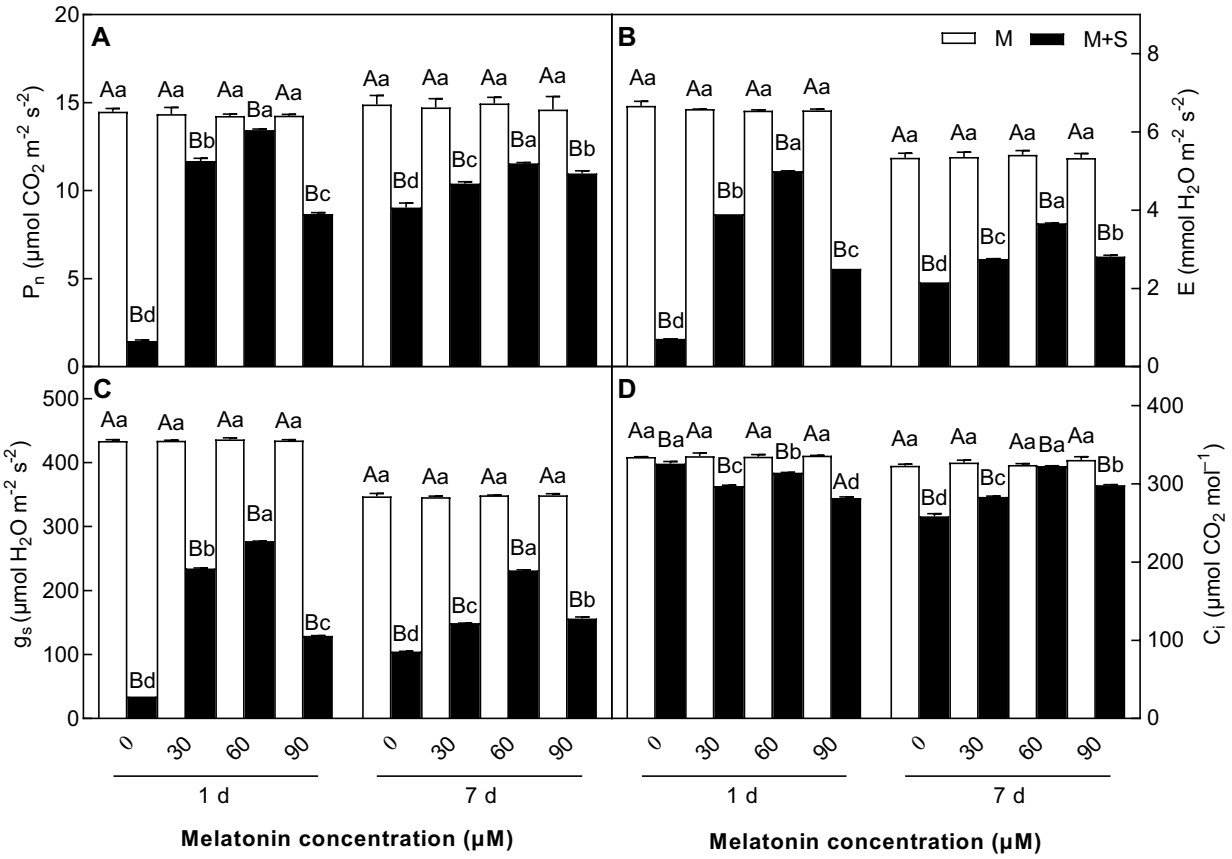

Fig. 5 Effects of different concentrations of melatonin on gas exchange parameters. **a** Net photosynthetic rate (P_N), **b** transpiration rate (E), **c** stomatal conductance (g_s), and **d** intercellular CO_2 concentration (C_i) of sugar beet seedlings with 0, 30, 60 and 90 μM melatonin treatments and exposure to salt stress for 1 and 7 days

The relationship between abiotic stress and RuBPcase activity is highly controversial. Studies have shown that leaves do not alter RuBPcase activity when they are within the acceptable RWC range (Bota et al. 2004; Lawlor 1995). However, there exist studies that hold the opposite view and believe that increasing the RuBPcase activity contributes to crop growth and can also increase the crop yield under adverse conditions (Ren et al. 2018). RuBPcase activity was not markedly changed during the initial salt treatment stage, which might be caused by the physiological drought of sugar beet (Fig. 3). Under long-term salt stress, the RuBPcase activity of sugar beet leaves was significantly reduced that improved with melatonin pretreatment.

A series of photoreaction processes caused by photosynthetic pigments (chlorophyll, carotenoids, etc.) in plants to absorb photosynthetically active radiation forms the basis of photosynthesis (Fig. 3). Studies have shown that salt stress reduces the photosynthetic pigment concentration of plant leaves. Melatonin maintains a high pigment concentration and enhances photosynthesis. Results from studies on corn and watermelon concluded the same (Chen et al. 2018; Li et al. 2017b).

F_v/F_m and Y(II) are indicative of the real and maximum light energy conversion efficiency of Photosystem II, respectively (Genty et al. 1989; Kitajima and Butler 1975). In the current study, the F_v/F_m and Y(II) of the leaves significantly increased compared with the non-melatonin treatment, and P_N, E, and g_s increased in the later stage of stress (Fig. 3), which indicated that melatonin exerted a positive regulatory effect on plant photosynthetic systems under salt stress. Chen et al. (2018) showed that melatonin treatment could alleviate oxidative damage in photosynthetic organs. Under salt stress, melatonin increased E or g_s; however, it did not decrease LRWC, indicating that melatonin could positively affect water conservation and photosynthesis protection (Ahmad et al. 2019; Chen et al. 2018).

Until date, only a few studies are available on the protective effect of melatonin on the oxidative defense system of sugar beet in the presence of salt exposure. Salt exposure frequently leads to the disturbance in ROS generation and scavenging, thereby increasing ROS accumulation and aggravating oxidative damage to proteins, lipids, and nucleic acids (Tahjib-Ul-Arif et al. 2019; Zhang et al. 2019). Excessive amounts of ROS within plant cells will induce lipid

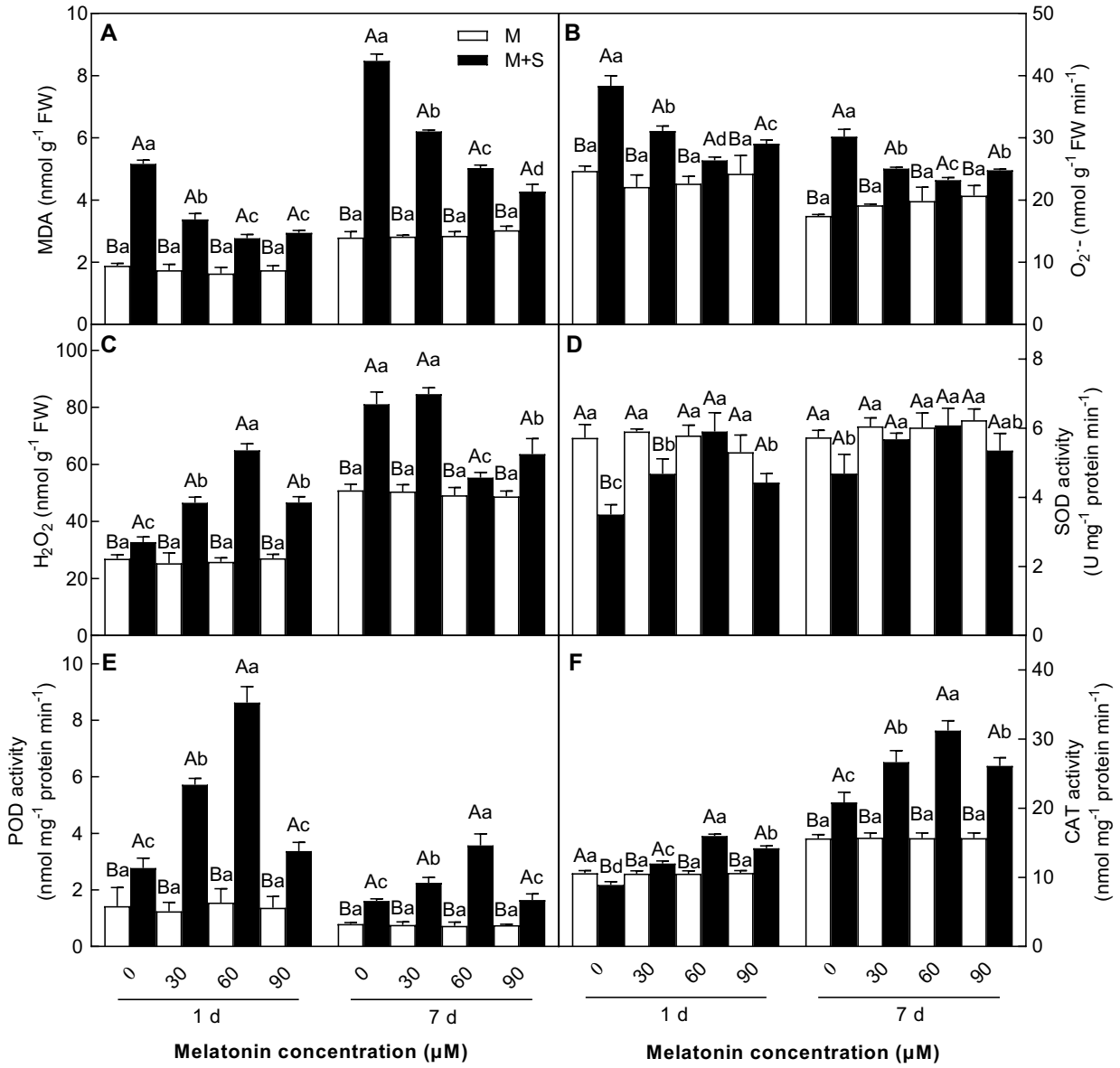

Fig. 6 Effects of different concentrations of melatonin on reactive oxygen species and the antioxidant system. **a** Malondialdehyde (MDA), **b** superoxide anion ($O_2 \cdot^-$), **c** catalase (H_2O_2), **d** superoxide dismutase (SOD), **e** peroxidase (POD) and **f** catalase (CAT) of sugar beet seedlings with 0, 30, 60, and 90 μM melatonin treatments and exposure to salt stress for 1 and 7 days

peroxidation while increasing H_2O_2, MDA and $O_2 \cdot^-$ levels (Farouk and Al-Amri 2019b, c). In the present study, melatonin-treated MDA and $O_2 \cdot^-$ accumulation was lower, indicating that melatonin attenuated the membrane lipid peroxidation under salt stress and reduced the oxidative damage of sugar beet seedlings under salt conditions (Fig. 6). In salt-stressed oat (Gao et al. 2019), corn (Chen et al. 2018), and soybean (Wei et al. 2015), melatonin was found to be involved in reducing the concentration of MDA, thus keeping the membrane permeability and integrity (Li et al. 2019). Critical antioxidant enzymes, including POD, CAT and SOD, usually exert important parts in preventing damage to plant cell membrane systems by ROS accumulation (Jaleel et al. 2009; Tahjib-Ul-Arif et al. 2019; You and Chan 2015). As shown in Fig. 6, the activity of SOD, POD, and CAT in sugar beet leaves was significantly increased in the current study, with POD playing the most crucial role, indicating that melatonin effectively decreased ROS accumulation and avoided membrane structural or functional degradation in sugar beet cells in the presence of salt exposure. These results are strengthened by those of the study that reports that melatonin effectively participates in physiologically regulating plants upon salt exposure (Li et al. 2019). In the present study, the flavonoid concentration following

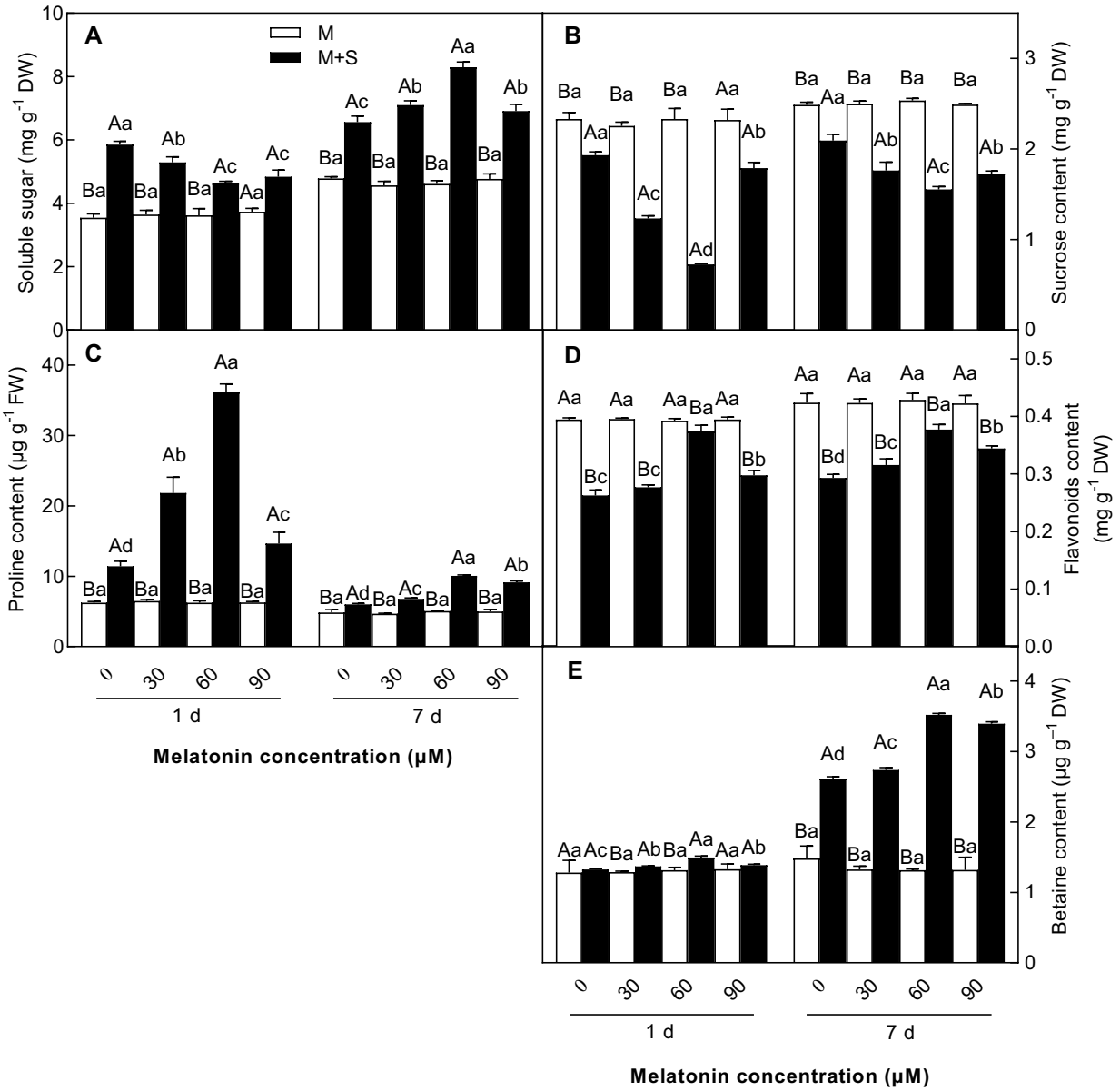

Fig. 7 Effects of different concentrations of melatonin on osmotic adjustment substances. **a** Soluble sugar concentration, **b** sucrose concentration, **c** proline concentration, **d** flavonoid concentration, and **e** betaine concentration of sugar beet seedlings with 0, 30, 60, and 90 μM melatonin treatments and exposure to salt stress for 1 and 7 days

melatonin treatment under salt stress was opposite to that of Y(NPQ). Flavonoids were inhibited by salt stress, and melatonin pretreatment protected the leaves by decreasing the reduction of flavonoids.

Recently, numerous studies have demonstrated that H_2O_2 acts as the signal transduction element in plants in the presence of biotic or abiotic stress (Li et al. 2018; Neto et al. 2005; Smirnoff and Arnaud 2019). In the present study, melatonin pretreatment significantly reduced MDA and $O_2\cdot^-$ levels in the early stage of stress; however, it increased H_2O_2 concentration in seedlings on day 1, which could be attributed to the fact that H_2O_2 as a signaling substance involved in plant responses to stress (Neto et al. 2005). On day 7, H_2O_2 contents in M60 + S and M90 + S were lower than that of M0 + S, suggesting that it was cleared by the antioxidant system induced by melatonin (Chen et al. 2018).

The cellular energy status has been suggested to be a vital modulator for plant growth as well as stress decrease (Jamsheer and Laxmi 2015). In our study, salt stress significantly reduced sucrose concentration; however, it increased the concentration of soluble sugar (Fig. 7). Melatonin treatment effectively promoted this process. According to certain

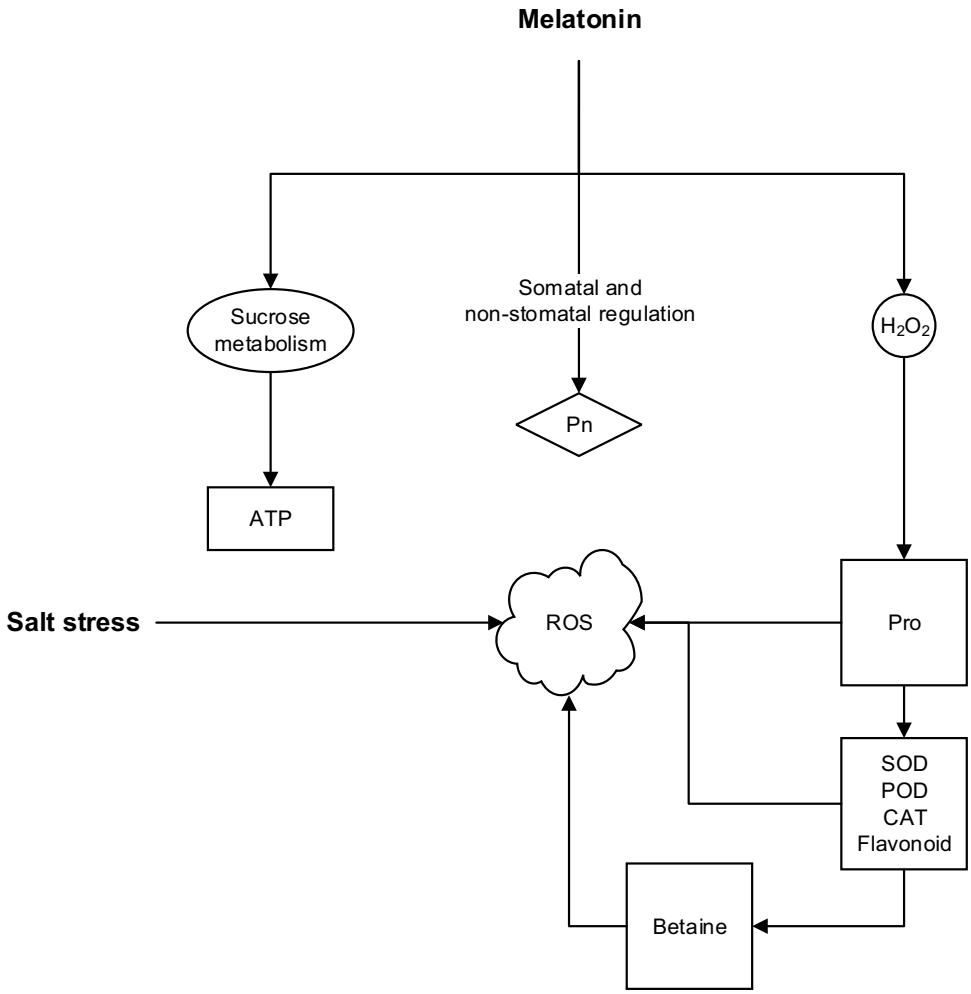

Fig. 8 The model showing that melatonin preconditioning reduces the potential mechanism of salinity-induced photosynthesis inhibition and oxidative stress in sugar beet

studies, plants are required to consume energy in response to stress (Crawford et al. 2018; De Block et al. 2005). In the early stages of salt stress, melatonin reduced the concentration of soluble sugar. We speculate that melatonin could cope with stress by enhancing energy metabolism in cells (Zhang et al. 2017a; Zhao et al. 2020). Cells can produce large amounts of ATP through sucrose metabolism and TCA cycling accompanied by the accumulation of several primary and secondary metabolites, such as proline, betaine, and flavonoids. The accumulation of soluble sugars, proline, and betaine ensures plant photosynthesis (Ashraf and Foolad 2007; Rady et al. 2018; Wei et al. 2015). In general, an increase in soluble sugar and proline concentration helps reduce damage under abiotic stress (Ben Ahmed et al. 2010; Yang et al. 2007).

Proline and betaine account for 2 main organic permeants accumulating within multiple plant varieties upon environmental stress (Farouk and Al-Amri 2019c). Generally, plants respond to abiotic stress on the basis of certain permeates production and accumulation. These permeations not only positively affect membrane and enzyme integrity but also exert vital parts in plant osmotic adjustment that mediates growth under stress conditions (Liu et al. 2018; Vicente et al. 2016). Our data suggest that melatonin could first act through responsive regulation of proline (early stage) and betaine (late stage) and subsequently activate an antioxidant defense system to combat cellular damage caused by salt stress (Fig. 8).

Conclusion

Our study demonstrated that exogenous application of melatonin could alleviate inhibition of the growth of sugar beet seedlings which exposed to salt stress. Melatonin may rapidly enhance the production of H_2O_2 as a signaling substance in sugar beet leaves, induce high levels of proline synthesis, and perform the osmotic adjustment. Simultaneously, melatonin could enhance the activity of protective enzymes to remove ROS.

Author contribution statement LL contributed to the experimental design, data analyses and manuscript writing; CFL and ZJG conceived the study, contributed to the experimental design, and revised the manuscript; DL, BW, ZYW, PFZ, XYL, JTC, SYZ, CLZ and YBW helped with the sample preparation and data analyses; and all the authors approved the manuscript.

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Declarations

Conflict of interest The authors declare no conflict of interests.

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