Journal of Soil Science and Plant Nutrition (2022) 22:3527–3540
https://doi.org/10.1007/s42729-022-00907-8

ORIGINAL PAPER

Antioxidant Machinery and Glyoxalase System Regulation Confers Salt Stress Tolerance to Wheat (*Triticum aestivum* L.) Plants Treated with Melatonin and Salicylic Acid

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Received: 19 March 2022 / Accepted: 14 June 2022 / Published online: 19 July 2022

Abstract
Plant growth regulators melatonin (MT) and salicylic acid (SA) have potent roles in plant salt tolerance. However, only a few reports have studied the influence of their combined treatment on plant salt tolerance. The current study, as a first investigation, was aimed to evaluate the effect of MT and SA combined treatment on the antioxidant and glyoxalase defense machineries of salt-stressed wheat plants. In the present study, the potential role of 70 µM MT and/or 75 mg l^{-1} SA on mitigating salt injury (6.0 and 12.0 dS m^{-1} salinity levels) was investigated in wheat (*Triticum aestivum* L. cv. Sids 14). Exogenously applied MT and/or SA improved the activity of ascorbate peroxidase, monodehydroascorbate reductase, dehydroascorbate reductase, glutathione reductase, glutathione peroxidase, and glutathione S-transferase, which effectively scavenged reactive oxygen species (ROS) in stressed plants. Excessive accumulation of the toxic methylglyoxal was reversed via the up-regulation of the glyoxalase system (glyoxalase I and glyoxalase II) and the ascorbate–glutathione cycle. Foliar applications also reduced membrane damage by lowering lipid peroxidation and protein oxidation due to effective ROS detoxification by antioxidants such as ascorbate, glutathione, phenols, and flavonoids. Moreover, exogenous MT and/or SA applications increased endogenous MT and SA levels under both non-saline and saline conditions. The combined treatment of MT and SA yielded the best results. Overall, this combined treatment regulated the antioxidant machinery and glyoxalase system, suggesting a role for it in salt stress mitigation. Therefore, it can be considered as an effective method for reducing salt toxicity in sustainable agricultural systems.

Keywords Antioxidant defense system · Glyoxalase defense system · Melatonin · Salicylic acid · Salt stress · Wheat (*Triticum aestivum* L.)

1 Introduction

Salt stress is one of the most damaging environmental stresses that causes significant losses in agricultural productivity. Approximately 20% of arable land throughout the world are affected by salt (Zörb et al. 2019). Therefore, it is important to increase crops salt tolerance for sustainable agricultural development. High salt concentrations induce the accumulation of ROS, which are highly toxic, causing lipid peroxidation, protein denaturalization, carbohydrates breakdown, enzyme inhibition, and finally cell death (Talaat 2015; Nahar et al. 2016; Kamran et al. 2020). Plants have evolved potential enzymatic and non-enzymatic antioxidant defense systems to combat oxidative stress and increase their tolerance to saline conditions. The enzymatic system includes superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (POD), ascorbate peroxidase (APX), glutathione peroxidase (GPX), glutathione reductase (GR), glutathione S-transferase (GST), monodehydroascorbate reductase (MDHAR), and dehydroascorbate reductase (DHAR), while the non-enzymatic antioxidants include glutathione (GSH), ascorbic acid (AsA), phenolic compounds, flavonoids, carotenoids, α-tocopherol, etc. (Singh et al. 2019; Shen et al. 2021; Shopova et al. 2021). Salt stress also stimulates methylglyoxal (MG) production. Excessive accumulation of MG

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causes oxidative damage to carbohydrates, proteins, and lipids (Nahar et al. 2016). To eliminate excessive MG, a GSH-dependent glyoxalase system, including glyoxalase I (Gly I) and glyoxalase II (Gly II) has evolved in plant cells. GSH-dependent glyoxalase system plays a vital role in stress tolerance by converting excess MG to D-lactate (Rahman et al. 2016; Kamran et al. 2020).

Melatonin (MT, *N*-acetyl-5-methoxytryptamine) is an important plant hormone. In 1995, the MT was detected in the plant kingdom (Dubbels et al. 1995) and now recognized as a ubiquitous biomolecule synthesized in all kingdoms, from prokaryotes to eukaryotes, from animals to plants (Arnao and Hernández-Ruiz 2014). Evidences indicate that MT is a plant master regulator that promote plant growth (Park et al. 2021; Yu et al. 2021) and crop yield (Zahedi et al. 2020). Moreover, MT plays an important role in plant stress tolerance (Sun et al. 2018; Khan et al. 2020; Shafi et al. 2021). It has an efficacious ROS scavenging capacity by reacting with hydroxyl and peroxy radicals (Khan et al. 2020; Pardo-Hernández et al. 2020). Previous studies reported that MT works in plant by regulating gene expression, activating antioxidant enzymes, scavenging ROS molecules, and stabilizing biological membranes (Debnath et al. 2019; Pardo-Hernández et al. 2020; Zhang et al. 2021a). Exogenous application of MT induces tolerance to salt stress via enhancing photosynthetic efficiency, nitrogen metabolism, ion uptake, and antioxidant defense system (Ahmad et al. 2021; Park et al. 2021; Talaat 2021a,b; Talaat and Shawky 2022). There are also reports that exogenous MT improves crop salt tolerance by improving redox homeostasis, transcription factors, and plant hormones (Chen et al. 2021; Zhang et al. 2021b; Yu et al. 2021). Treatment with exogenous MT has been shown to elevate the endogenous MT content (Li et al. 2018a; Ni et al. 2018). Endogenously synthesized MT performs a vital role in coping with salt stress (Ke et al. 2018; Zahedi et al. 2020).

Salicylic acid (SA, 2-hydroxybenzoic acid) as a plant growth regulator, can stimulate several biochemical events, resulting in a new metabolic state (Wani et al. 2017). It has been shown that exogenously applied SA can significantly increase plant growth under both non-saline and saline conditions (Hoang et al. 2020; Kamran et al. 2020; Es-sbihi et al. 2021). Several studies evidenced that SA modulates plant response to various abiotic stresses (Zanganeh et al. 2019; Bukhat et al. 2020; Hediji et al. 2021). SA-regulated plant salt tolerance could contribute to maintaining cellular detoxification through the regulation of antioxidant and glyoxalase defense systems (Kamran et al. 2020; Kaya et al. 2020a; Shamili et al. 2021). Furthermore, SA is involved in the regulation of important plant physiological processes such as photosynthesis, nitrogen metabolism, and nutrient acquisition to counteract saline conditions (Bukhat et al. 2020; Talaat 2021a, b; Talaat and Shawky 2022).

The MT-SA crosstalk plays an important role in regulating plant stress responses. Interestingly, MT and SA biosynthesis pathways initiate from common precursor—chorismic acid (Hernández-Ruiz and Arnao 2018). Exogenous MT up-regulates the expression of SA-related genes in *Arabidopsis* and leads to an increased resistance to the pathogen (Weeda et al. 2014). A previous study by Lee et al. (2015) also showed that the increased MT levels in plants leads to enhanced SA levels, since MT induces SA biosynthetic genes. It appears that MT is a key player, directly or indirectly enhancing SA, which finally active plant stress tolerance (Hernández-Ruiz and Arnao 2018; Park et al. 2021). Therefore, it is important to study the influence of MT and SA combined treatment to develop an effective method for crop improvement under saline conditions.

Wheat (*Triticum aestivum* L.) is one of the most important grain crops in the world. It faces severe losses in its productivity due to soil salinization (Talaat 2019). Some reports have investigated the effect of single application of MT or SA on plants exposed to salt stress; however, only a few have studied the influence of their combined treatment on plant salt tolerance. To fill this gap, we conducted this investigation to evaluate the effect of MT and SA combined treatment on the antioxidant and glyoxalase defense machineries of salt-stressed wheat plants. Hence, we hypothesized that the co-application of MT and SA might improve wheat salt tolerance via counteracting the injury effects of salt-induced oxidative stress. To verify this hypothesis, the response of antioxidant machinery including activity of antioxidant enzymes (APX, DHAR, MDHAR, GR, GPX, GST), content of antioxidant molecules (AsA, GSH, phenolic compounds, flavonoids), lipid peroxidation, protein oxidation, and 2,2-diphenyl-1-picrylhydrazyl (DPPH) activity along with the response of glyoxalase defense system including activity of (Gly I, Gly II) and content of MG were evaluated under the impact of MT and/or SA foliar applications on wheat plants grown under both non-saline and saline conditions. We have also evaluated the endogenous MT and SA levels in wheat plants. The results will contribute to further understanding of the role played by MT and/or SA in ameliorating salt stress.

2 Materials and Methods

2.1 Plant Material and Experimental Design

A pot experiment was carried out twice, on September 10th, of 2019 and 2020 in the greenhouse of Plant Physiology Department, Faculty of Agriculture, Cairo University, Egypt, under natural light and temperature conditions with 65% relative humidity and 22/16 ± 2 °C (day/night) temperature. Wheat (*Triticum aestivum* L. cv. Sids 14) grains

were obtained from the Wheat Research Department, Agriculture Research Center, Ministry of Agriculture, Egypt. The pots (30 cm in diameter and 35 cm in height) were filled with 15 kg of clay loamy soil (sand 37%, silt 28%, clay 35%). Ammonium nitrate (33.5% N), calcium superphosphate (15.5% P_2O_5), and potassium sulfate (48% K_2O) were applied at the rates of 2.0, 2.0, and 0.5 g pot^{-1}, respectively. In addition, 30 days after planting, 2.0 g pot^{-1} ammonium nitrate was added. Table S1 shows the soil chemical analysis, which performed according to Cottenie et al. (1982).

Pots were divided into three groups before sowing. The first group was allocated as a control (non-saline; 0.1 dS m^{-1}), while the other two were assigned as two levels of salinity treatment (6.0 and 12.0 dS m^{-1} salinity level; obtained by adding to the soil a mixture of NaCl, $CaCl_2$, and $MgSO_4$ at the molar ratio of 2:2:1, respectively) (Talaat 2021a, b; Talaat and Shawky 2022).

Wheat plants at 45- and 90-day-old were foliar sprayed with 0.00 (distilled water; DW), 70 µM MT, 75 mg L^{-1} SA, and 70 µM MT + 75 mg L^{-1} SA. The concentrations of 70 µM MT and 75 mg L^{-1} SA were the most effective concentrations according to preliminary experiment within a range from 0 to 100 µM for MT and from 0 to 100 mg L^{-1} for SA. Tween-20 (0.05%) was used as a surfactant at the time of treatment.

Twelve treatments consisting of three salinity levels [0.1 dS m^{-1} (non-saline), 6.0, and 12.0 dS m^{-1}] and four foliar applications (distilled water, 70 µM MT, 75 mg L^{-1} SA, and 70 µM MT + 75 mg L^{-1} SA) were implemented. The layout of the experiment was a completely randomized design using two factors (salinity levels and foliar treatments) and four replicates.

2.2 Plant Growth Measurements

Shoot height, total leaf area, and shoot dry weight were recorded in 70-day-old wheat plants. A portable leaf area meter (LI-COR 3000, Lambda Instruments Corporation, Lincoln, NE, USA) was used to measure the total leaf area. Shoot dry weight was measured after 48 h of oven drying at 70 °C. Data were collected from four replicates, each of which contained six plants gathered from the same pot. Grain yield was recorded at maturity.

The physiological and biochemical traits, including DPPH activity, lipid peroxidation, protein oxidation, antioxidant enzymes activity, antioxidant molecules content, MG content and its relevant enzymes, and endogenous MT and SA levels were determined in 70-day-old (after 25 days of MT and/or SA first application) wheat leaves. Data were collected from four replicates, each of which contained six plants gathered from the same pot.

2.3 Evaluation of DPPH Radical-Scavenging Activity

Antioxidant activities were measured by a DPPH radical-scavenging assay according to the method described by Sharma and Bhat (2009). In brief, fresh wheat leaves (0.25 g) were homogenized in 2.5 mL of methanol, and the homogenates were centrifuged at 8000 × *g* for 15 min at 4 °C. Then, 0.4 mL of the supernatant was mixed with 1.1 mL of a 50 mM methanolic DPPH solution. The absorbance was read at 517 nm after 30 min of incubation at 30 °C in the dark. Inhibitory percentage on free radical DPPH was calculated with the following equation: Radical scavenger effect (%) = [(A—B)/A] × 100, where A is the absorbance of the control reaction and B is the absorbance of the sample.

2.4 Antioxidant Enzymes Extraction and Assay

Fresh leaf samples (0.5 g) were ground with 5 mL of ice-cold 100 mM phosphate buffer (pH 7.4) containing 1% polyvinyl pyrrolidine and 1 mM EDTA, then centrifuged at 15,000 × *g* for 10 min at 25 °C. The supernatant was collected and used for the determination of enzymatic activity. According to the method of Ramel et al. (2009), the APX activity was determined by measuring the decrease in absorbance at 290 nm caused by ascorbate oxidation. NADH oxidation at 340 nm was used to determine MDHAR activity (Hossain et al. 1984). The DHAR activity was measured by examining ascorbate formation at 265 nm (Doulis et al. 1997). The oxidation of NADPH at 340 nm was used to measure GR activity (Foyer and Halliwell 1976). The activities of GPX and GST were evaluated according to (Nagalakshmi and Prasad 2001). 1-Chloro-2,4-dinitrobenzene (CDNB) was used to start the reaction, and the absorbance change at 340 nm was measured to calculate GPX and GST activity.

2.5 Reduced (GSH) and Oxidized (GSSG) Glutathione as well as Reduced (AsA) and Oxidized (DHA) Ascorbate Measurements

Fresh leaf samples (0.5 g) were homogenized in 5 mL of 5% (w/v) sulfosalicylic acid and centrifuged at 20,000 × *g* for 20 min at 4 °C. The GSH was oxidized using the 5,5′-dithio-bis-nitrobenzoic acid to give GSSG and TNB (5-thio-2-nitrobenzene). GSSG was reduced to GSH by the action of GR and NADPH. GSSG was assayed from the sample after removal of GSH by 2-vinylpyridine and triethanolamine derivatizations. Changes in absorbance due to the rate of TNB formation were measured at 412 nm. The amount of GSH was the difference between total glutathione and GSSG (Hernandez et al. 2010). The assay of AsA is based on the reduction of Fe^{3+} to Fe^{2+} by ascorbic acid in acidic solution. The Fe^{2+} and dipyridine produce a colored complex that absorbs at 525 nm.

DHA was reduced to AsA by pre-incubating the sample with dithiothreitol (DTT). The excess DTT was removed with *N*-ethylmaleimide, and the total ascorbate was determined. The amount of DHA was the difference between total ascorbate and the AsA (Hernandez et al. 2010).

2.6 Determination of MG Content as well as Gly I and Gly II Activities

The content of MG was measured using the technique of Yadav et al. (2005). Leaf samples (0.5 g) were homogenized in 5% perchloric acid and centrifuged at 11,000 × *g* for 15 min. The homogenates were decolorized, neutralized, and then mixed with nacetyl-L-cysteine and sodium dihydrogen phosphate. The formation of N-α-acetyl-S-(1-hydroxy-2-oxo-prop-1-yl) cysteine was measured spectrophotometrically at 288 nm after 10 min. The MG content was calculated using a standard curve and was expressed as μg g^{-1} FW. As previously described by Li et al. (2018b), the activities of Gly I and Gly II were estimated. The enzyme extraction buffer was the same as that used for antioxidant enzymes. The absorbance of the assay mixture was determined at 240 nm and 412 nm for Gly I and Gly II, respectively. Enzymes activities were expressed as μmol min^{-1} mg^{-1} protein.

2.7 Quantification of Malondialdehyde (MDA) and Protein Oxidation

Fresh wheat leaves (0.1 g) were homogenized with 900 μL buffer to estimate MDA, following the instructions described in MDA kit, according to the procedure given by Nawaz et al. (2018). At wavelength of 532 nm, the content of MDA, was measured. Protein oxidation level was determined by using 5, 5′-dithiobis (2-nitrobenzoate) reagent (Ellman 1959). At a wavelength of 412 nm, it was recorded.

2.8 Determination of Phenolic and Flavonoid Compounds

Total phenolic compounds and total flavonoids were measured as described by Wang and Huang (2015). Briefly, 0.2 g fresh wheat leaves were extracted with a mixture of cold 70% (v/v) methanol, 2% (v/v) formic acid, and 28% (v/v) ethanol. The homogenate was centrifuged at 10,000 *g* for 10 min at 4 °C. Total phenolic compounds were assayed quantitatively by A_{765} with Folin–Ciocalteau reagent. The absorption of total flavonoids was measured at 510 nm. The concentrations of phenols and flavonoids were represented as mg caffeic acid g^{-1} DW and mg rutin g^{-1} DW, respectively.

2.9 Quantification of Endogenous MT Level

Frozen dried leaf samples (0.5 g) were ground with liquid nitrogen, then extracted overnight at 4 °C with 5 mL chloroform. After centrifuging at 10,000 *g* for 15 min at 4 °C, the supernatant was dried by nitrogen gas. The dried samples were dissolved in 1 mL 42% methanol and passed through a 0.45 μm membrane for high-performance liquid chromatography (HPLC). Aliquots of 400 μL were subjected to HPLC with fluorescence detection. The samples were separated on a Shim-pack VP-ODS column (4.6 × 150 mm, Shimadzu) using a gradient elution profile (from 42% methanol to 50% methanol in 0.1% formic acid for 27 min, then isocratic elution with 50% methanol in 0.1% formic acid for 18 min at a flow rate of 0.15 mL/min) as described by (Byeon and Back 2014). MT was detected at excitation wavelength of 280 nm and emission wavelength of 348 nm.

2.10 Quantification of Endogenous SA Content

The content of SA was determined using the method of (Enyedi et al. 1992; Seskar et al. 1998). Frozen dried leaf samples (0.3 g) were ground with liquid nitrogen, then extracted with methanol (90 and 100%) by centrifuging at 12,000 *g* for 15 min at 4 °C. The methanol extracts were vacuum-dried. The dried residue was dissolved in 5% trichloroacetic acid and centrifuged for 10 min at 10,000 *g*. The supernatant was partitioned with ethyl acetate/cyclopentane/isopropanol (49.5:49.5:1, v/v). The aqueous solution's top layer was dried and utilized for SA quantification using HPLC.

2.11 Statistical Analysis

Four replicates per treatment were employed in a completely randomized design. Because the findings of the two growing seasons followed a similar pattern, a combined analysis was performed. The two-way ANOVA test was used to statistically assess all measured parameters, where the first factor was the salt treatments, and the second was the foliar application treatments. The least significant difference (LSD) test at a level of significance $p < 0.05$ was used to determine whether there were any differences between the treatments. The data are presented as means ± standard error (SE). The SAS software (SAS Inc., Cary, NC) was used for the statistical analysis.

3 Results

3.1 Foliar Applications of MT and/or SA Alleviate the Growth and Yield Reduction Induced by Salt Stress

Salt stress (6.0 and 12.0 dS m^{-1}) suppressed wheat growth and development, resulting in significant ($p < 0.05$) reductions in the shoot height, leaf area, shoot dry weight, and grain yield (Table S2). Conversely, foliar applications of MT and/or SA enhanced these parameters, and the dual application (70 µM MT + 75 mg L^{-1} SA) conferred salt tolerance by significantly reducing the negative impact of saline conditions (Table 1). The combined treatment significantly increased the shoot height (65.8%), leaf area (80.0%), shoot dry weight (107.7%), and grain yield (130.8%) in wheat plants subjected to 12.0 dS m^{-1} salinity level, compared with control plants.

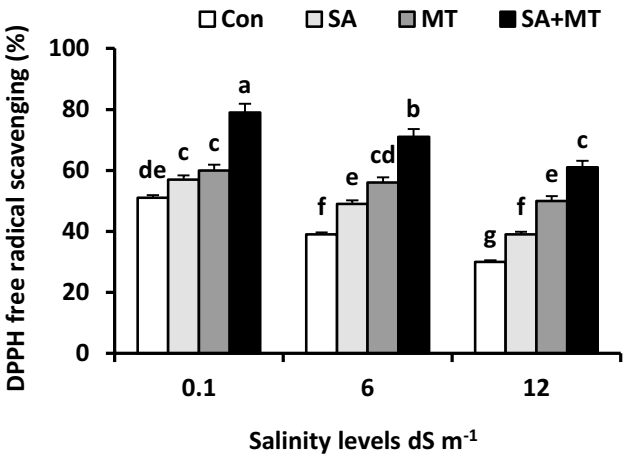

Fig. 1 Influence of salicylic acid (SA) and/or melatonin (MT) on 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity under salt stress (6 and 12 dS $m.^{-1}$). Values show the means ± standard error ($n = 4$). Different letters indicate significant differences at $p < 0.05$ level according to LSD test

3.2 Exogenous Treatments of MT and/or SA Raise DPPH Radical Scavenging Activity Under Saline Conditions

The antioxidant capacity of wheat plants was determined by DPPH radical scavenging assay. Compared with unstressed wheat plants, salt stress treatments decreased DPPH activity, whereas MT and/or SA applications significantly ($p < 0.05$) increased its activity (Fig. 1). Plants grown under 6.0 and 12.0 dS m^{-1} salinity levels and treated with the combined MT and SA treatment showed strong enhancement in the DPPH activity compared with plants subjected to individual application. Exogenous MT + SA improved the activity of DPPH in plants grown under 6.0 and 12.0 dS m^{-1} salinity level by 82.1% and 103.3% respectively, compared to those in the control plants.

3.3 Spraying of MT and/or SA Up-regulate Antioxidant Enzymes Activity in Salt-Stressed Plants

As shown in Fig. 2a, b, e, and f, soil salinization significantly increased the APX, GR, GPX, and GST activities in wheat plants. Moreover, plants grown under 6.0 and 12.0 dS m^{-1} salinity levels and subjected to the combined treatment

Table 1 Influence of salicylic acid (SA) and/or melatonin (MT) on shoot height, total leaf area, shoot dry weight, and grain yield under salt stress (6 and 12 dS $m.^{-1}$)

Salinity levels Electrical conductivity (EC; dS m^{-1}) + foliar applications	Shoot height (cm)	Total leaf area $plant^{-1}$ (cm^2)	Shoot dry weight $plant^{-1}$ (g)	Grain yield $plant^{-1}$ (g)
0.1	61 ± 0.61 de	112.0 ± 3.2 ef	5.8 ± 0.15 ef	4.98 ± 0.17 de
0.1 + SA	70 ± 0.99 c	137.7 ± 3.4 c	7.2 ± 0.14 c	6.33 ± 0.24 bc
0.1 + MT	74 ± 1.21 b	149.3 ± 3.3 b	7.9 ± 0.16 b	6.88 ± 0.26 b
0.1 + SA + MT	84 ± 1.11 a	166.7 ± 4.4 a	8.9 ± 0.28 a	7.83 ± 0.18 a
6.0	49 ± 0.77 g	89.0 ± 2.5 g	4.5 ± 0.15 g	3.09 ± 0.15 g
6.0 + SA	59 ± 1.23 e	117.3 ± 3.9 de	6.0 ± 0.17 e	4.38 ± 0.17 ef
6.0 + MT	62 ± 0.99 d	125.1 ± 2.8 d	6.5 ± 0.13 d	4.47 ± 0.13 ef
6.0 + SA + MT	72 ± 1.01 bc	143.5 ± 3.5 bc	7.8 ± 0.29 b	5.68 ± 0.19 cd
12.0	38 ± 0.66 h	60.0 ± 1.2 h	2.6 ± 0.11 h	2.11 ± 0.11 h
12.0 + SA	49 ± 0.79 g	88.0 ± 1.8 g	4.4 ± 0.17 g	3.45 ± 0.11 g
12.0 + MT	53 ± 1.03 f	94.0 ± 1.4 g	4.7 ± 0.13 g	3.79 ± 0.13 fg
12.0 + SA + MT	63 ± 0.86 d	108.0 ± 2.9 f	5.4 ± 0.19 f	4.87 ± 0.19 e

Values show the means ± standard error ($n = 4$). Different letters indicate significant differences at $p < 0.05$ level according to LSD test

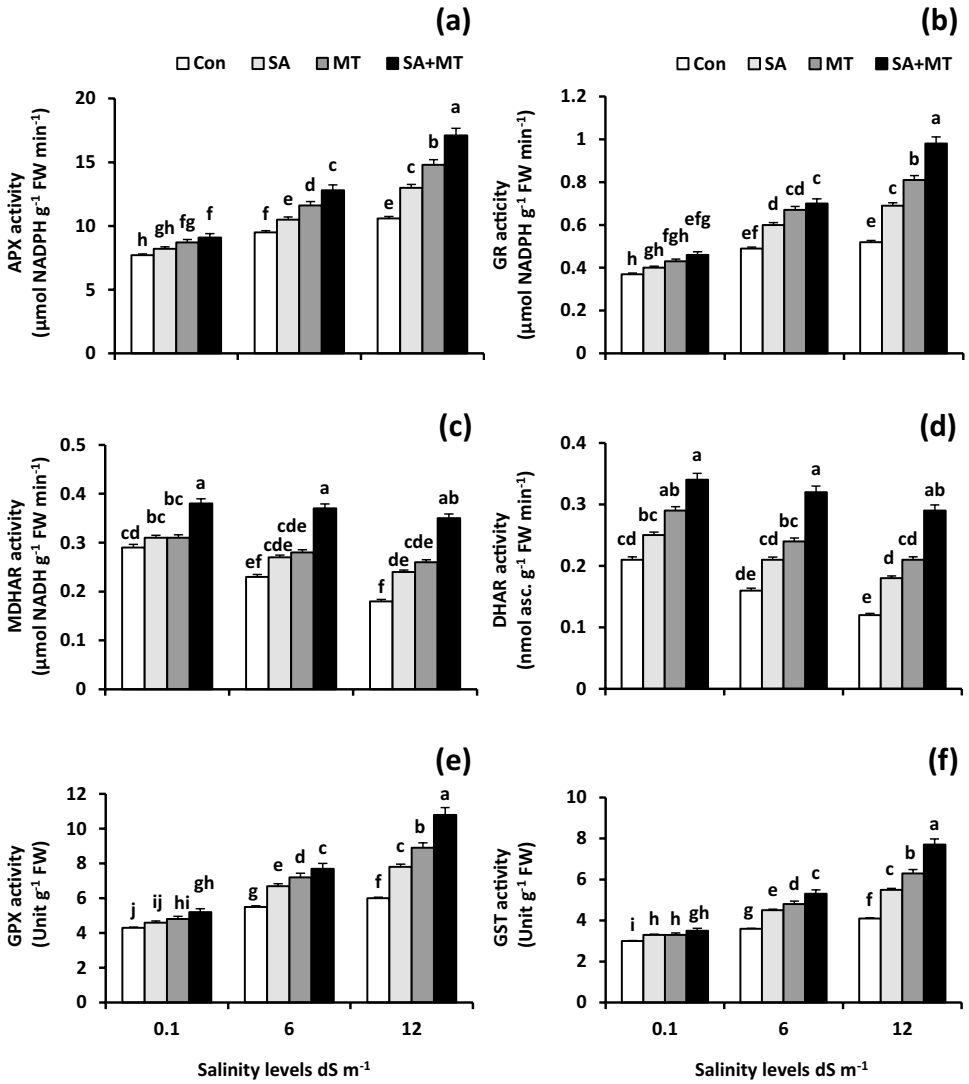

Fig. 2 Influence of salicylic acid (SA) and/or melatonin (MT) on **a** ascorbate peroxidase (APX), **b** glutathione reductase (GR), **c** monodehydroascorbate reductase (MDHAR), **d** dehydroascorbate reductase (DHAR), **e** glutathione peroxidase (GPX), and **f** glutathione S-transferase (GST) activities under salt stress (6 and 12 dS $m.^{-1}$). Values show the means ± standard error ($n = 4$). Different letters indicate significant differences at $p < 0.05$ level according to LSD test

exhibited a significant increase in APX, GR, MDHAR, DHAR, GPX, and GST activity (Table S2 and Fig. 2a, b, c, d, e, and f). Co-application of MT and SA led to an increase in APX (61.3%), GR (88.5%), MDHAR (94.4%), DHAR (141.7%), GPX (80.0%), and GST (87.8%) activity in wheat plants grown under 12.0 dS m^{-1} salinity level, compared with control plants.

3.4 Exogenously Applied MT and/or SA Maintain Redox Homeostasis Under Saline Conditions

An increase in GSH and GSSG content was observed under saline conditions (Fig. 3a and c). However, exogenous treatments considerably increased GSH content and decreased GSSG content at 6.0 and 12.0 dS m^{-1} salinity levels. Meanwhile, a higher ratio of GSH/GSSG was detected under 12.0 dS m^{-1} salinity level in plants supplemented with the combined treatment (Fig. 3e). Dual application of MT and SA led to an increase in GSH/GSSG (634.3%) ratio in wheat plants grown under 12.0 dS m^{-1} salinity level, comparison with control plants ($p < 0.05$).

Furthermore, As shown in Fig. 3b and d, AsA content and DHA content were both increased under salt stress treatments. However, exogenously applied MT and/or SA significantly increased AsA content, while they decreased DHA content at 6.0 and 12.0 dS m^{-1} salinity levels. Additionally, a higher ratio of AsA/DHA was detected under 12.0 dS m^{-1} salinity level in plants treated with the combined treatment (Fig. 3f). Dual application led to an increase in AsA/DHA (595.4%) ratio in wheat plants subjected to 12.0 dS m^{-1} salinity level, compared with control plants ($p < 0.05$).

3.5 Foliar Applications of MT and/or SA Ameliorate Glyoxalase Machinery Under Salinity

MG content was significantly increased by salt stress, while exogenous applications of MT and/or SA reduced its content (Fig. 4a). Dual application of MT and SA under 6.0 and

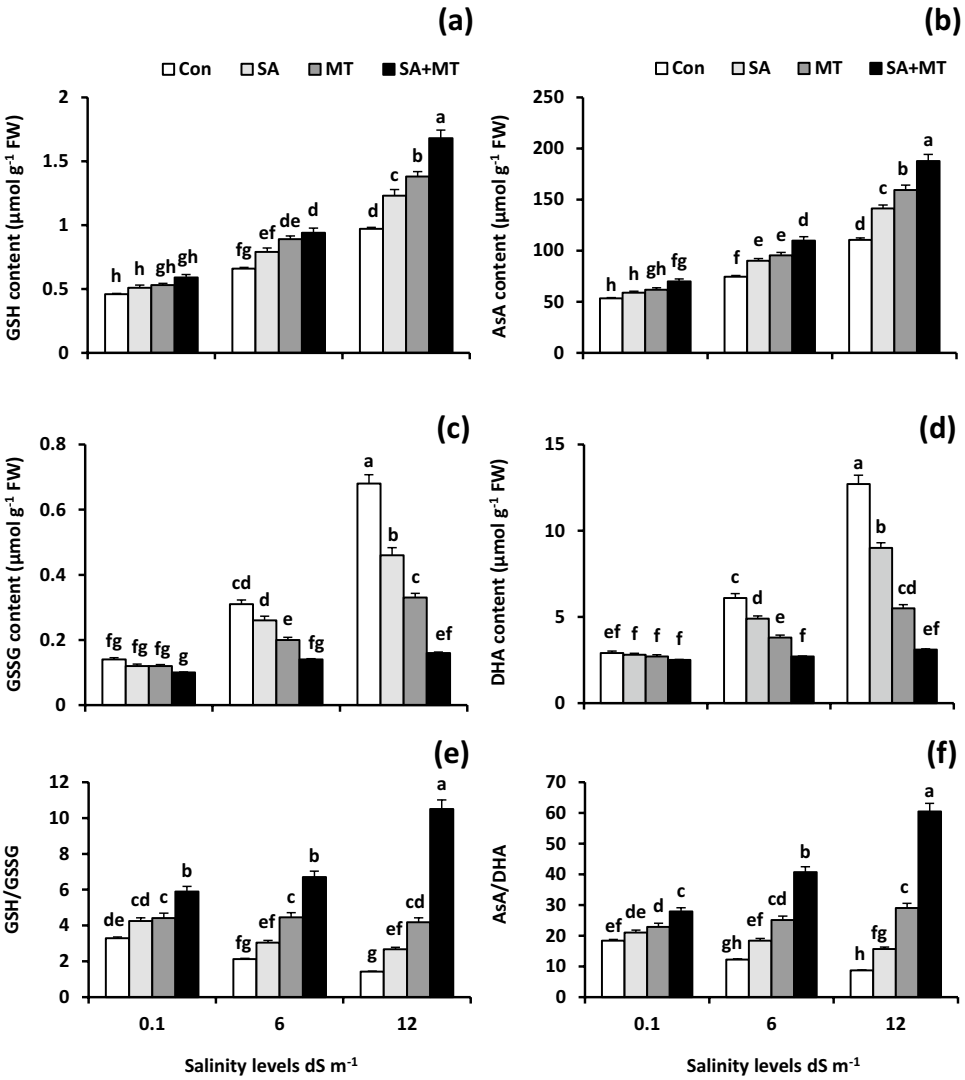

Fig. 3 Influence of salicylic acid (SA) and/or melatonin (MT) on **a** reduced glutathione (GSH), **b** ascorbate (AsA), **c** oxidized glutathione (GSSG), and **d** dehydroascorbate (DHA) contents, as well as **e** GSH/GSSG and **f** AsA/DHA ratios under salt stress (6 and 12 dS m.$^{-1}$). Values show the means ± standard error ($n = 4$). Different letters indicate significant differences at $p < 0.05$ level according to LSD test

12.0 dS m^{-1} salinity levels resulted in MG content decreases by 47.3% and 59.6%, respectively, compared with control plants. Furthermore, salt stress significantly reduced the activity of Gly I and Gly II in wheat leaves. Foliar spraying MT and/or SA significantly increased their activities under salt stress (Fig. 4b and c). Application of combined treatment under 6.0 and 12.0 dS m^{-1} salinity levels led to an increase in Gly I activity by 49.2% and 70.0%, and Gly II activity by 74.4% and 128.9%, respectively, relative to control plants.

3.6 Exogenous Applied MT and/or SA Protect Leaf Cell Membrane Structure

As shown in Table 2, salt stress increased lipid peroxidation and protein oxidation, whereas these increases were significantly attenuated by exogenous MT and/or SA applications. MT and SA co-application had the greatest ameliorative effect. It significantly ($p < 0.05$) reduced the lipid peroxidation by 21.0% and 31.5% and protein oxidation by 18.3% and 29.7% when compared to values of control plants at 6.0 and 12.0 dS m^{-1} salinity levels, respectively.

3.7 MT and/or SA Treatments Improve the Accumulation of Phenolic and Flavonoid Compounds

The phenolic and flavonoid levels were significantly increased in wheat plants under salt stress. Application of MT and/or SA further increased their accumulation in salt-stressed plants (Table 2). Wheat plants grown under 12.0 dS m^{-1} salinity level and supplemented with the combined treatment accumulated much more phenols and flavonoids than other treated plants. The combined treatment significantly enhanced phenolic and flavonoid concentrations by 46.4% and 57.5% respectively, in plants grown under 12.0 dS m^{-1} salinity level, compared with control plants.

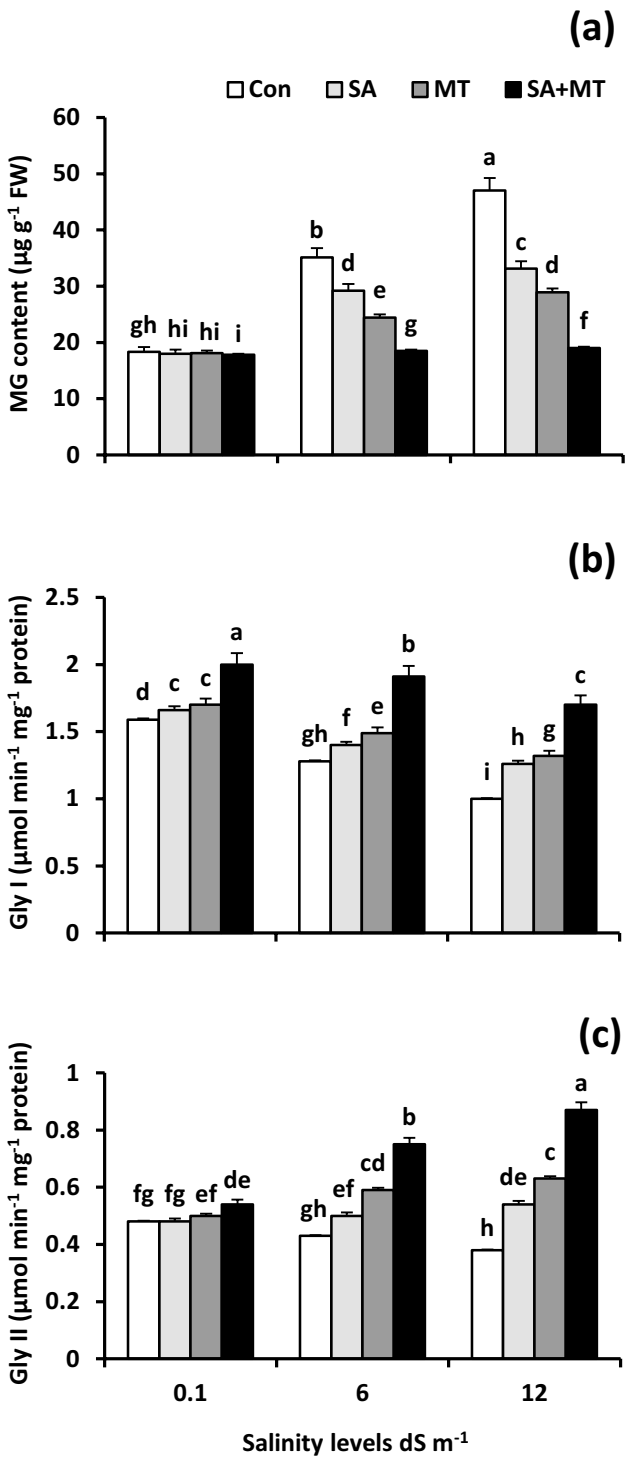

Fig. 4 Influence of salicylic acid (SA) and/or melatonin (MT) on **a** methylglyoxal (MG) content, as well as **b** glyoxalase I (Gly I) and **c** glyoxalase II (Gly II) activities under salt stress (6 and 12 dS m.$^{-1}$). Values show the means ± standard error ($n = 4$). Different letters indicate significant differences at $p < 0.05$ level according to LSD test

3.8 Exogenous MT and/or SA Supplementations Increase Endogenous MT and SA Levels in Salt-Stressed Plants

As shown in Fig. 5a and b, salt stress significantly increased endogenous MT and SA levels in wheat leaves compared to values in the non-stressed ones; moreover MT and/or SA treatments led to further increases in their concentrations. Salt-stressed plants grown under 6.0 and 12.0 dS m^{-1} salinity levels and subjected to the combined treatment showed more increments in the MT and SA concentrations compared with plants subjected to individual application. In combined treatment-treated plants, the concentrations of MT (82.0%), and SA (61.5%) were higher than untreated ones under 12.0 dS m^{-1} salinity level.

4 Discussion

Salt stress is one of the most damaging environmental stresses that can cause significant restraints in agricultural production (Talaat and Shawky 2012; Zörb et al. 2019). It leads to excessive accumulation of toxic ROS and MG that disrupt cellular redox homeostasis (Kamran et al. 2020). Previous studies have shown that exogenous application of MT or SA enhances plant salt tolerance (Ahmad et al. 2021; Chen et al. 2021; Es-sbihi et al. 2021; Shamili et al. 2021; Talaat and Shawky 2022). However, our knowledge regarding the mechanisms involved in their combined treatment-mediated salt tolerance still remains fragmentary. In the present work, we examined the effect of exogenously applied MT and/or SA on ROS metabolism in wheat under saline conditions. Our results clearly revealed that co-application of MT and SA can alleviate the detrimental effects of salt stress on wheat growth and productivity, through improved activation of the antioxidant and glyoxalase defense machineries.

In the current study, salt stress significantly reduced wheat growth and development. This decrease can be attributed to the overproduction and accumulation of ROS and MG, which consequently cause oxidative damages, impaired cell membranes structure, and suppressed metabolic activities. On the contrary, in agreement with reports of (Bukhat et al. 2020; Kamran et al. 2020; Zahedi et al. 2020; Park et al. 2021; Yu et al. 2021), we observed that exogenous applications of MT and/or SA alleviate deleterious effects of salt stress by increasing DPPH activity, improving enzymatic and non-enzymatic antioxidant machinery, stimulating methylglyoxal detoxification system, changing biological membrane structure, and elevating the endogenous MT and SA levels. These results provide evidence that MT and SA can involve in wheat salt tolerance by maintaining optimal redox homeostasis and blocking ROS burst.

Table 2 Influence of salicylic acid (SA) and/or melatonin (MT) on lipid peroxidation (nmol thiobarbituric acid reactive substances g^{-1} dry weight), protein oxidation (μm sulfhydryl g^{-1}), as well as the concentrations of total phenols (mg caffeic acid g^{-1} dry weight) and total flavonoids (mg rutin g^{-1} dry weight) under salt stress (6 and 12 dS $m.^{-1}$)

Salinity levels Electrical conductivity (EC; dS m^{-1}) + foliar applications	Lipid peroxidation	Protein oxidation	Total phenols	Total flavonoids
0.1	164 ± 3.81 efg	50 ± 1.17 fgh	52.3 ± 1.18 f	6.9 ± 0.31 e
0.1 + SA	157 ± 3.19 fg	48 ± 1.24 gh	55.9 ± 1.46 ef	7.3 ± 0.27 de
0.1 + MT	154 ± 3.99 fg	48 ± 1.11 gh	56.9 ± 1.33 ef	7.2 ± 0.23 de
0.1 + SA + MT	148 ± 2.61 g	46 ± 1.22 h	60.0 ± 1.29 ef	7.7 ± 0.21 de
6.0	200 ± 3.77 bcd	60 ± 0.99 cd	69.3 ± 1.55 de	8.4 ± 0.25 d
6.0 + SA	185 ± 3.53 cde	56 ± 1.31 de	78.0 ± 1.03 cd	10.0 ± 0.34 c
6.0 + MT	177 ± 2.91 def	54 ± 1.42 ef	81.2 ± 1.17 cd	9.9 ± 0.36 c
6.0 + SA + MT	158 ± 2.21 fg	49 ± 0.77 gh	88.7 ± 1.27 c	10.7 ± 0.29 c
12.0	248 ± 4.06 a	74 ± 1.02 a	92.3 ± 1.61 c	11.3 ± 0.31 c
12.0 + SA	215 ± 2.89 b	67 ± 1.38 b	112.0 ± 2.14 b	14.1 ± 0.23 b
12.0 + MT	209 ± 2.73 bc	61 ± 1.14 c	119.7 ± 2.16 ab	15.0 ± 0.39 b
12.0 + SA + MT	170 ± 2.76 efg	52 ± 1.09 efg	135.1 ± 2.25 a	17.8 ± 0.39 a

Values show the means ± standard error ($n = 4$). Different letters indicate significant differences at $p < 0.05$ level according to LSD test

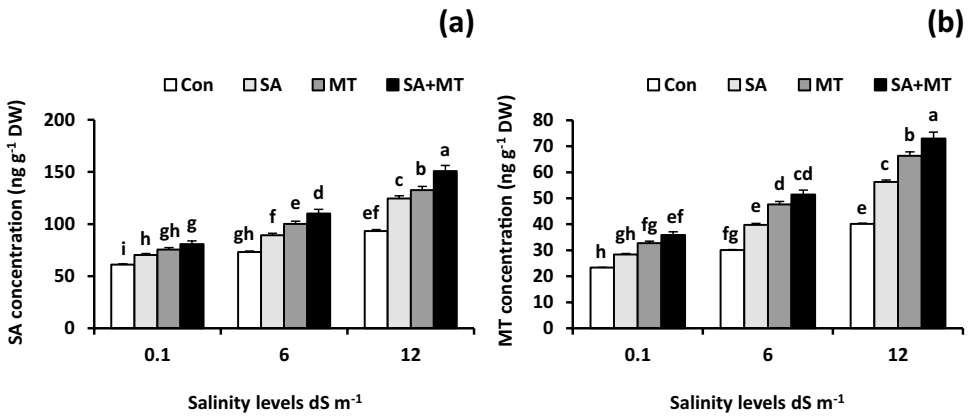

Fig. 5 Influence of salicylic acid (SA) and/or melatonin (MT) on endogenous (**a**) salicylic acid and (**b**) melatonin concentrations under salt stress (6 and 12 dS $m.^{-1}$). Values show the means ± standard error (n = 4). Different letters indicate significant differences at $p < 0.05$ level according to LSD test

Antioxidant defense system plays an important role in plant's response to saline conditions. It protects stressed plants from oxidative damage. DPPH radical scavenging activity is a measure of non-enzymatic antioxidant activity. In this study, we found that salt stress decreases the DPPH radical scavenging activity. Nevertheless, exogenous MT and/or SA applications significantly increased its activity, suggesting a possible role in the cellular defense against oxidative stress in wheat plants. Higher levels of DPPH radical scavenging activity in plant tissues have been correlated with enhanced stress tolerance (Zhou et al. 2018). Strong evidences have demonstrated that MT has an efficacious ROS scavenging capacity by reacting with hydroxyl and peroxy radicals to directly scavenge ROS molecules (Khan et al. 2020; Pardo-Hernández et al. 2020). Its scavenging ability can be attributed to its surprisingly exceptional competence in ROS detoxification by (i) acting as an antioxidant, (ii) improving the mitochondrial electron transport chain efficiency, (iii) controlling the expression of genes involved in salt stress-related signal transduction, (iv) interacting with ROS to generate MT-derivatives that improve MT's ability to detoxify ROS, and/or (v) interacting with ROS to form a complex redox network (Debnath et al. 2019; Khan et al. 2020; Chen et al. 2021; Zhang et al. 2021b).

On the other hand, some studies have shown that MT is unable to directly scavenge ROS (Bonnefont-Rousselot et al. 2011), suggesting that MT may reduce the oxidative damage caused by salt stress by directly enhancing the antioxidant enzymes activity. This is consistent with our results, we showed that the protective effects of MT and/or SA on stress-induced ROS accumulation could be related to antioxidant enzymes activation. The observed changes in the enzymes activity by MT could be considered due to its critical role as a mediator in different antioxidant pathways such as the AsA-GSH cycle, POD, SOD, and CAT (Ahmad et al. 2021; Chen et al. 2021; Park et al. 2021). Several studies have pointed out that MT plays a major role in regulating the expression of antioxidant-related

gene under stress conditions (Li et al. 2018a; Sun et al. 2018; Zhang et al. 2021b). Other reports showed that SA application significantly boosted antioxidant enzymes activity in salt-stressed plants (Bukhat et al. 2020; Kaya et al. 2020a). This is in line with Hediji et al. (2021), who postulated that SA may operate directly as an antioxidant to scavenge ROS and/or indirectly by triggering antioxidant responses. The present study confirmed that saline environments enhance ROS production, which causes oxidative damage, and in order to deter this, MT and/or SA induce battery of antioxidant enzymes.

In order to remove ROS, MT and SA can induce the accumulation of some representative non-enzymatic antioxidants, such as AsA and GSH (Kaya et al. 2020a; Pardo-Hernández et al. 2020). Both AsA and GSH are involved in scavenging ROS and controlling cellular redox potential (Talaat 2019; Singh et al. 2019). This is consistent with our data, we found that foliar applications of MT and/or SA significantly maintain high contents of AsA and GSH, as well as high ratios of AsA/DHA and GSH/GSSG in salt-stressed wheat plants. These findings reveal that MT and SA functions are closely related to the upregulation of antioxidant enzymes activity involved in AsA-GSH pathway. Previous studies have postulated that exogenous MT could maintain higher levels of AsA and GSH by speeding up the conversion of DHA to AsA and GSSG to GSH (Cui et al. 2017). Moreover, MT may boost the expression of genes involved in AsA-GSH cycle (Liang et al., 2018). Other reports showed that SA can increase AsA and GSH synthesis as well as their redox potential by up-regulating the activities of AsA-GSH cycle related enzymes in plants under saline stress (Kaya et al. 2020a). Similar evidence that SA improves AsA and GSH pool by stimulating the activities of enzymes associated with AsA-GSH cycle has been observed in salt-stressed *Brassica parachinensis* (Kamran et al. 2020) and Cd-stressed *Phaseolus vulgaris* (Hediji et al. 2021). Our findings suggest that MT and/or SA can improve cellular redox homeostasis by activating the entire antioxidant system in plants to protect cells from salt stress-induced oxidative stress.

Salt stress can also trigger an oxidative damage on plants by overproduction and accumulation of MG (Nahar et al. 2016; Kamran et al. 2020). Consistent with these reports, our study indicated a significant increase in wheat MG content under 6.0 and 12.0 dS m^{-1} salinity levels. Salinity might decrease the activity of enzymes responsible for eliminating MG (Gly I and Gly II) or might disrupt the glyoxalase system, which ultimately causes oxidative stress through reduction of GSH content. On the contrary, we found that exogenously applied MT and/or SA significantly improve the effectiveness of the MG detoxification system by raising Gly I and Gly II activities and improving GSH content, which is in line with the findings of Rahman et al. (2016) and Kamran et al. (2020). GSH is a part of glyoxalase system and an enhancement in its content presumably assists in detoxification of MG in plants pretreated with SA (Zanganeh et al. 2019). Moreover, report by Kaya et al. (2020b) indicated that SA improves maize tolerance by upregulating the AsA-GSH cycle and glyoxalase system. Our study suggests that MT and/or SA have an important role in alleviating salt damage by boosting ROS and MG detoxification through stimulating the antioxidant and glyoxalase defense machineries in wheat plants.

Our results showed that salt stress-induced ROS and MG accumulation was consistent with the increase in lipid peroxidation and protein oxidation, indicating that excessive ROS and MG production might be responsible for the salt-induced membrane damage. However, exogenous MT and/or SA applications ameliorated ROS- and MG-caused membrane damage and maintained cellular membranes structure. MT, located in hydrophilic side of the lipid bilayer, prevents biological membrane from the lipid peroxidation by directly neutralizing the toxic reactants (de Lima et al. 2010). Several studies have demonstrated that MT acts as an antioxidant and provides protection against oxidative stress by scavenging most ROS directly or indirectly, which is helpful to maintain the integrity of the membrane structure (Khan et al. 2020; Shafi et al. 2021; Yu et al. 2021). Moreover, MT increases the activity of antioxidant enzymes, thus reducing ROS and, as a result, protecting cell membranes from oxidative damages (Ahmad et al. 2021; Chen et al. 2021; Park et al. 2021; Zhang et al. 2021a). SA also mediates the ROS detoxification and acts as a natural signal molecule for the activation of plant defense responses (Hoang et al. 2020; Kamran et al. 2020; Kaya et al. 2020a). Consistently, Li et al. (2014) showed that SA foliar application mitigates salt stress in *Torreya grandis* through boosting the expression of antioxidant genes, thus restoring cell membrane integrity. Overall, our results clearly indicate that ROS-caused membrane damage was alleviated by exogenous applications of MT and SA.

The phenolic and flavonoids compounds can act as a secondary ROS scavenging system in plant cells. They can protect membrane lipids from peroxidation via inhibiting lipoxygenase activity and preventing oxidative chain reactions (Panche et al. 2016). In the present study, the exogenous applications of MT and/or SA significantly boosted their accumulation under saline conditions, implying that higher phenols and flavonoids synthesis could be one of the protective response of wheat plants to salty soils. Several studies have indicated that MT treatment enhances the accumulation of phenolic and flavonoids compounds by regulating the expression of genes involved in their biosynthesis

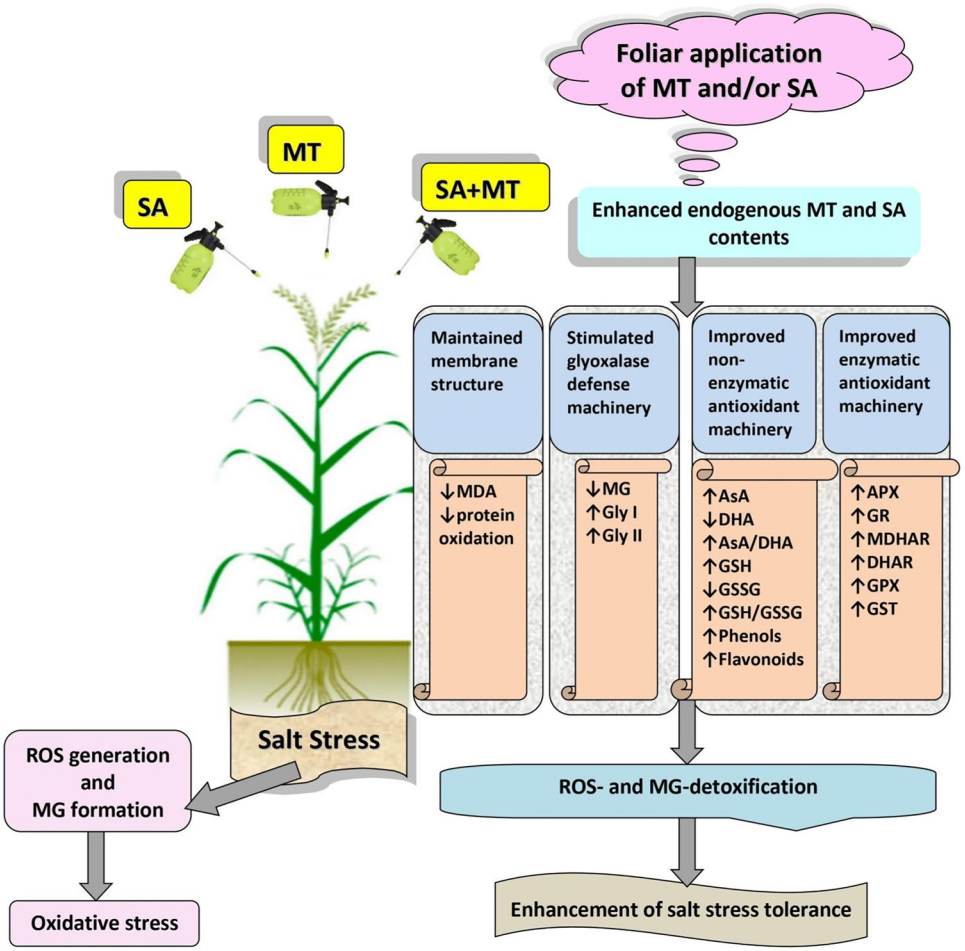

Fig. 6 A model showing salt stress induced oxidative stress in wheat plant by increasing reactive oxygen species (ROS) generation and methylglyoxal (MG) formation. Meanwhile, melatonin (MT) and/or salicylic acid (SA) reduced salt stress damage to the plant by affecting the activity of ascorbate peroxidase (APX), glutathione reductase (GR), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), glutathione peroxidase (GPX), glutathione S-transferase (GST), glyoxalase I (Gly I), and glyoxalase II (Gly II) as well as the content of ascorbate (AsA), dehydroascorbate (DHA), reduced glutathione (GSH), oxidized glutathione (GSSG), phenols, flavonoids, methylglyoxal (MG), malondialdehyde (MDA), and protein oxidation

(Liang et al. 2018; Coskun et al. 2019). Furthermore, Liang et al. (2018) postulated that there is a direct or indirect crosstalk between MT and flavonoids. There are also reports that exogenous SA improves crop salt tolerance by increasing total phenolics and flavonoids content and antioxidant activity (Hoang et al. 2020; Shamili et al. 2021). Indeed, SA treatment induces an increase in the expression level of genes involved in the flavonoid metabolism (Gondor et al. 2016). According to the results of this study, we can conclude that MT and/or SA alleviate the salt-induced crop yield inhibition by scavenging ROS and MG via stimulating a battery of enzymatic and non-enzymatic detoxification systems as well as improving glyoxalase defense machinery.

Phytohormones such as MT and SA not only influence the plant growth and development, but they also protect plants against saline conditions (Kamran et al. 2020; Es-sbihi et al. 2021; Park et al. 2021; Yu et al. 2021). It has been reported that environmental stresses induce high endogenous MT and SA levels, helping to activate various other defensive compounds (Wani et al. 2017; Hernández-Ruiz and Arnao 2018). In the present study, we found that both salt stress and foliar application treatments significantly increase MT and SA levels in wheat plants, indicating their possible role as alleviating-stressor agents under saline conditions. Consistent with our results, exogenously applied MT significantly elevates endogenous MT content and enhances plant stress tolerance (Ke et al. 2018; Li et al. 2018a; Wang et al. 2021). Exogenous MT triggers the endogenous MT biosynthetic activity by up-regulating the expression of MT-biosynthetic genes (Ni et al. 2018). Furthermore, MT could potentially interact with other plant hormones, employing beneficial roles in stress management. Several studies have confirmed the association between MT and SA under stress conditions (Weeda et al. 2014; Lee et al. 2015; Hernández-Ruiz and Arnao 2018; Park et al. 2021). MT can enhance SA level by positively regulating its biosynthetic genes (Lee et al. 2015). Our findings were consistent with previous works and postulated that MT applications increase endogenous SA levels. Overall, exogenously applied MT and/or SA increase endogenous MT and SA levels that promotes enzymatic and non-enzymatic antioxidant defense systems, improves glyoxalase defense machinery, maintains cellular membranes stability, facilitating prompt removal of excess ROS and MG, and thereby enhancing wheat salt tolerance (Fig. 6).

5 Conclusions

The present study reveals that melatonin co-applied with salicylic acid appears to be a promising candidate for improving wheat salt tolerance. Their interaction strengthened the antioxidant and glyoxalase defense machineries, which in turn improved the reactive oxygen species and methylglyoxal detoxification. These findings provide novel insight into the effect of melatonin and salicylic acid combined treatment against salt stress that occurs through reducing reactive oxygen species burst and methylglyoxal toxicity, decreasing lipid peroxidation and protein oxidation, increasing the activities of antioxidative and glyoxalase enzymes, enhancing the levels of antioxidant metabolites, as well as maintaining the stability of cellular membranes. As a result, the ecologically acceptable application of melatonin and salicylic acid can function as a robust elicitor against adverse environmental circumstances in agronomic and horticultural crops.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s42729-022-00907-8.

Author Contribution NBT and DT conceptualized the research. NBT designed and carried out the experiments, generated and analyzed the data, and wrote the manuscript.

Funding Open access funding provided by The Science, Technology & Innovation Funding Authority (STDF) in cooperation with The Egyptian Knowledge Bank (EKB). This research was supported by the Academy of Scientific Research and Technology in Egypt and the Bulgaria-Egypt Joint Research Project.

Declarations

Conflict of Interest The authors declare no competing interests.

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