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Ascorbic acid is involved in melatonin-induced salinity tolerance of maize (*Zea mays* L.) by regulating antioxidant and photosynthetic capacities

M. ZHU[*,†], T. GUO[*,†], Y.B. LIU[*], R. XIAO[*], T. YU[*], J.X. HUANG[*], W.L. DU[*], X.M. ZHONG[*], B. SONG[**,+], and F.H. LI[*,+]

College of Agronomy, Specialty Corn Institute, Shenyang Agricultural University, 110866 Shenyang, Liaoning Province, China[*]
Affiliated Experimental Field, Shenyang Agricultural University, 110866 Shenyang, Liaoning Province, China[**]

Abstract

Melatonin (MT), an indole compound, can boost plant growth under abiotic stress conditions. This experiment aims to elucidate the synergistic effect of MT and ascorbic acid (AsA) in mitigating salinity stress by assessing the photosynthetic and antioxidant capacity of the maize inbred lines H123 and W961. The results indicated that exogenous MT and AsA significantly improved photosynthetic efficiency and biomass of maize under salinity stress. Additionally, exogenous MT and AsA also improved antioxidant enzyme activities, promoted regeneration of AsA and GSH, decreased reactive oxygen species contents, suppressed Na^+ accumulation, and improved the K^+/Na^+ ratio of maize seedlings. Additionally, the AsA inhibitor lycorine decreased the endogenous content of AsA and eliminated the positive effects of MT, while the MT inhibitor *p*-chlorophenyl alanine (CPA) reduced the endogenous content of MT, which could not eliminate the promoting effects of AsA. The results suggested that AsA may act as a downstream signal involved in the regulatory effects of MT on maize under salinity stress.

Keywords: AsA–GSH cycle; ascorbic acid; ionic homeostasis; maize; melatonin; salinity stress.

Introduction

Salinity stress, one of the most significant abiotic stresses, limits worldwide grain production (Munns and Tester 2008). The response mechanisms of salinity stress mainly include osmotic stress, ion toxicity, and reactive oxygen species (ROS) (Wang *et al*. 2021a). At the early stage of salinity stress, osmotic stress is a rapid process that increases the Na^+ and Cl^- ion content around the root system, reducing the cell water potential (Zhan *et al*. 2019). Hence, plants reduce salinity damage by excluding ions, controlling ions accumulation around the roots, limiting the translocation to aboveground organs of plants, and accumulation of soluble osmotic compounds (El-Katony *et al*. 2020). Maize, as a salt-sensitive crop, exhibits sensitivity to salinity stress especially during

Highlights

- Salinity stress induces oxidative stress and inhibits the growth of maize
- Melatonin improves the balance of ROS in maize under salinity stress
- Ascorbic acid mitigates salinity stress by enhancing antioxidant capacities

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[+]Corresponding authors
e-mail: dongyakj@163.com (B. Song)
lifenghai@126.com (F.H. Li)

Abbreviations: APX – ascorbate peroxidase; AsA – ascorbic acid; CAT – catalase; Chl – chlorophyll; C_i – intercellular CO_2 concentration; DHA – oxidized ascorbic acid; DHAR – dehydroascorbate reductase; *E* – transpiration rate; ETR – electron transport rate; FM – fresh mass; F_v/F_m – maximum quantum yield of PSII photochemistry; g_s – stomatal conductance; GR – glutathione reductase; GSH – glutathione; GSSG – oxidized glutathione; MDA – malondialdehyde; MDHAR – dehydroascorbate reductase; MT – melatonin; NPQ – nonphotochemical quenching; $O_2^{\bullet-}$ – superoxide anion; P_N – net photosynthetic rate; POD – superoxide dismutase; q_p – photochemical quenching coefficient; ROS – reactive oxygen species; SOD – superoxide dismutase; Φ_{PSII} – effective quantum yield of PSII photochemistry.

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[†]These authors contributed equally to this study.

Conflict of interest: The authors declare that they have no conflict of interest.

seed germination and seedling growth stages, when the salinity concentration reaches 100 mmol L^{-1}, the biomass of maize seedlings could decrease by approximately 50% (Cao *et al*. 2018). Plants typically respond to abiotic stress by triggering internal protective mechanisms, such as improving the antioxidant defense system. Nevertheless, in cases of abiotic stress, the endogenous protective mechanisms may prove insufficient to counteract oxidative damage (Nie *et al*. 2023). Many methods and technologies have been used to improve plant salinity tolerance. One of the most recent methods to reduce the effect of biotic and abiotic stresses on plants is an exogenous application of biostimulators or growth hormones (Kamran *et al*. 2018). Therefore, improving the salinity stress tolerance of plants through the regulation of exogenous plant growth substances has become a research hot spot.

Melatonin (MT), discovered in plants in 1995, functions as a potent antioxidant and mediates various adverse stresses (Dubbels *et al*. 1995, Gao *et al*. 2022a, Hasan *et al*. 2023). Currently, melatonin has been found in many plant species, mainly synthesized in plant mitochondria and chloroplasts, and is present in various organs in plants (Wang *et al*. 2017). Research has found that melatonin has multiple functions in plants, including induction of callus tissue, seed germination, flowering, root growth, and prevention of leaf senescence (Li *et al*. 2012). Previous research indicated that exogenous melatonin can improve photosynthesis by increasing chlorophyll (Chl), carotenoids, as well as the maximum quantum yield of PSII photochemistry (F_v/F_m), in tomatoes (*Solanum lycopersicum*) under salinity stress (Zhou *et al*. 2022). Furthermore, exogenous melatonin could have scavenged excessive ROS and directly enhanced the antioxidant system (Zhang *et al*. 2022). Additionally, under salinity stress, the application of melatonin increases the content of K^+, maintains the dynamic balance of cellular Na^+/K^+, and enhances the salinity tolerance of plants (Sezer *et al*. 2021).

Ascorbic acid (AsA), widely recognized as vitamin C, serves as a primary nonenzymatic antioxidant, enhancing the stress resistance of plants (Akhlaghi *et al*. 2014). Various studies have shown that ascorbic acid enhances photosynthesis and alleviates the inhibition of growth and development of plants under salinity stress (Hassan *et al*. 2021). Under high salinity conditions, exogenous AsA effectively promotes the plant's absorption of soil nutrients, reduces the content of Na^+, increases the content of K^+, and maintains the ionic homeostasis within the cells (Hasanuzzaman *et al*. 2023). Additionally, exogenous AsA could scavenge ROS directly or indirectly through enzymatic reactions that improve the activities of superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione reductase (GR) in plant chloroplasts, also promote the synthesis of AsA and glutathione (GSH) and mitigate the salinity stress on chloroplasts (Aziz *et al*. 2021).

Luo *et al*. (2022) indicated that the application of MT can promote the content of endogenous AsA in kiwifruit. Tomato root system irrigated with MT significantly increased the content of AsA and lycopene in tomato fruits (Liu *et al*. 2016). Furthermore, Shah *et al*. (2024) found that MT could enhance the salt tolerance of rapeseed by upregulating the expression of AsA–GSH genes under salinity stress and increasing the content of AsA and the activity of APX. The main aims of the study were as follows: (*1*) to determine whether MT and AsA could mitigate the salinity stress of maize; (2) to elucidate the alleviating role of MT and AsA in maize under salinity stress by evaluating photosynthetic capacity, membrane lipid peroxidation, and antioxidant enzyme activities. The research could highlight the potential application of MT and AsA in agricultural production, and provide a new method for the cultivation of maize in saline soils.

Materials and methods

Plant material and experimental design: This experiment was performed at the Research and Education Center of Agronomy of Shenyang Agricultural University (Shenyang, China) in 2023. The salt-resistant H123 and salt-sensitive inbred line W961 were provided by the Specialty Corn Institute, Shenyang Agricultural University. On 25 May, seeds were sown in pots with a bottom diameter of 8 cm and a height of 12 cm, each containing 0.58 kg of soil. The concentrations of NaCl (100 mM) and MT (100 μM) in the current experiment were determined based on our preliminary experimental settings (Wang *et al*. 2021a). The concentration of AsA used was 2 mM (unpublished). Additionally, the MT inhibitor CPA (10 μM) and the AsA inhibitor lycorine (0.3 mM) were added to validate the upstream and downstream relationships between MT and AsA.

The experiment was divided into six treatments: (*1*) CK: normal control; (*2*) NaCl: 100 mM NaCl; (*3*) NaCl + MT: 100 mM NaCl + 100 μM melatonin; (*4*) NaCl + AsA: 100 mM NaCl + 2 mM ascorbic acid; (*5*) NaCl + MT + lycorine: 100 mM NaCl + 100 μM melatonin + 0.3 mM lycorine; (*6*) NaCl + AsA + CPA: 100 mM NaCl + 2 mM ascorbic acid + 10 μM CPA. Each treatment was set up with three replicates. When the third leaf of the maize had fully expanded, for six consecutive days, the treatment group was irrigated with 200 ml of 100 mM NaCl every day, and the control group was watered with 200 ml of distilled water every day. On the 4th and 6th day of salinity stress, 200 ml of 100 μM MT, 2 mM AsA, 10 μM CPA, and 0.3 mM lycorine containing 0.01% *Tween-20* were sprayed on the maize leaves with a handheld sprayer. At 9:00 h on the 7th day, photosynthetic indicators were measured on the third leaf of maize. Subsequently, the third leaf was harvested and placed in a –80°C freezer for further analysis.

Sampling and determination of growth parameters: The shoot length of the maize seedlings was measured using a ruler with a 1-mm scale. Then the entire seedlings were harvested, and the fresh mass (FM) of the roots and seedlings was determined separately. Subsequently, the maize seedlings were placed in an oven at 105°C for 2 h, then the temperature was adjusted to 80°C to dry the samples to a constant mass.

Gas-exchange parameters: The gas-exchange parameters of maize were measured using a portable photosynthesis system, *LI-COR 6800* (*Li-COR*, *Inc*., NE, USA). The instrument parameters were set to a leaf chamber area of 1 × 3 cm, 400 μmol(CO_2) mol^{-1}, a relative humidity of 50%, and a photosynthetic photon flux density of 1,500 μmol m^{-2} s^{-1}. Three seedlings were randomly selected for measurement for each treatment, using the third leaf as the leaf position. Measurements of net photosynthetic rate (P_N), stomatal conductance (g_s), intercellular CO_2 concentration (C_i), and transpiration rate (E) were taken between 9:00 and 11:00 h after 6 d of NaCl treatment (Wang *et al*. 2021b).

Chl fluorescence parameters: The third leaf of the seedling was selected. After 30 min of dark treatment, the maximum photochemical quantum yield (F_v/F_m), nonphotochemical quenching coefficient (NPQ), electron transport rate (ETR), and photochemical quenching coefficient (q_p) were measured using a portable fluorometer *PAM-2500* (*Walz*, Germany). For each treatment, three plants were randomly selected for measurement (Huang *et al*. 2024).

Chl content: Fresh leaves (0.1 g) were harvested from each treatment with three replicates. All samples were extracted with 15 mL of 95% ethanol and incubated under dark conditions for 8 h. The absorbance was determined at 645 and 663 nm using a multifunctional microplate reader (*Multiskan GO*, *Thermo Fisher Scientific*, USA) with 95% ethanol as the blank. The following equations were used to calculate the Chl content (Lichtenthaler and Wellburn 1983):

$$\text{Chl } a \text{ [mg g}^{-1}\text{(FM)]} = (12.7\ OD_{663} - 2.69\ OD_{645}) \times V/(1{,}000 \times M)$$

$$\text{Chl } b \text{ [mg g}^{-1}\text{(FM)]} = (22.9\ OD_{645} - 4.68\ OD_{663}) \times V/(1{,}000 \times M)$$

$$\text{Chl } (a + b) = \text{Chl } a + \text{Chl } b$$

where OD_{663} and OD_{645} are the absorbances of the extract solution at 663 and 665 nm; V is the total volume of the extract, 15 mL; M is the mass of the fresh sample, 0.1 g.

MDA, H_2O_2, and $O_2^{\bullet-}$ content: Malondialdehyde (MDA) [μmol g^{-1}] was determined using Heath and Packer's method (Heath and Packer 1968). 0.1 g of leaf tissue and 1 mL of 10% trichloroacetic acid (TCA) were ground into a homogenate using a mortar and pestle. After being centrifuged at 4,000 × *g* for 10 min at 4°C, 1 mL of the supernatant was taken and mixed well with 1 mL of TCA solution containing 0.6% (w/v) thiobarbituric acid (TBA). The mixed solution was placed in a boiling water bath for 20 min, after which the sample was allowed to cool and was centrifuged again. A multifunctional microplate reader (*Multiskan GO*, *Thermo Fisher Scientific*, USA) was used to measure the absorbance at 450, 532, and 600 nm.

The content of H_2O_2 [μmol g^{-1}] was assayed by corresponding kits (*Beinong Yuhe Science and Technology Development Co.*, *Ltd.*, Beijing, China) according to the instructions. A multifunctional microplate reader (*Multiskan GO*, *Thermo Fisher Scientific*, USA) was used to measure the absorbance at 415 nm.

The content of $O_2^{\bullet-}$ [μmol g^{-1}] was assayed by corresponding kits (*Prone Technology Co.*, *Ltd.*, Tianjin, China) according to the instructions. A multifunctional microplate reader (*Multiskan GO*, *Thermo Fisher Scientific*, USA) was used to measure the absorbance at 530 nm.

Antioxidant enzyme activities: The leaf tissue (0.1 g) was homogenized with liquid nitrogen using a mortar and pestle. Then, 500 μL of 50 mM PBS (pH 7.8, containing 1% PVP) was added and mixed well. Subsequently, it was centrifuged at 4°C, 12,000 rpm for 5 min, and all the supernatant was collected into a centrifuge tube for the subsequent determination of antioxidant enzymes.

The activity of SOD (EC 1.15.1.1) [U g^{-1}] in maize leaves was determined according to the method proposed by Abedi and Pakniyat (2010). 50 μL of enzyme solution, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 5 mM nitroblue tetrazolium, and 5 mM riboflavin were included in the reaction system. After 10 min, the absorbance was measured at 560 nm.

The activity of CAT (EC 1.11.1.6) [U g^{-1}] was determined according to the method of Wang (1995). 0.1 mL of crude enzyme extract was added to 2 mL of sodium acetate buffer solution and 1 mL of 0.25% (w/v) guaiacol solution. Then, 0.1 mL of 0.1 mol L^{-1} H_2O_2 was added. The absorbance of the mixture at 470 nm was measured with a multifunctional microplate reader for 3 min.

The activity of POD (EC 1.11.1.7) [U g^{-1}] was determined according to the method of Rao *et al*. (1996). 0.1 mL of crude enzyme extract was added to 2 mL of sodium acetate buffer solution and 1 mL of 0.25% (w/v) guaiacol solution, then finally 0.1 mL of 0.1 mol L^{-1} H_2O_2 was added. The absorbance of the mixture at 470 nm was measured with a multifunctional microplate reader for 3 min.

The activity of APX (EC 1.11.1.11) [U g^{-1}] was determined according to the method of Nakano and Asada (1981). The reaction mixture (1.5 ml) contained 50 mmol L^{-1} phosphate buffer (pH 7.8), 0.1 mM Na_2-EDTA, 0.3 mM ascorbic acid, 0.06 mM H_2O_2, and 0.1 mL of enzyme crude extract. The absorbance at 290 nm was measured at 25°C.

AsA–GSH cycle: The concentration of AsA and oxidized ascorbic acid (DHA) was assayed by corresponding kits (*Herbal Biotechnology Co*., *Ltd*., Nanjing, China) according to the instructions. The concentration of GSH and GR was assayed by corresponding kits (*Meisenxun Biotechnology Co*., *Ltd*., Shenyang, China) according to the instructions.

The activity of monodehydroascorbate reductase (MDHAR) (EC 1.6.5.4) and dehydroascorbate reductase (DHAR) (EC 1.8.5.1) were assayed by corresponding kits (*Huayue Ruike Trade Co*., *Ltd*., Shenyang, China) according to the instructions.

The concentration of oxidized glutathione (GSSH) was assayed by corresponding kit (*Prone Technology Co*., *Ltd*., Tianjin, China) according to the instructions. The absorbance for each indicator was measured using a multifunctional microplate reader (*Multiskan GO*, *Thermo Fisher Scientific*, USA). The content of various indicators in plant samples was calculated according to the above method [μmol g^{-1}].

MT content: The fresh leaf was taken in an amount of 0.1 g. The concentration of MT was assayed using the corresponding kit (*Herbal Source Biotechnology Co*., *Ltd*., Nanjing, China), and the sample was processed according to the instructions. A multifunctional microplate reader (*Multiskan GO*, *Thermo Fisher Scientific*, USA) was used to measure the absorbance at 450 nm. The content of MT [ng L^{-1}] in the plant samples was calculated according to the above method.

K^+ and Na^+ content was determined according to the method of Munns *et al*. (2010), with improvements made. First, 0.1 g of dry leaves was taken and ground into a powder. The sample was placed into a test tube and positioned in a digestion pot preheated to 370°C. Then 10 ml of concentrated H_2SO_4 was added, and digestion was carried out for 45 min. Afterward, the test tube rack was removed and placed in a fume hood for 10 min, 10 ml of H_2O_2 was added, and digestion was continued for another 10 min until the solution in the test tube became transparent. After being cooled, it was diluted to a final volume of 100 ml with distilled water. Subsequently, the absorbance was measured using a flame spectrophotometer *M410* (*Sherwood M410*, UK). Standard curves were prepared with K^+ standard concentrations of 0, 5, 10, 20, 30, 40 mg ml^{-1}, and Na^+ standard concentrations of 0, 2, 4, 6, 8, 10 mg ml^{-1}. The content of K^+ and Na^+ in the plant samples was calculated based on the standard curves.

Statistical analysis: All data were analyzed using *IBM SPSS 22.0* software, employing a one-way analysis of variance (*ANOVA*). *Duncan*'s test was used to compare differences between treatments. All results were expressed as the mean ± standard deviation (SD). Graphing was performed using *Origin 2024* software.

Results

Plant growth parameters: The phenotypic differences between different treatments of both inbred lines are shown in Fig. 1S (*supplement*). The NaCl treatment significantly inhibited the growth and development of both maize inbred lines (Table 1). Compared with H123, W961 was more sensitive to salinity stress. Exogenous MT and AsA resulted in a significant increase in the growth parameters of both inbred lines compared to NaCl treatment. Additionally, lycorine inhibited the positive effects of MT, while CPA did not eliminate the positive effect of AsA.

Gas-exchange parameters: Compared to CK, salinity stress significantly reduced the gas-exchange parameters of both inbred lines. The net photosynthetic rate (P_N), stomatal conductance (g_s), and transpiration rate (E) of H123 and W961 decreased by 43.9, 57.1, 46.4%, and 27.8, 52.0, 41.7%, respectively (Fig. 1). Compared with the NaCl treatment, exogenous MT and AsA significantly increased the gas-exchange parameters of maize seedlings. H123 increased by 53.4, 92.4, 47.4%, and 51.5, 128.6, and 74.4%, respectively; W961 increased by 25.7, 57.3, 49.5%, and 25.0, 75.2, 63.9%, respectively. Compared to the NaCl treatment, the application of lycorine showed no significant difference between the inbred lines, indicating that lycorine decreased the positive effects of MT. In contrast, AsA continued to play a positive role even after applying CPA under salinity stress.

Chl fluorescence parameters: Compared to CK, salinity stress significantly reduced the F_v/F_m, q_p, and ETR values

Table 1. Effects of melatonin (MT) and ascorbic acid (AsA) on the growth and development of maize under salinity stress. CK: normal control; NaCl: 100 mM NaCl; NaCl + MT: 100 mM NaCl + 100 μM melatonin; NaCl + AsA: 100 mM NaCl + 2 mM ascorbic acid; NaCl + MT + lycorine: 100 mM NaCl + 100 μM melatonin + 0.3 mM lycorine; NaCl + AsA + CPA: 100 mM NaCl + 2 mM ascorbic acid + 10 μM CPA. Values are expressed as mean ± SD of three replicates. For each variable, means with *different lowercase letters* were significantly different (p<0.05).

Autogamy	Treatment	Plant height [cm]	Shoot fresh mass [g]	Root fresh mass [g]	Shoot dry mass [g]	Root dry mass [g]
H123	CK	29.93 ± 1.64^{a}	4.23 ± 0.25^{a}	4.81 ± 0.13^{a}	0.43 ± 0.01^{a}	0.43 ± 0.02^{a}
	NaCl	21.53 ± 0.47^{d}	2.06 ± 0.15^{e}	1.97 ± 0.01^{c}	0.22 ± 0.03^{c}	0.18 ± 0.01^{d}
	NaCl + MT	28.23 ± 0.83^{b}	3.72 ± 0.19^{b}	3.59 ± 0.25^{b}	0.40 ± 0.01^{a}	0.38 ± 0.04^{b}
	NaCl + AsA	29.70 ± 0.09^{ab}	3.17 ± 0.34^{c}	3.95 ± 0.04^{b}	0.34 ± 0.01^{b}	0.42 ± 0.03^{a}
	NaCl + MT + lycorine	24.17 ± 1.09^{c}	2.67 ± 0.17^{d}	1.91 ± 0.36^{c}	0.25 ± 0.01^{c}	0.29 ± 0.02^{c}
	NaCl + AsA + CPA	28.13 ± 0.40^{b}	3.28 ± 0.31^{c}	3.76 ± 0.51^{b}	0.34 ± 0.05^{b}	0.42 ± 0.03^{ab}
W961	CK	27.73 ± 1.09^{a}	4.11 ± 0.27^{a}	2.98 ± 0.00^{a}	0.43 ± 0.03^{a}	0.41 ± 0.02^{a}
	NaCl	20.43 ± 0.41^{d}	1.96 ± 0.30^{e}	1.43 ± 0.16^{d}	0.19 ± 0.03^{d}	0.13 ± 0.01^{e}
	NaCL + MT	25.50 ± 0.43^{b}	3.09 ± 0.07^{b}	2.29 ± 0.09^{b}	0.28 ± 0.00^{c}	0.24 ± 0.00^{c}
	NaCl + AsA	27.36 ± 0.40^{a}	3.20 ± 0.31^{b}	2.38 ± 0.11^{b}	0.36 ± 0.02^{b}	0.27 ± 0.02^{b}
	NaCl + MT + lycorine	21.63 ± 0.75^{c}	2.33 ± 0.16^{c}	1.76 ± 0.19^{c}	0.22 ± 0.01^{d}	0.15 ± 0.00^{d}
	NaCl + AsA + CPA	26.57 ± 0.64^{ab}	3.19 ± 0.27^{b}	2.18 ± 0.22^{b}	0.31 ± 0.01^{bc}	0.25 ± 0.00^{bc}

of the inbred lines H123 and W961 (Fig. 2). Compared to NaCl treatment, exogenous MT and AsA significantly increased the F_v/F_m, q_p, and ETR of both inbred lines. H123 increased by 17.2, 45.5, 68.9%, and 15.6, 38.1, 52.6%. W961 increased by 10.9, 17.1, 41.6%, and 9.8, 29.6, 72.7%, respectively. The regulation of Chl fluorescence parameters in H123 under salinity stress by exogenous MT and AsA was more pronounced. Additionally, AsA effectively alleviated the inhibitory effect of CPA, which increased the F_v/F_m, q_p, and ETR values in both inbred lines. The application of lycorine significantly decreased the positive effects of MT. Furthermore, the application of MT, AsA, and CPA + AsA resulted in significant decreases in the NPQ values of H123 and W961 by 37.5, 23.7, 26.8%, and 35.3, 27.7, 17.9%, respectively.

Chl content: Compared to CK, NaCl treatment significantly decreased the contents of Chl *a*, Chl *b*, and total Chl (*a* + *b*) in both inbred lines, with H123 decreased by 58.8, 34.0, and 50.9%, and W961 decreased by 58.7, 32.3, and 49.9%, respectively (Fig. 3). Compared to salinity stress, exogenous MT and AsA significantly increased the content

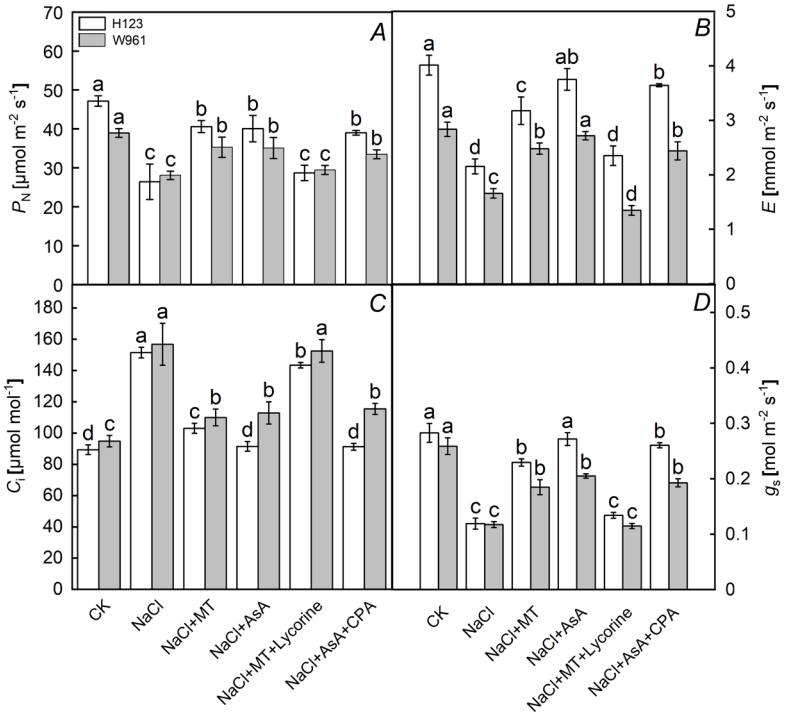

Fig. 1. Effects of melatonin (MT) and ascorbic acid (AsA) on the gas-exchange parameters of maize under salt stress. CK: normal control; NaCl: 100 mM NaCl; NaCl + MT: 100 mM NaCl + 100 μM melatonin; NaCl + AsA: 100 mM NaCl + 2 mM ascorbic acid; NaCl + MT + lycorine: 100 mM NaCl + 100 μM melatonin + 0.3 mM lycorine; NaCl + AsA + CPA: 100 mM NaCl + 2 mM ascorbic acid + 10 μM CPA. C_i – intercellular CO_2 concentration; E – transpiration rate; g_s – stomatal conductance; P_N – net photosynthetic rate. Values are expressed as mean ± SD of three replicates. For each variable, means with *different lowercase letters* were significantly different (p<0.05).

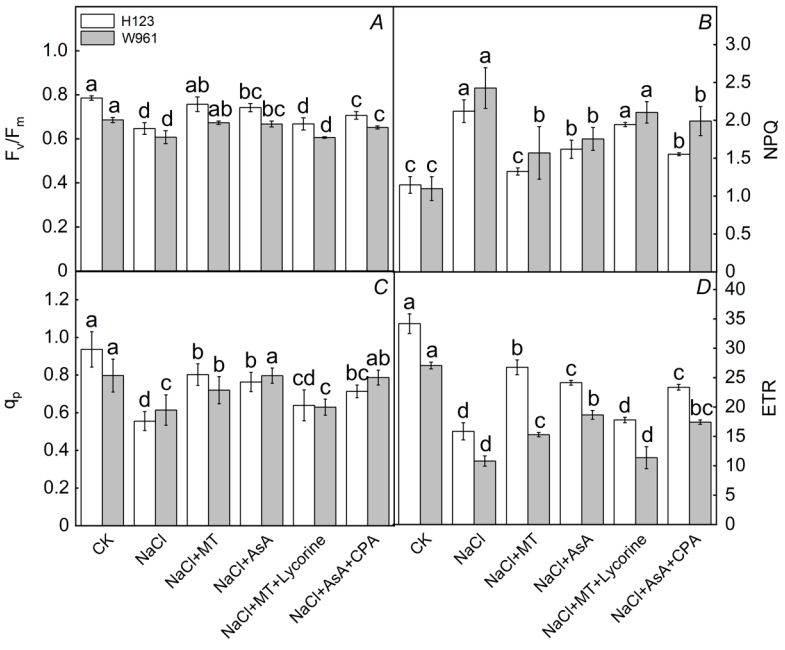

Fig. 2. Effects of melatonin (MT) and ascorbic acid (AsA) on chlorophyll fluorescence parameters of maize under salinity stress. CK: normal control; NaCl: 100 mM NaCl; NaCl + MT: 100 mM NaCl + 100 μM melatonin; NaCl + AsA: 100 mM NaCl + 2 mM ascorbic acid; NaCl + MT + lycorine: 100 mM NaCl + 100 μM melatonin + 0.3 mM lycorine; NaCl + AsA + CPA: 100 mM NaCl + 2 mM ascorbic acid + 10 μM CPA. ETR – electron transport rate; F_v/F_m – maximum quantum yield of PSII photochemistry; NPQ – nonphotochemical quenching; q_p – photochemical quenching coefficient. Values are expressed as mean ± SD of three replicates. For each variable, means with *different lowercase letters* were significantly different (p<0.05).

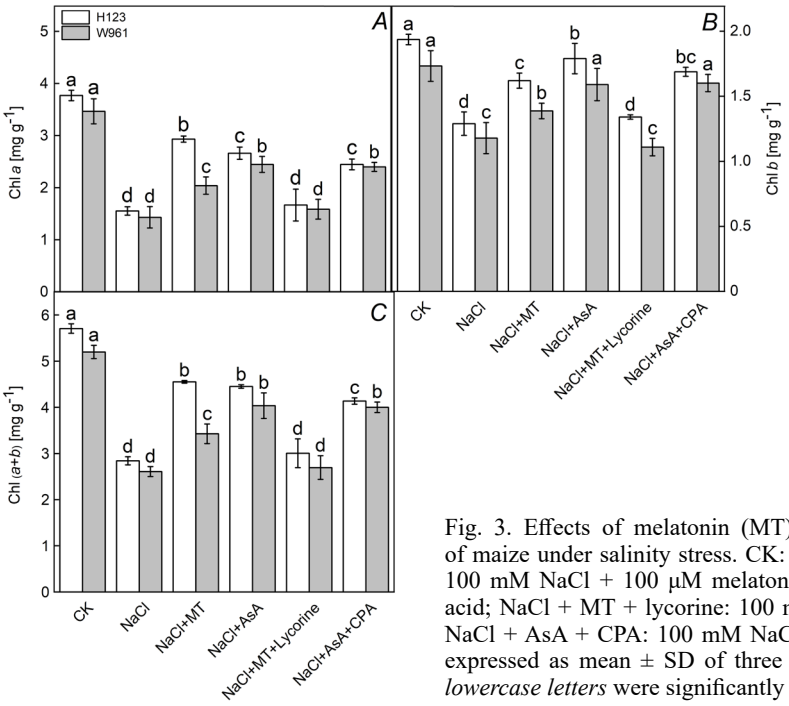

Fig. 3. Effects of melatonin (MT) and ascorbic acid (AsA) on chlorophyll content of maize under salinity stress. CK: normal control; NaCl: 100 mM NaCl; NaCl + MT: 100 mM NaCl + 100 μM melatonin; NaCl + AsA: 100 mM NaCl + 2 mM ascorbic acid; NaCl + MT + lycorine: 100 mM NaCl + 100 μM melatonin + 0.3 mM lycorine; NaCl + AsA + CPA: 100 mM NaCl + 2 mM ascorbic acid + 10 μM CPA. Values are expressed as mean ± SD of three replicates. For each variable, means with *different lowercase letters* were significantly different (p<0.05).

of Chl *a*, Chl *b*, and total Chl (*a* + *b*) in H123 by 89.0, 25.6, 60.2%, and by 71.5, 38.8, 56.6% respectively; for W961, it was 42.6, 17.6, 31.3%, and by 71.0, 34.9, 54.7% respectively. Compared to W961, the increase in Chl content was greater in H123 under the influence of MT and AsA. The NaCl + MT + lycorine treatment group showed no significant difference from the NaCl treatment group, indicating that lycorine eliminated the promoting effect of MT on the synthesis of photosynthetic pigments. In contrast, applying AsA effectively alleviated the inhibitory effect of CPA and promoted the synthesis of photosynthetic pigments.

MDA, H_2O_2, and $O_2^{\bullet-}$ content: As shown in Fig. 4, compared to CK, the contents of H_2O_2, $O_2^{\bullet-}$, and MDA significantly increased under salinity stress conditions, which indicated that salinity stress accelerated lipid peroxidation of the membrane. Exogenous MT and AsA significantly decreased the H_2O_2, $O_2^{\bullet-}$, and MDA content of both inbred lines under salinity stress, and reduced membrane peroxidation. Additionally, the addition of CPA did not inhibit the ability of AsA to scavenge reactive oxygen species. The application of lycorine reduced MT's ability to scavenge reactive oxygen species.

Antioxidant enzyme activities: As shown in Fig. 4, compared to CK, the SOD, POD, and CAT activities in both inbred lines significantly increased under salt stress. Compared to NaCl treatment alone, the SOD, POD, and CAT activities were significantly enhanced under NaCl + MT, NaCl + AsA, and NaCl + AsA + CPA treatments. AsA was capable of eliminating the inhibitory effect of CPA. Lycorine eliminated the positive effects of MT.

AsA–GSH cycle: Compared to CK, salinity stress significantly reduced the contents of AsA and GSH, and rations of AsA/DHA and GSH/GSSG. H123 declined by 51.6, 40.4, 71.3, and 56.3%. W961 decreased by 64.9, 42.9, 38.9, and 80.4%, respectively. It indicated that salinity stress had a greater harmful effect on the AsA–GSH cycle in W961 (Fig. 2S, *supplement*). However, compared to NaCl, MT decreased the inhibitory effect of salinity stress on both inbred lines and increased the content of endogenous AsA. At the same time, exogenous AsA effectively alleviated the inhibitory effect of CPA, resulting in a significant increase of reduced nonenzymatic metabolites and a reduction of oxidized substances. Exogenous MT and lycorine showed no significant differences in the content of nonenzymatic metabolites in the AsA–GSH cycle of both inbred lines compared to the salinity treatment group, indicating that MT could not alleviate the inhibitory effect of lycorine.

As shown in Fig. 5, compared to CK, the APX activity in both inbred lines significantly increased under salinity stress, with H123 increased by 189.7% and W961 by 103.6%. At the same time, the activities of GR, MDHAR, and DHAR in H123 and W961 decreased by 48.9, 12.9, 58.9%, and 69.9, 32.5, and 50.2%, respectively. Exogenous application MT and AsA significantly increased the activities of APX, GR, MDHAR, and DHAR, with H123 increased by 160.7, 33.3, 19.6, 165.7%, and 69.4, 66.7, 16.4, 129.9% respectively. W961 increased by 47.0, 133.3, 8.3, 105.6%, and 81.7, 166.7, 29.4, and 102.2% respectively. Under salinity stress, AsA decreased the inhibitory effect of CPA and enhanced the activities of key enzymes in the AsA–GSH cycle. Additionally, lycorine significantly suppressed the promoting effect of MT.

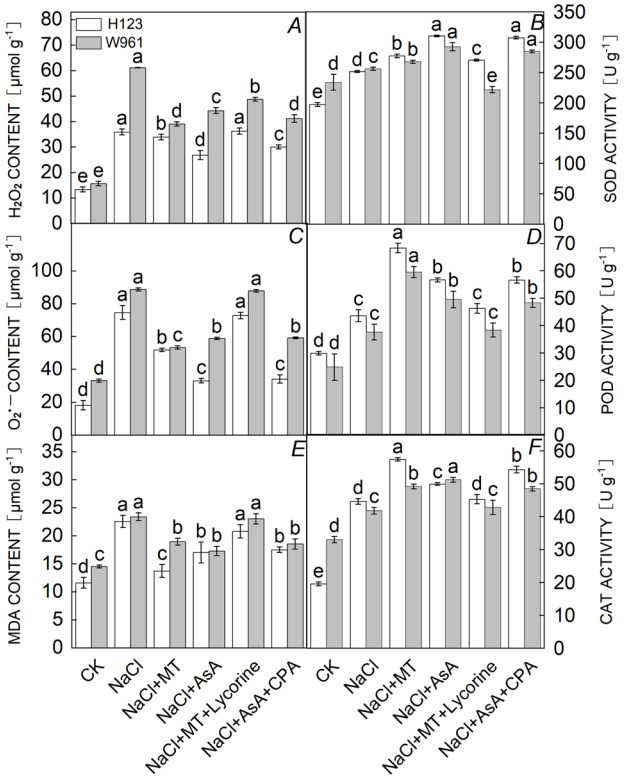

Fig. 4. Effects of melatonin (MT) and ascorbic acid (AsA) on the content of reactive oxygen species and the activity of antioxidant enzymes in maize under salinity stress. CK: normal control; NaCl: 100 mM NaCl; NaCl + MT: 100 mM NaCl + 100 μM melatonin; NaCl + AsA: 100 mM NaCl + 2 mM ascorbic acid; NaCl + MT + lycorine: 100 mM NaCl + 100 μM melatonin + 0.3 mM lycorine; NaCl + AsA + CPA: 100 mM NaCl + 2 mM ascorbic acid + 10 μM CPA. CAT – catalase; MDA – malondialdehyde; POD – superoxide dismutase; SOD – superoxide dismutase. Values are expressed as mean ± SD of three replicates. For each variable, means with *different lowercase letters* were significantly different (p<0.05).

Melatonin content: Compared to CK, the endogenous melatonin content in W961 significantly increased under salinity stress (Fig. 6). Compared to NaCl, MT increased the endogenous MT content in both inbred lines, with the content in H123 increased by 32.6% and in W961 by 25.1%. Exogenous AsA increased the endogenous MT in H123 and W961 by 17.8% and 16.1%, respectively. Applying the AsA inhibitor lycorine did not inhibit the promotion of endogenous MT synthesis in both inbred lines by exogenous MT. However, The CPA significantly inhibited the synthesis of endogenous MT in both inbred lines.

K^+ and Na^+ content: As shown in Fig. 7, the Na^+ content of both inbred lines under salinity stress significantly increased compared to CK, while the K^+ content significantly decreased (with H123 decreasing by 60.0% and W961 decreasing by 65.8%), which led to an increase in the Na^+/K^+ ratio. W961 experienced more severe ionic stress than H123. Compared to the NaCl treatment, exogenous MT and AsA significantly increased the K^+ content, and reduced the Na^+ content, thus lowering the Na^+/K^+ ratio. The application of lycorine inhibited the positive regulatory effect of MT on ionic balance. However, CPA did not suppress the promoting effect of AsA.

Discussion

MT and AsA, as growth regulators, played a positive role in alleviating the damage of salinity stress to plants (Bawa *et al.* 2020, Niu *et al.* 2022). In this study, the growth parameters of both inbred lines significantly decreased under salinity stress compared to CK. Exogenous MT and AsA significantly reduced the damage of salinity stress to inbred lines H123 and W961, and improved growth and development indicators such as plant height (Table 1). This indicated that MT and AsA could effectively alleviate

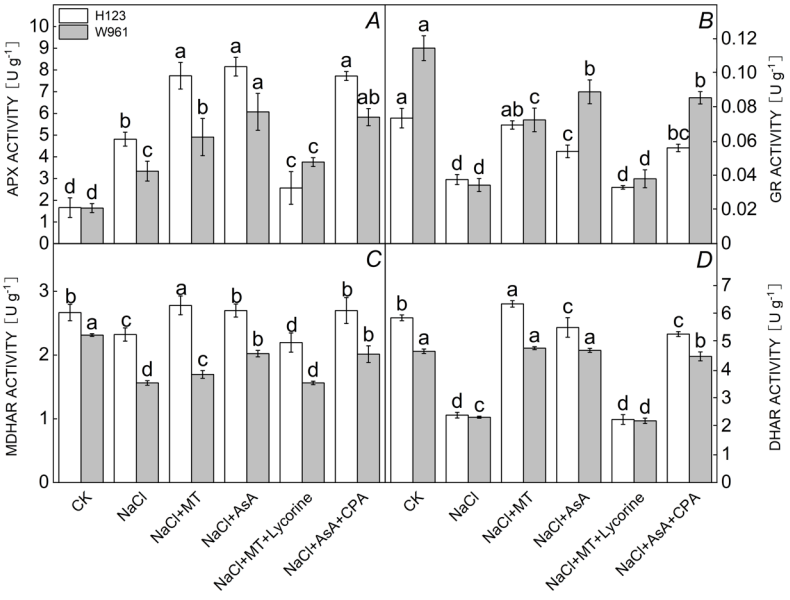

Fig. 5. Effects of melatonin (MT) and ascorbic acid (AsA) on APX, GR, MDHAR, and DHAR activities of maize under salinity stress. CK: normal control; NaCl: 100 mM NaCl; NaCl + MT: 100 mM NaCl + 100 μM melatonin; NaCl + AsA: 100 mM NaCl + 2 mM ascorbic acid; NaCl + MT + lycorine: 100 mM NaCl + 100 μM melatonin + 0.3 mM lycorine; NaCl + AsA + CPA: 100 mM NaCl + 2 mM ascorbic acid + 10 μM CPA. APX – ascorbate peroxidase; DHAR – dehydroascorbate reductase; GR – glutathione reductase; MDHAR – dehydroascorbate reductase. Values are expressed as mean ± SD of three replicates. For each variable, means with *different lowercase letters* were significantly different (p<0.05).

the harmful effects of salinity stress on plants (Barzegar *et al*. 2018, Zhang *et al*. 2022).

Chlorophyll is the most important pigment for plant photosynthesis, participating in the absorption and transfer of light energy (Wu *et al*. 2021). Consistent with previous studies, under salinity and flooding stress, MT significantly increased the Chl content in plants (Siddiqui *et al*. 2019). AsA improved photosynthesis and promoted the synthesis of Chl *a* and Chl *b* (Bybordi *et al*. 2012). The gas-exchange parameters of hibiscus (*Hibiscus syriacus* L.) under drought stress were effectively improved by the increase in Chl content (Yan *et al*. 2024). Salinity stress significantly reduced the g_s, P_N, and E of both inbred lines in this study (Fig. 1). In addition, salinity stress also lowered significantly the Chl *a*, Chl *b*, and total Chl (*a* + *b*), which indicated that salinity stress accelerated the degradation of photosynthetic pigments and led to a reduction of photosynthetic rate (Fig. 3). However, the application of MT and AsA effectively alleviated this damage.

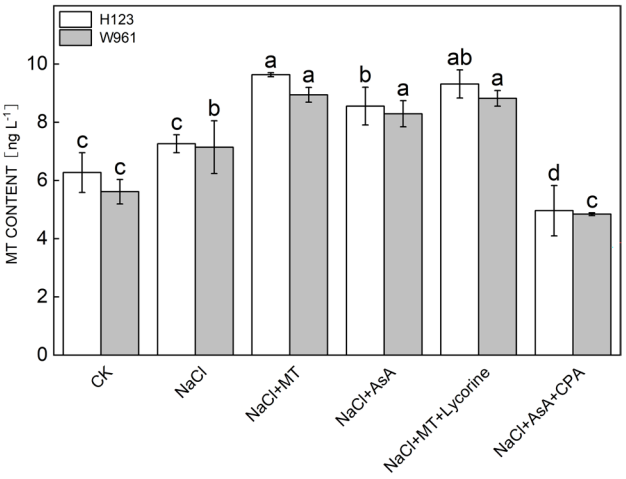

Fig. 6. Effects of melatonin (MT) and ascorbic acid (AsA) on MT content of maize under salinity stress. CK: normal control; NaCl: 100 mM NaCl; NaCl + MT: 100 mM NaCl + 100 μM melatonin; NaCl + AsA: 100 mM NaCl + 2 mM ascorbic acid; NaCl + MT + lycorine: 100 mM NaCl + 100 μM melatonin + 0.3 mM lycorine; NaCl + AsA + CPA: 100 mM NaCl + 2 mM ascorbic acid + 10 μM CPA. Values are expressed as mean ± SD of three replicates. For each variable, means with *different lowercase letters* were significantly different (p<0.05).

The content of Chl directly affected PSII's light energy efficiency. Previous research indicated that salinity stress significantly decreased the q_p, ETR, and F_v/F_m of mung bean, ultimately reducing photosynthesis (Ahmad *et al*. 2019). Studies by Azeem *et al*. (2023) have shown that under salinity stress, the application of MT and AsA positively affects the light energy utilization rate of PSII in tobacco and sorghum seedlings. In this experiment, salinity stress reduced the q_p, ETR, and F_v/F_m of both inbred lines and increased the NPQ value (Fig. 2). This indicates that the chloroplast thylakoid membrane and the PSII reaction center were damaged, accompanied by a blockage of electron transport function, with most of the captured light energy being converted into heat and dissipated (Wang *et al*. 2022). However, exogenous MT and AsA improved the F_v/F_m, q_p, and ETR of both inbred lines, which indicated that MT and AsA could alleviate the impact of salinity stress on the photosynthetic capacity and the PSII reaction center of both inbred lines, thereby enhancing

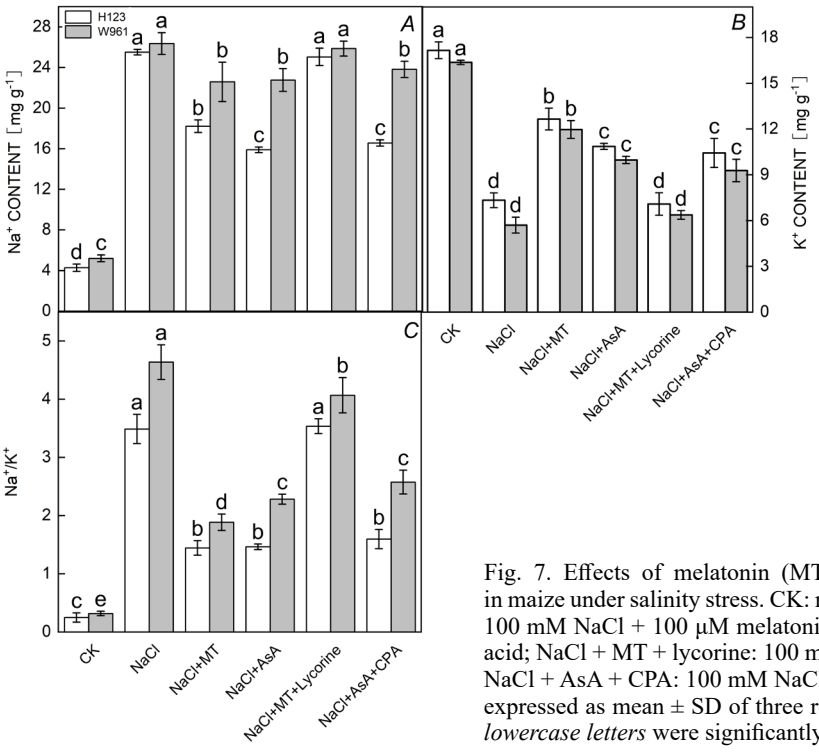

Fig. 7. Effects of melatonin (MT) and ascorbic acid (AsA) on K^+-Na^+ content in maize under salinity stress. CK: normal control; NaCl: 100 mM NaCl; NaCl + MT: 100 mM NaCl + 100 μM melatonin; NaCl + AsA: 100 mM NaCl + 2 mM ascorbic acid; NaCl + MT + lycorine: 100 mM NaCl + 100 μM melatonin + 0.3 mM lycorine; NaCl + AsA + CPA: 100 mM NaCl + 2 mM ascorbic acid + 10 μM CPA. Values are expressed as mean ± SD of three replicates. For each variable, means with *different lowercase letters* were significantly different (p<0.05).

the photosynthetic electron transport efficiency within the leaves. This was consistent with the findings of Hao *et al*. (2017) and Chen *et al*. (2021), who demonstrated that MT improved Chl fluorescence parameters in watermelon under salinity stress, while AsA enhanced light energy utilization and stability of PSII in tomato seedlings subjected to salinity stress. Huang *et al*. (2024) found that the addition of CPA somewhat inhibited the alleviating effect of MT on the damage to maize seedlings under nicosulfuron stress. In this experiment, AsA alleviated the inhibitory effects of CPA, which led to the inference that the protective effect of MT was dependent on AsA.

Excessive ROS in plant cells led to membrane lipid peroxidation damage under salinity stress (Okamoto *et al*. 2001, Mittler *et al*. 2006). Plants use antioxidant defense systems to counteract the excessive ROS (Alscher *et al*. 2002, Ros-Barceló *et al*. 2002). Additionally, MDA, as the intermediate product of lipid peroxidation, could reflect the degree of damage to biological membranes caused by oxidative stress (Vafadar *et al*. 2020). Research has shown that MT and AsA can directly scavenge ROS and free radicals by enhancing the activity of antioxidant enzymes in a variety of plants, such as tea plants (Li *et al*. 2019, Fu *et al*. 2023) and rice (*Oryza sativa* L.) (Wang *et al*. 2018, Khan *et al*. 2024). This experiment is consistent with previous research. Exogenous MT and AsA had a protective effect on cell membrane damage under salinity stress and inhibited the production of ROS (Fig. 4). However, the application of lycorine increased the contents of ROS, indicating that the synthesis of AsA may be inhibited, thereby impeding the action of MT. In addition, exogenous MT and AsA also enhanced the antioxidant enzyme activities under salinity stress, improved the ability to eliminate reactive oxygen, and prevented membrane lipid peroxidation (Fig. 4). The application of lycorine produced results that aligned with the observed trends in ROS contents. However, CPA did not inhibit the positive effects of AsA, which indicates that MT's protective effect depends on AsA.

Previous studies showed that the content of nonenzymatic metabolites and the key enzyme activities in the AsA–GSH cycle were significantly reduced under salinity stress. Exogenous MT significantly increased AsA and GSH content, and the key enzyme activities in sorghum seeds under salinity stress (Nie *et al*. 2023). Additionally, exogenous AsA could also eliminate ROS through enzymatic reactions, increase the content of endogenous AsA and GSH, reduce the degree of lipid peroxidation of the chloroplast membrane, and alleviate the damage of salinity stress to the chloroplasts in *C. procera* (Aziz *et al*. 2021). The AsA–GSH cycle is an effective antioxidant system that eliminates excess ROS by maintaining the ratios of AsA/DHA and GSH/GSSG (Wang *et al*. 2021b). In this study, the endogenous contents of AsA, GSH, AsA/DHA, and GSH/GSSG significantly decreased under salinity stress, while the contents of DHA and GSSG significantly increased. The results have been consistent with those observed in the stressed *Nitraria tangutorum* Bobr. (Gao *et al*. 2022b) and rapeseed (Hasanuzzaman *et al*. 2017). This indicated that the accumulation of ROS exceeded the AsA–GSH cycle system eliminating capacity, which reduced the plant's defensive capabilities. Yin *et al*. (2019) reported that exogenous MT increased the activities of APX, GR, MDHAR, and DHAR in tomatoes under salinity stress, thereby enhancing the efficiency of the AsA–GSH cycle. In this experiment, exogenous MT and AsA significantly increased the activities of APX, GR, MDHAR, and DHAR under salinity stress, promoted the regeneration of endogenous AsA and GSH, and significantly increased the ratios of AsA/DHA and GSH/GSSG, thereby alleviating the oxidative damage caused by salinity stress (Fig. 2S, Fig. 5). It was reported that lycorine acted as an inhibitor by suppressing the activity of the terminal enzyme in AsA biosynthesis, galactono-1,4-lactone dehydrogenase (Davey *et al*. 1998). In this experiment, lycorine inhibited the synthesis of endogenous AsA and inhibited the protective effect of MT under salinity stress. It is inferred that the action of MT depends on the response of AsA. The application of CPA did not inhibit the function of AsA, which suggested that AsA may participate in the defense against oxidative stress under salinity stress through the stability of the AsA–GSH cycle (Fig. 2S).

The influx of Na^+ into cells depolarized the plasma membrane and led to a continuous outward flow of K^+ under salinity stress. The increase in the Na^+/K^+ ratio severely damaged many physiological processes in the cell (Shabala *et al*. 2006). In this study, salinity stress significantly increased the content of Na^+ in plants and decreased the content of K^+, which was associated with the rise of the Na^+/K^+ ratio (Fig. 7). The result was consistent with the studies of Ghorbani *et al*. (2019). It was also reported that treatment with 100 μM MT significantly reduces Na^+ concentrations in tomato leaves under salinity stress while increasing K^+ content (Altaf *et al*. 2021). The exogenous application of AsA decreased Na^+ concentrations in barley (*Hordeum vulgare* L.) under salinity stress while significantly increasing K^+ content (Noreen *et al*. 2021). The results of this experiment are consistent with previous research, the addition of exogenous MT and AsA promoted an increase in K^+ and a reduction in Na^+, which helped re-establish ionic homeostasis within the cells and alleviated ionic toxicity in maize. However, the addition of lycorine inhibited the positive effects of MT, while the addition of CPA did not inhibit the action of AsA.

Conclusions: The MT and AsA promoted the maize growth parameters under salinity stress, and improved the PSII photoreaction center, thereby enhancing the photosynthetic capacity of maize. MT and AsA also enhanced the antioxidant enzyme activities. They promoted the efficiency of the AsA–GSH cycle system, which increased the ability to scavenge excessive ROS and maintained the dynamic balance of Na^+/K^+ ions. However, under salinity stress, lycorine decreased the content of endogenous AsA and reversed the positive effects of MT. The application of CPA reduced the content of endogenous MT without restraining the function of AsA. It suggested that AsA acted as a downstream signal involved in MT-mediated salinity resistance of maize.

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