Exogenous Melatonin Strengthens Saline-alkali Stress Tolerance in M9-T337 Seedlings by Initiating a Variety of Physiological and Biochemical Pathways

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Abstract

Melatonin (MT) is an important phytohormone that significantly regulates the growth and development of plants. Previous studies confirmed the effectiveness of MT in improving plant stress tolerance. In this study, annual M9-T337 seedlings were selected as subjects and five treatments were applied: conventional control (CK), in which only half the concentration of Hoagland was applied; Saline-alkali stress treatment (SA, 100 mmol·L⁻¹ Saline-alkali solution); melatonin treatment (MT, CK + 200 μmol·L⁻¹ exogenous MT); Saline-alkali + melatonin treatment (MS, SA + 200 μmol·L⁻¹ exogenous MT); and Saline-alkali stress + melatonin + inhibitor treatment (HS, additional 100 μmol·L⁻¹ p-CPA treatment to MS). The results showed that Saline-alkali stress negatively affected the growth of M9-T337 seedlings by reducing photosynthetic capacity, increasing Na⁺, promoting reactive oxygen species such as H₂O₂, and changing the osmotic content and antioxidant system. However, the application of exogenous MT effectively alleviated Saline-alkali damage and significantly promoted the growth of M9-T337 seedlings. It significantly increased plant height, diameter, root length, root surface area, volume and activity. Furthermore, MT alleviated osmotic stress by accumulating proline, soluble sugars, soluble proteins and starch. Furthermore, MT improved photosynthetic capacity by delaying chlorophyll degradation and regulating gas exchange parameters as well as fluorescence parameters in leaves. Furthermore, MT improved the Na⁺/K⁺ ratio to reduce ion toxicity by upregulating the expression of Na⁺ transporter genes (MhCAX5, MhCHX15, MhSOS1, and MhALT1) and downregulating the expression of K⁺ transporter genes (MhSKOR and MhNHX4). In addition, MT can increase antioxidant enzyme activity (SOD, POD, CAT, AAO, APX and MDH) in the ASA-GSH cycle and increase AsA, GSH and GSSG levels to counteract the accumulation of reactive oxygen species (ROS) such as H₂O₂ and O₂⁻, reducing oxidative damage. Exogenous MT promotes root growth under salt-alkaline stress by increasing root activity and responding synergistically with IAA, GA₃ and ZT to salt-alkaline stress. Our results confirm that MT has the potential to alleviate Saline-alkali stress by promoting root growth, increasing biomass accumulation and photosynthetic capacity, strengthening the antioxidant defense system, maintaining ionic balance, the ascorbate-glutathione cycle and the Osmoregulation facilitates and regulates endogenous hormone levels in M9-T337 seedlings.

Introduction

As a global environmental problem, soil salinization is a major factor hindering the sustainable development of agriculture [1]. With the intensification of the greenhouse effect and global warming, soil drought occurs, which leads to increased soil salinity [2]. Studies have shown that about 950 million hectares are affected by salinization worldwide, with China's saline land area being about 9.9 × 10⁷ hectares. These areas are mainly distributed in the northwestern inland and eastern coastal areas [3]. However, apples, as an important crop in China, are subject to severe soil salinization in their main growing regions, which has a significant impact on their growth and development [4]. Therefore, it is crucial to improve the resistance of apple trees to saline and alkaline conditions to ensure sustainable development in the apple industry.
Saline-alkali stress is one of the most important abiotic stress factors, and its damage to plants is mainly reflected in four aspects: osmotic stress, ion toxicity, high pH damage and reactive oxygen stress [5]. Osmotic stress mainly reflects that Saline-alkali stress increases the osmotic pressure of the environment of plant roots, so that the water potential of the environment of roots decreases, resulting in water leakage in plants [6]. The main manifestation of ion toxicity is that due to soil salinization, a large amount of Na\(^+\) accumulates in plants, which greatly reduces the uptake of K\(^+\) ions by plants, resulting in a series of toxicities in plants, such as: destroying the cell membrane structure, inhibiting the synthesis of related enzymes, weakening photosynthesis and hindering signal transmission in vivo [7]. The harm of high pH is mainly reflected in that Saline-alkali stress increase the pH of the rhizosphere environment of plants, thereby damaging the root environment of plants and reducing the vitality of plant roots. At the same time, there is also precipitation of a large amount of metal ions such as Ca\(^{2+}\) and Mg\(^{2+}\) in the soil, causing soil compaction, which ultimately leads to a reduction in plant absorption of Ca, Mg and other mineral nutrients, which affects the normal growth and development of plants impaired [6]. Active oxygen stress is mainly reflected in the accumulation of a large amount of ROS in plants under Saline-alkali stress, which aggravates the degree of lipid peroxidation of plant cell membrane and produces a large amount of the toxic substance MDA, ultimately leading to the destruction of plant cell membrane structure and protein synthesis, as well as the disruption of the electron transport chain in the photosynthetic respiratory pathway, which affects plant growth and development [8]. Under Saline-alkali stress, plants also alleviate salt-base damage through physiological and molecular regulatory mechanisms. The physiological mechanism of regulation is mainly manifested during saline-alkali stress: the plant itself accelerates the synthesis of organic substances such as proline, betaine and polyols [9], increases the degree of Na\(^+\) efflux and Na\(^+\) compartmentalization, and promotes absorption Ca\(^{2+}\) [10] promotes the secretion of organic acids in plants [11] and the rapid response of enzyme protection systems and non-enzyme protection systems, and accelerates the synthesis of endogenous hormones such as auxin (IAA), cytokinin (ABA), and gibberellin (GA\(_3\)) in plants [12, 13]. The molecular mechanism is mainly manifested in the regulation of salt-base stress: plants first transmit salt-base stress signals through the ABA pathway, the protein kinase pathway and the SOS pathway, and then the transcription factors are accepted upstream Transduce saline-alkali stress signals and regulate the expression of related salt-base tolerance genes downstream [14, 15]. Including the induction of plant osmoregulation, ion transport, antioxidants and other related genes [16, 17, 18]. Studies have found that the application of exogenous plant growth regulators under stress can improve plant resistance [19, 20, 21, 22]. Melatonin, a hormone-like substance widely distributed in higher plants, plays an important role in alleviating abiotic stress. Studies have shown that the application of exogenous melatonin under drought conditions [23], Saline-alkali conditions [24] and low temperatures [25] is effective in promoting seed germination and dry matter accumulation in plants, alleviating leaf senescence and Increase chlorophyll content of the leaves. At the same time, it can also promote the synthesis of amino acids and osmotic regulatory substances in plants. Application of exogenous melatonin under manganese stress significantly increased MDA and H\(_2\)O\(_2\) contents in tobacco seedlings, thereby increasing their antioxidant capacity [26]. Exogenous melatonin sprayed under high temperature stress can significantly promote the
synthesis of endogenous hormones such as auxins, cytokinins and abscisic acid in cherries, thereby increasing their heat resistance [27]. Appropriate concentrations of melatonin can also effectively increase the content of photosynthetic pigments, osmotically regulating substances and antioxidant enzyme activity in rapeseed seedlings under salt stress, thereby alleviating the damage to seedlings caused by saline-alkali stress [28]. In summary, the application of exogenous MT under various stresses has been reported in pepper [29], tomato [30], potato [31] and other plants. However, there are few reports on the effect of exogenous MT on the growth of apple plants under Saline-alkali stress and its mechanism. Therefore, there is an urgent need to investigate whether it has the same regulatory mechanism as apple rootstocks.

In this study, dwarf apple rootstock M9-T337 seedlings were used as materials to study the effects of exogenous MT on plant growth under saline-alkali stress, and the regulatory mechanism of MT was revealed from four aspects: ion homeostasis, osmotic regulation, antioxidant system and pH balance to provide a theoretical basis for the application of MT to M9-T337 in response to saline-alkaline stress.

Materials and Methods

Test materials

In May 2022, M9-T337 virus-free apple seedlings cultivated in tissue culture were procured from Shandong Huinong Horticulture Co., Ltd. The seedlings were initially nurtured for one week in the Laboratory of Fruit Tree Stress Physiology at the College of Horticulture, Gansu Agricultural University and subsequently transplanted to a rain shelter at Gansu Agricultural University to facilitate standardized cultivation management procedures. Following acclimation for a period of 10 days, experimental treatments were initiated.

Treatment and Experimental Design

Based on the previous studies of the group of test subjects, The optimal concentrations of MT and Saline-alkali were determined. This experiment consisted of five treatments: 1) Control (CK): irrigation with half concentration of Hoagland nutrient solution; 2) Saline-alkali stress (SA): Application of a compound saline-alkaline solution with a concentration of 100 mmol·L\(^{-1}\) based on the control; 3) Melatonin treatment (MT): application of 200 µmol·L\(^{-1}\) exogenous MT on a control basis; 4) saline-alkali stress + melatonin treatment (MS): application of 200 µmol·L\(^{-1}\) exogenous MT based on SA; 5) saline-alkali stress + melatonin + p-CPA treatment (HS): Application of 100 µmol·L\(^{-1}\) p-CPA based on MS. Three replicates were used for each treatment and six plants were used for each replicate. Seedlings were evenly spaced for each treatment to ensure consistent light exposure. Melatonin was applied every 3 days from 7:00–8:00 p.m. until the leaves dripped, for a total of 3 applications. On day 15, the functional leaves of M9-T337 seedlings were harvested for relevant measurements.

Growth parameter
The measurements of plant height, stem diameter, leaf area, leaf circumference, fresh weight and dry weight were conducted on three plants collected from each replicate. Each treatment was repeated 3 times.

Each plant was selected for processing and placed in the root system scanner (STD-4800 company, Canada) to capture images of the root system. Subsequently, the obtained root images were analyzed using WinRHIZO5.0 software (Regent Instruments Inc., Hydro Quebec, Canada) to determine various indices including length, total root surface area, average root diameter, total root volume, and number of root tips.

The root tip tissue weighing 0.5g was assessed for root activity using the triphenyltetrazolium chloride (TTC) method by Comas et al. [32]. Each treatment was repeated 3 times.

**Photosynthesis**

The chlorophyll a (Chla), chlorophyll a + b (Chla + b), chlorophyll a (Chla), and ca-rotenoids contents of the leaves were measured according to the method described by Arnon et al.[33].

As described by Lin et al. [34], The net photosynthetic rate ($P_n$), transpiration rate ($T_r$), intercellular CO$_2$ concentration ($C_i$), and stomatal conductance ($G_s$) of the third leaf from the top to bottom of each plant were quantified using a portable photofluorometric apparatus (CIRAS-2, PP-system, UK). Each treatment was repeated 3 times.

As described by Yuan et al. [35], M9-T337 seedlings were subjected to a 30 minutes period of darkness using a modulated leaf green fluorescence imager (image-PAM, WALZ, Germany). The third fully developed leaf was selected from the bottom of the plant for determining initial fluorescence (F0), maximum fluorescence (Fm), maximum photochemical quantum yield (Fv/Fm), and photochemical quenching coefficient (qP) following dark adaptation. Each treatment was repeated 3 times.

**Antioxidant system**

Fresh leaves (0.1 g) were homogenized with 1 mL of PBS solution with pH 7.8 on ice and centrifuged for 10 minutes at 4°C and 12,000 rpm. The resulting supernatant was used to determine the activity levels of superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) using a kit from Beijing Solarbio Technology Co., Ltd. used. Each treatment was repeated three times.

**Lipid peroxidation**

Relative electrical conductivity (REC) was measured using a DDS-307 conductivity meter, as described by Yuan et al. [36]. Malondialdehyde (MDA) content was determined using the thiobarbituric acid (TBA) colorimetric method [37](37Predieri et al. 1995). Each treatment was repeated 3 times.

**ROS**
Superoxide anion free radicals (O$_2^-$) were measured using the hydroxylamine oxidation method\[38\]. Fresh leaves (1 g) were weighed and placed in a mortar. Phosphate buffer (65 mmol·L$^{-1}$, 3 mL) was added. Homogenization was carried out by grinding, and the resulting homogenate was transferred to a centrifuge tube. The homogenate was centrifuged at 10,000 r/min for 15 min, and the supernatant was used for measurement. The hydrogen peroxide (H$_2$O$_2$) content was determined using the xylenol orange method \[39\].

**ASA-GSH cycle**

Fresh leaves (0.1 g) were homogenized with 1 mL of extract on ice, and the resulting homogenate was placed into a centrifuge tube. The homogenate was centrifuged at 12,000 rpm for 10 min at 4°C, and the supernatant was used to determine the levels of reduced glutathione (GSH), reduced ascorbic acid (AsA), oxidized glutathione (GSSG), ascorbic acid oxidase (AAO), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), and glutathione reductase (GR) using assay kits from Beijing So-leibao Technology Co., Ltd. Each treatment was repeated 3 times.

**Osmotic adjustment**

Soluble sugars (SS) were measured using the 3,5-dinitrosalicylic acid method, as described by Cut et al. \[40\], while soluble proteins (SP) were measured using the Coomassie brilliant blue G-250 staining method, as described by Sevket et al. \[41\]. Free proline (Pro) was measured using the acid ninhydrin method, as described by Wang et al. \[42\], and starch was measured using the anthrone colorimetric method, as described by Eros et al. \[43\].

**Endogenous hormone**

Endogenous hormone contents were determined according to Zheng et al. \[44\]. method. The endogenous hormone levels of auxin (IAA), gibberellin (GA$_3$), abscisic acid (ABA), and zeatin (ZT) were determined in 0.5 g leaves using ultra-high-performance liquid chromatography-tandem mass spectrometry (UHPLC–MS/MS). The chromatographic column used was a Symmetry C18 column (4.6 mm 250 mm, 5 µm) at a temperature of 30°C with a mobile phase consisting of a 1:9 ratio of methanol to 0.1% phosphoric acid at a flow rate of 1.0 mL/min. An injection volume of 10 µL was used.

**Na$^+$ K$^+$ and Ca$^{2+}$ contents**

The leaf powder samples (5 g) were precisely weighed and dried, followed by digestion with H$_2$SO$_4$-H$_2$O$_2$. The concentrations of Na$^+$, K$^+$, and Ca$^{2+}$ were determined using an inductively coupled plasma-optical emission spectrometer (Perkin Elmer, Wal-tham, Massachusetts, USA), following the methodology described by Kuang et al. \[45\]. Each treatment was repeated 3 times.

**qRT–PCR**

Total RNA was extracted from leaves using the Plant RNA Extraction Kit and reverse transcribed with the RT Master Mix Reverse Transcription Kit, both provided by Takara Clontech Biochemicals in Shanghai. An
internal reference gene, the apple actin gene (Accession No. MDPO000774288), was used. Using RNA-seq results from plants treated with salt-alkali compound stress (NCBI No. PRJNA588566), nine apple salt-responsive genes were selected and detected via fluorescence quantification with qRT-PCR. The reaction system, consisting of 20 µL, included 2 µL each of upstream and downstream primers, 2 µL of cDNA template, 10 µL of Trans Start Top Green qPCR Super Mix, and 6 µL of ddH₂O. Shanghai Sangon Biotech designed and synthesized the qPCR primers, which were checked for specificity using BLAST in the Apple genome database (https://www.rosaceae.org). See Table S1 for a list of the primers used. Each treatment was repeated 3 times.

**Statistical analysis**

Data processing was performed using Microsoft Office Excel 2019, and the graphs were plotted using Origin 2022 software. Statistical analysis was carried out using the IBM SPSS Statistics 25 program (SPSS Inc., Chicago, IL, USA). An analysis of the variance (ANOVA) was used to compare mean values between samplings. The Duncan post-hoc test was used to determine differences between treatments. Significance levels of 95% (P < 0.05) are indicated in figure legends.

**Results**

**Effects of exogenous MT on leaves and roots of M9-T337 seedlings under salt-alkali stress**

After 15 days of saline-alkaline stress treatment, the phenotype of M9-T337 seedlings was observed as shown in Fig. 1A. Compared to the control (CK), the leaves showed chlorosis and wilted under saline-alkali stress (SA). In contrast, when treated with exogenous melatonin (MT) alone, leaves retained their green color and appeared large. When melatonin was sprayed on seedlings under salt-alkaline (MS) stress, the leaves turned visibly green and grew well. However, spraying a combination of melatonin and p-CPA treatment (HS) under saline-alkali stress resulted in poor leaf greening, without significant differences compared to SA. After exogenous MT treatment, the deleterious effects of saline-alkali stress on root damage were significantly alleviated, total root length, mean root diameter, total root volume, total root surface area, number of root tips and total activity increased by 43.22%, 10.71%, 96.06%, 73.75% and 9.70%, respectively (Table S2). However, coapplication of MT inhibitor (p-PCA) under saline-alkali stress could not alleviate the impairment of root growth in M9-T337 seedlings (Fig. 1B).

**Effects of exogenous MT on growth parameters of M9-T337 seedlings under saline-alkali stress**

The growth rate of M9-T337 seedlings under saline-alkali stress was significantly reduced, as shown in Fig. 2. Under saline-alkali stress, the growth of M9-T337 seedlings was hindered, resulting in significantly reduced plant height, stem diameter, leaf area and leaf circumference compared to the control group (CK). Specifically, these measurements were only 73.76%, 52.23%, 40.71% and 61.98% of the control group,
respectively. However, when 200 µmol·L\(^{-1}\) exogenous MT (MS) was sprayed, plant height, stem diameter, leaf area and leaf circumference of M9-T337 seedlings showed a significant increase compared to SA treatment. These measurements were 1.11 times, 1.59 times, 1.43 times, and 1.33 times higher than those of the SA treatment, respectively. Furthermore, when p-CPA (HS) was applied in addition to MS treatment, the plant height, stem diameter, leaf area and leaf circumference of M9-T337 seedlings were significantly lower compared to MS treatment. However, when exogenous MT was sprayed based on CK treatment, the plant height, stem diameter, leaf area and leaf circumference of M9-T337 seedlings were slightly higher than those in CK treatment, although the difference was not statistically significant.

The biomass accumulation of M9-T337 seedlings under saline-alkali (SA) stress was significantly inhibited, as shown in Fig. S1. Both the fresh weight and dry weight of both aboveground and belowground parts were lower than that of control (CK) and were only 42.38%, 75.28%, 76.12% and 50.82% of CK. However, when exogenous melatonin (MS) was sprayed at a concentration of 200 µmol·L\(^{-1}\) after SA treatment, there was a significant increase in the fresh weight and dry weight of M9-T337 seedlings by about 1.81-fold, 1.17-fold 1.56-fold, and 1.71-fold, compared with SA treatment. On the other hand, when p-CPA was applied after MS treatment, there was a significant decrease in fresh and dry weight of both above-ground and below-ground parts, which was only about 1.45 g, 2.11g, 0.53g, and 0.35g, compared to MS treatment alone. When spraying exogenous melatonin at a concentration of 200 µmol·L\(^{-1}\) based on the CK, the fresh weight and dry weight of the M9-T337 seedlings were slightly higher than that of the control, however, the difference was not significant.

**Effects of exogenous MT on photosynthesis in leaves of M9-T337 seedlings under saline-alkali stress**

The effects of exogenous melatonin (MT) on photosynthetic pigments in the leaves of M9-T337 seedlings under saline-alkali stress are shown in Fig. 3. Compared to the control group (CK), the levels of Chl a (Fig. 3A), Chl b (Fig. 3B), total chlorophyll (Chl a + b) (Fig. 3C) and carotenoids (Car) (Fig. 3D) in the leaves of M9-T337 seedlings exposed to 100 mmol·L\(^{-1}\) saline-alkali stress were significantly reduced by 36.25%, 53.19%, 41.09% and 46.27%, respectively. However, after foliar application of exogenous MT at a concentration of 200 µmol·L\(^{-1}\), the contents of Chl a, Chl b, Chl a + b and Car in the leaves of M9-T337 seedlings treated with MS were significantly higher than those treated with SA, showing an increase of 26.74%, 60.86%, 34.44% and 58.31%, respectively.

As shown in Fig. 4, the net photosynthetic rate (\(Pn\)), transpiration rate (\(Tr\)), and stomatal conductance (\(Gs\)) of M9-T337 seedlings significantly decreased under saline-alkali (SA) stress. Compared to the control (CK), \(Pn\), \(Tr\) and \(Gs\) decreased by 30.67%, 45.19% and 33.07%, respectively, while the \(Ci\) increased significantly to 1.67 times the CK concentration. After spraying exogenous melatonin (MT) at a concentration of 200 µmol·L\(^{-1}\) based on SA treatment, leaf \(Pn\), \(Tr\) and \(Gs\) values significantly increased by 30.66%, 39.93% or 49.91%, while \(Ci\) decreased significantly by 23.52%. However, after application of p-PCA based on MS treatment, the leaf \(Pn\), \(Tr\) and \(Gs\) of M9-T337 seedlings significantly reduced to 10.55
µmol·m$^{-2}$·s$^{-1}$, 2.72 µmol ·m$^{-2}$·s$^{-1}$ and 10.30 mol · m$^{-2}$·s$^{-1}$, respectively, while $Ci$ increased significantly to 410.55 µmol · mol$^{-1}$. Finally, when exogenous MT was sprayed at a concentration of 200 µmol·L$^{-1}$ based on CK, there was no significant change in $Pn$, $Tr$, $Gs$ and $Ci$ of M9-T337 seedling leaves.

The parameter values F0, Fm, Fv/Fm and qP of the CK and MT treatments showed no significant difference. However, after SA treatment, the F0, Fm, Fv/Fm and qP parameter values of M9-T337 seedlings decreased significantly. This trend was changed by MT spraying. Nevertheless, the combined application of MT and p-CPA had no significant effect on alleviating saline-alkali stress (Fig. S2).

**Effect of exogenous MT on membrane lipid peroxidation degree of M9-T337 seedlings under saline-alkali stress**

According to the results shown in Fig. 5A-B, the H$_2$O$_2$ content and O$_2^-$ production rate of M9-T337 seedlings under saline-alkali (SA) stress showed a significant increase, reaching a value of 1.38-fold and 2.01-fold that of the control group (CK). However, when exogenous melatonin (MT) was applied at a concentration of 200 µmol·L$^{-1}$ after SA treatment, a significant decrease in H$_2$O$_2$ content and O$_2^-$ production rate of M9-T337 seedling leaves was observed. Specifically, the decreases were 13.72% and 41.24%, respectively, compared to the SA treatment. Conversely, when p-PCA treatment was applied after MT treatment, a significant increase in H$_2$O$_2$ and O$_2^-$ contents was observed in the leaves of M9-T337 seedling, compared to MT treatment, the increases were 11.13% and 65.46%, respectively. Although there was a slight increase in H$_2$O$_2$ and O$_2^-$ contents in the leaves of M9-T337 seedlings at the CK level when spraying with only exogenous MT, no significant difference was observed compared to CK treatment.

As shown in Fig. 5C-D, application of saline-alkali (SA) treatment resulted in a significant increase in REC and MDA contents in the leaves of M9-T337 seedlings. Specifically, the REC and MDA concentrations were measured at 47.21% and 8.21 µmmol·g$^{-1}$, respectively. Compared to the control group (CK), these increases were 60.74% and 102.45%, respectively. Nevertheless, application of exogenous melatonin (MT) at a concentration of 200 µmol·L$^{-1}$ after SA treatment resulted in a significant decrease in REC and MDA contents in the leaves of M9-T337 seedlings. More specifically, these contents decreased by 14.55% and 26.99%, respectively, compared to the SA treatment. In contrast, p-PCA treatment resulted in a significant increase in REC and MDA contents in the leaves of M9-T337 seedlings after MS treatment. Compared to MS treatment, these increases were 15.39% and 31.51%, respectively. Finally, application of exogenous melatonin (MT) at a concentration of 200 µmol·L$^{-1}$ after CK treatment did not result in any significant change in REC or MDA.

As shown in Fig. 5E-G, the activities of superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) in the leaves of M9-T337 seedlings showed a significant increase under saline-alkali. Specifically, they were 2.13 times, 1.36 times and 1.42 times higher than those of the control group (CK), respectively. When exogenous melatonin (MT) was applied at a concentration of 200 µmol·L$^{-1}$ following SA treatment, the activities of SOD, POD and CAT in M9-T337 seedling leaves further experienced an increase
of 36.76%, 12.45% and 34.04% compared to SA treatment. However, when p-PCA was applied based on MS processing, the activities of SOD, POD and CAT in M9-T337 seedling leaves significantly decreased by 25.05%, 7.69% and 22.46%, respectively. Finally, application of exogenous MT at a concentration of 200 µmol·L⁻¹ after CK treatment did not result in significant changes in the activities of SOD, POD or CAT in M9-T337 seedling leaves.

**Effects of exogenous MT on ASA-GSH cycle of M9-T337 seedlings under saline-alkali stress**

Figure 6A shows a significant decrease in ascorbic acid oxidase (AAO) activity in the leaves of M9-T337 seedlings when subjected to saline-alkali stress (SA) treatment. In contrast, the activities of ascorbate peroxidase (APX) (Fig. 6B), glutathione reductase (GR) (Fig. 6C), and monodehydroascorbate reductase (MDHAR) (Fig. 6D) showed a significant increase. The respective activities were 0.21 mg·g⁻¹, 1.27 mg·g⁻¹, 0.33 mg·g⁻¹ and 0.64 mg·g⁻¹. Subsequently, when 200 µmol·L⁻¹ exogenous MT (MS) was sprayed onto the SA treatment, the activities of AAO, APX, GR and MDHAR in the leaves showed a significant increase. Specifically, these activities were 2.6 times, 1.89 times, 2.31 times, and 1.65 times the SA, respectively. In contrast, the activities of AAO, APX, GR and MDHAR in the leaves of M9-T337 seedlings showed a significant decrease after the application of p-PCA (HS) based on MS treatment. The declines were 56.41%, 44.07%, 53.73% and 37.79%, respectively. Finally, spraying 200 µmol·L⁻¹ exogenous MT onto the CK treatment resulted in only a slight increase in the activities of AAO, APX, GR and MDHAR in the leaves. These activities only increased by 15.00%, 3.15%, 3.03% and 3.15%, respectively, compared to CK.

Figure 6E-G clearly shows that saline-alkali (SA) treatment significantly reduced the content of reduced ascorbic acid (AsA) (Fig. 6E) and reduced glutathione (GSH) (Fig. 6F) in the leaves of M9-T337 seedlings. The content of AsA decreased by 57.02% to 4.62 mg·g⁻¹, while the content of GSH decreased by 34.77% to 25.94 µg·g⁻¹ compared to the control group (CK). In contrast, the oxidized glutathione (GSSG) content (Fig. 6G) increased significantly, reaching a value 2.69 times higher than that of the CK group. However, when exogenous melatonin (MT) was sprayed at a concentration of 200 µmol·L⁻¹ after SA treatment, the content of AsA and GSH in leaves significantly increased by 198.48% and 21.13%, respectively, during the GSSG content reduced significantly and fell significantly by 47.23%. Conversely, when p-PCA treatment was applied after MS treatment, the contents of AsA and GSH in the leaves of M9-T337 seedlings significantly decreased to 5.01 mg·g⁻¹ and 26.84 µg·g⁻¹, respectively. the content of GSSG increased significantly by 41.17 µg·g⁻¹. Finally, spraying exogenous MT at a concentration of 200 µmol·L⁻¹ after CK treatment did not result in significant changes in AsA, GSSG or GSH content in the leaves of M9-T337 seedlings.

**Effects of exogenous MT on Osmotic adjustment of M9-T337 seedlings under saline-alkali stress**
As shown in Fig. 7, the contents of proline (Pro) (Fig. 7A), soluble sugars (SS) (Fig. 7B), soluble proteins (SP) (Fig. 7C), and starch (St) (Fig. 7D) in the leaves of M9-T337 seedlings showed a significant increase when exposed to saline-alkali stress (SA) treatment. Specifically, they were found to be 2.15-fold, 1.19-fold, 1.26-fold and 0.48-fold higher than those of the control group (CK), respectively. Subsequently, after the application of exogenous melatonin (MT) at a concentration of 200 µmol·L$^{-1}$ after SA treatment, the concentrations of Pro, SS, SP and St in the leaves of M9-T337 seedlings further increased. Achieving 1.99-fold, 1.37-fold, 1.14-fold and 1.43-fold, respectively, the values obtained from SA treatment. However, when p-PCA was applied based on MS processing, a significant decrease in the amounts of Pro, SS, SP and St was observed in the leaves of M9-T337 seedlings, which reached 20.66 µg·g$^{-1}$, 23.78 mg·g$^{-1}$, 17.06 mg·g$^{-1}$ and 4.91 mg·g$^{-1}$, respectively. Finally, although a slight increase in Pro, SS, SP and St levels was observed in the leaves of M9-T337 seedlings upon spraying with exogenous MT on the CK level, there was no significant difference compared to the CK treatment.

**Effects of exogenous MT on endogenous hormone content in leaves of M9-T337 seedlings under saline-alkali stress**

According to the results shown in Fig. 8, the concentrations of zeatin (ZT), gibberellin (GA$_3$), auxin (IAA), and cytokinin (ABA) in the leaves of M9-T337 seedlings showed a significant increase under saline-alkali stress (SA) Treatment. These increases were 17.43%, 33.10%, 117.88% and 118.83%, respectively, compared to the control group (CK). After application of 200 µmol·L$^{-1}$ exogenous MT (MS) in SA treatment, the concentrations of ZT, GA$_3$ and IAA in leaves showed a further increase of 64.85%, 46.69% and 42.90, respectively % compared to the values observed under SA. However, the ABA concentration decreased by 20.66% compared to SA. Subsequently, the concentrations of ZT, GA$_3$ and IAA in the leaves of M9-T337 seedlings showed a significant decrease after the application of p-PCA based on MS treatment, while the concentration of ABA increased significantly. Finally, spraying 200 µmol·L$^{-1}$ exogenous MT onto the CK treatment did not result in significant changes in the concentrations of ZT, GA$_3$, IAA, or ABA in leaves.

**Effects of exogenous melatonin on Na$^+$, K$^+$ and Ca$^{2+}$ contents in leaves of M9-T337 seedlings under saline-alkali stress**

According to Fig. 9A, the Ca$^{2+}$ content in the leaves of M9-T337 seedlings significantly decreased by 22.19% under saline-alkali stress (SA) treatment compared to the control (CK). In contrast, Na$^+$ (Fig. 9B) and K$^+$ (Fig. 9C) contents increased significantly compared to CK, with an increase of 13.50-fold and 2.45-fold, respectively. After spraying 200 µmol·L$^{-1}$ exogenous MT (MS) on SA seedlings, leaf Ca$^{2+}$ and K$^+$ contents increased significantly by 1.18-fold and 1.67-fold, respectively, during the Na$^+$ content decreased significantly by 20.45% compared to those on SA. When p-PCA was applied to MS-treated seedlings, Ca$^{2+}$ and K$^+$ contents in leaves decreased significantly, while Na$^+$ content increased significantly. However, there were no significant changes in Ca$^{2+}$, K$^+$ and Na$^+$ contents in the leaves of seedlings treated with 200 µmol·L$^{-1}$ exogenous MT compared to those under CK.
According to Fig. 9D, the Na\(^+\)/K\(^+\) ratio in the leaves of M9-T337 seedlings under saline-alkali stress treatment (SA) showed a significant increase of 453.71% compared to the control (CK). However, the Na\(^+\)/K\(^+\) ratio in leaves decreased significantly to 0.35 after spraying 200 \(\mu\)mol·L\(^{-1}\) exogenous MT (MS) to SA-treated seedlings. Subsequently, application of p-PCA to the MS-treated seedlings resulted in a significant increase of 57.14% in Na\(^+\)/K\(^+\) ratio compared to those under MS. Notably, there were no significant changes in the Na\(^+\)/K\(^+\) ratio in the leaves of seedlings treated with only 200 \(\mu\)mol·L\(^{-1}\) exogenous MT compared to CK.

**Effects of exogenous melatonin on saline-alkali response gene expression in M9-T337 seedlings**

Compared to the control group, the expression levels of Na\(^+\) transporter genes (\textit{MhCAX5}, \textit{MhSOS}, \textit{MhALT1}), K\(^+\) transporter genes (\textit{MhSKOR}, \textit{MhNHX4}) and antioxidant enzyme regulatory genes (\textit{MhPOD}, \textit{MhCAT}) showed no significant changes after MT treatment alone. However, SA treatment resulted in significant downregulation of 4 Na\(^+\) transporter genes and \textit{MhSKOR} expression levels, while the expression of \textit{MhNHX4} was significantly upregulated. This phenomenon was reversed by MS processing. Importantly, application of p-PCA impaired the ability of MT to alleviate salt-base stress (Fig. S3).

**Correlation and principal component analysis of various indexes of M9-T337 seedlings under different treatments**

Figure 10A shows the results of a correlation analysis performed on 35 physiological indicators of M9-T337 seedlings after treatment. The results show that the $Pn$ of M9-T337 seedlings has a highly significant positive correlation with $Tr$, $Gs$, $F0$, $Fm$, $Fv/Fm$, $qP$, Chl a, Chl b, Chl a + b, Cal, SP, ST, AsA, GSH and root activities ($P < 0.01$). Furthermore, it was significantly positively correlated with AAO ($P < 0.05$), but showed extremely negative correlation with $Ci$, MDA, REC, $H_2O_2$, $O_2^-$, GSSG and ABA ($P < 0.01$). Furthermore, it was negatively correlated with SOD, POD and IAA ($P < 0.05$).

To comprehensively evaluate the physiological response of M9-T337 seedlings to exogenous MT under saline-alkaline stress, we performed principal component analysis for 48 indices after treatment. Two principal components with eigenvalues greater than 1 were extracted. The differential contribution rates of the first and second principal components were 75.1% and 20.7%, respectively, and the cumulative variance contribution rate was 95.8%. Comprehensive ranking found that MS treatment was the most effective (Fig. 10B).

**Discussion**

When plants are exposed to saline-alkali stress, the roots respond first and transmit stress signals to the above-ground parts through their own regulatory activities, thus affecting the normal growth and development of plants [46]. saline-alkali stress can accelerate the decomposition of light and pigments of plant leaves, ultimately leading to leaf senescence, wilting and a decrease in photosynthetic capacity [47].
Exogenous MT can effectively delay the degradation of chlorophyll content in tomato seedling leaves under saline-alkali stress, thereby improving their photosynthetic capacity [48]. In this experiment, under saline-alkali stress, the Chl a, Chl b, Chl a + b and Car contents of M9-T337 seedling leaves were significantly reduced, while growth indicators such as plant height, stem thickness, and dry and fresh weights were also reduced were significantly reduced. This is similar to the results of Ashraf et al. [49] and Dombrowsk et al. [50] and it may be due to the closure of stomata in M9-T337 seedlings under saline-alkali stress, which leads to a decrease in photosynthetic capacity and destroys the thylakoid membrane of various photosynthetic pigments [51]. After treatment with 200 µmol·L⁻¹ exogenous MT, its biological accumulation increased significantly and the Chl a, Chl b, Chl a + b and Car contents as well as the root vitality of leaves were significantly improved, which promoted root development, this may be because exogenous MT can regulate the expression levels of chlorophyll-related genes, thereby increasing the Chl a, Chl b, Chl a + b and Car contents of M9-T337 seedling leaves under saline-alkali stress is increased and photosynthetic capacity is increased. and promoting biomass accumulation and root development to alleviate the damage caused by saline-alkali stress [52]. The stability of photosynthetic gas exchange parameters plays an important regulatory role in plant photosynthesis [53]. The correlation analysis in this experiment showed that $P_n$ is strongly positively correlated with Chl a, Chl b, Chl a + b and Car, suggesting that the decrease in light and pigment content may be an important factor in the weakening of photosynthesis. Studies have shown that both stomatal and non-stomatic limitations can lead to a decline in leaf $P_n$ in plants [54]. When leaf $G_s$ decreases while $C_i$ remains unchanged or even increases, non-stomatic factors such as a decrease in the assimilation capacity of mesophyll cells are the main cause of the decrease in plant photosynthetic rate [55]. In this experiment, saline-alkali stress caused a decrease in the $G_s$ of M9-T337 seedling leaves while $C_i$ increased, indicating that the decrease in $P_n$ of M9-T337 seedling leaves under saline-alkali stress is caused by non-stomatic limiting factors became. After treatment with exogenous MT, $P_n$, $T_r$ and $G_s$ of M9-T337 seedlings, leaf area increased significantly while $C_i$ decreased significantly. This may be because exogenous MT can inhibit the excessive decline in photosynthetic activity of mesophyll cells, maintain the stability of photosynthetic gas exchange parameters in plants, and increase the photosynthetic efficiency of plants under stress, which is consistent with the results of Zhang et al. [56] resembles made of cotton.

Changes in chlorophyll fluorescence parameters may reflect the degree of damage to photosynthetic organs of plants under stress [57]. $F_0$ represents the initial fluorescence of PSII, $F_m$ represents the maximum fluorescence of PSII, $F_v/F_m$ represents the maximum photochemical efficiency of PSII, and $q_P$ represents the photochemical quenching coefficient of PSII. This study found that saline-alkali stress significantly reduced $F_0$, $F_m$, $F_v/F_m$ and $q_P$ of leaves of M9-T337 seedlings, suggesting that the potential active site of PSII in M9-T337 seedlings under saline-alkali stress also affected the degradation of PSII receptor-associated electron transfer proteins, ultimately leading to a reduction in light energy utilization and inhibition of the light response [58, 59, 60]. After treatment with exogenous MT, the $F_0$, $F_m$, $F_v/F_m$ and $q_P$ parameters of M9-T337 seedling leaves all increased significantly, which is consistent with the results of Yan et al. [61]. This may be because exogenous MT can reduce the relative efficiency of
electron transfer, consume a large amount of light intensity in heat dissipation, and effectively alleviate the damage of saline-alkali stress to PSII.

Saline-alkali stress can disrupt the balance between plant photosynthetic electron transfer and the Calvin cycle, causing the electrons to be transferred from chloroplasts and mitochondria to oxygen molecules due to a lack of electron transfer transport, resulting in the production of a large amount of reactive Oxygen species (ROS), with $\text{H}_2\text{O}_2$ and $\text{O}_2^{-}$ being the main products. The accumulation of a large amount of ROS can trigger membrane lipid peroxidation, damage the cytoplasmic membrane, and cause plant cell damage or even death [62, 63]. Relative electrical conductivity (REC) and malondialdehyde content (MDA) can be used to assess the extent of cell membrane damage [64]. This study found that the accumulation of $\text{H}_2\text{O}_2$ and $\text{O}_2^{-}$ in M9-T337 seedling leaves increased significantly under saline-alkali stress, and the REC and MDA content of leaves also increased significantly, which is consistent with the results of Weisany et al. [65], in soybean and High et al. [66]. After treatment with exogenous MT, the accumulation of $\text{H}_2\text{O}_2$ and $\text{O}_2^{-}$ in the leaves of M9-T337 seedling decreased significantly, and the REC and MDA contents of the leaves also decreased significantly. This is similar to the results of Liang et al. [67]; this may be because exogenous MT can induce ROS scavenger gene activity, thereby participating in the ROS scavenging process in M9-T337 seedlings under saline-alkali stress, inhibiting the degree of membrane lipid peroxidation, and maintaining the balance between photosynthetic electron transfer and the Calvin cycle [26].

Plants require the support of antioxidant enzymes (SOD, POD and CAT) to remove excess ROS from their body, and the activity of antioxidant enzymes can be used to determine the level of ROS scavenging [68]. This experiment showed that saline-alkali stress significantly increased SOD, POD and CAT activities in M9-T337 seedling leaves, which may be due to the spontaneous regulation of plant physiological activities to increase the activity of SOD, POD and CAT CAT in leaves. After treatment with exogenous MT, SOD, POD and CAT activities in M9-T337 seedling leaves further increased and were significantly higher than those under saline-alkali stress. This is similar to the results of Chen et al. [69], in maize and it may be due to the fact that exogenous MT can regulate the high expression of genes encoding antioxidant enzymes, remove excess ROS in a timely manner, and increase the activity of SOD, POD and CAT in leaves [70, 71, 72].

The AsA-GSH cycle, an important ROS scavenging system in plants, consists of two antioxidants (AsA and GSH) and four key enzymes (AAO, APX, MDHAR and GR). APX reduces $\text{H}_2\text{O}_2$ to $\text{H}_2\text{O}$, producing the unstable starting product MDHA, which is then reduced to AsA by MDHAR. GSH can be oxidized to GSSG, which is then converted back to GSH by GR through the reduction of coenzyme II, thereby maintaining cellular redox balance [73, 74]. In this study, we found that saline-alkali stress significantly reduced the contents of AsA and GSH in the leaves of M9-T337 seedlings, while the GSSG content significantly increased. After exogenous MT treatment, the contents of AsA and GSH in leaves increased significantly, while the content of GSSG decreased significantly. This is similar to the results of Wu et al. [75], this may be because exogenous MT promotes the conversion of AsA to DHA and regulates the exchange between
GSH and GSSG, thereby maintaining redox homeostasis and playing an important role in the salt-alkaline tolerance of M9-T337 seedlings.

The efficient operation of the AsA-GSH cycle requires the synergistic participation of various enzymes such as AAO, APX, MDHAR and GR. AAO and APX catalyze the reaction between AsA and H$_2$O$_2$ to form MDHA, which is then reduced to AsA by MDHAR. Meanwhile, GSSG can be reduced to GSH by GR [76]. The results of this study showed that under saline-alkali stress, the activities of APX, MDHAR and GR in M9-T337 seedling leaves increased to different extents, while the activity of AAO decreased significantly. This is similar to the results of Xu et al. [77]. After exogenous MT treatment, the activities of APX, MDHAR and GR in leaves significantly decreased, while the activity of AAO increased significantly. This is similar to the results of Wu et al. 75(2019), the may be due to the ability of exogenous MT to regulate the expression of genes related to the AsA-GSH cycle to alter corresponding enzyme activities, enhance antioxidant defense and improve oxidative stress tolerance of plants.

Osmotic regulation is an important physiological response mechanism of plants to salt-alkaline stress. The osmotic regulators mainly include proline, soluble sugars, soluble proteins, starch and other substances [78]. Under saline-alkali stress, plants increase the synthesis of osmotic regulators such as proline, soluble sugars, soluble proteins and starch to reduce cell water potential and maintain normal plant growth [79]. The results of this study showed that the contents of proline, soluble sugars, soluble proteins and starch in M9-T337 seedling leaves increased significantly under saline-alkali stress, and the contents of these four osmotic regulators in leaves further increased after exogenous MT treatment. This is similar to the results of Zhang et al. [79] in sugar beet, the may be due to the positive effects of exogenous MT on the synthesis and accumulation of osmotic regulators in plants, improving their adaptability to saline-alkali stress.

Endogenous hormones, as important signaling molecules for hormone response in plants, play an important regulatory role in normal plant growth under stress [80]. The results of this study showed that under saline-alkali stress, the content of ZT, GA$_3$ and IAA in the leaves of M9-T337 seedlings increased significantly, while the content of ABA significantly decreased. After exogenous MT treatment, the contents of ZT, GA$_3$, IAA and ABA in the leaves increased significantly. This is similar to the results of Jia et al. [81] in cherries and may be due to the ability of exogenous MT to regulate the levels of endogenous hormones in plants, thereby increasing their tolerance to saline-alkali stress.

During saline-alkali stress, there is a significant increase in sodium (Na$^+$) and potassium (K$^+$) levels in the cytoplasm of plants [82]. Nevertheless, these indicators decreased significantly after treatment with melatonin. At the same time, the expression of four sodium (Na$^+$) transport genes (MhCAX5, MhCHX15, MhSOS1 and MhACT1) was upregulated in M9-T337 seedlings, while the expression of two potassium (K$^+$) transport genes (MhSKOR and MhNHX4) was downregulated. These results suggest that melatonin maintains sodium and potassium homeostasis in M9-T337 seedlings by increasing sodium transport (Na$^+$) and potassium efflux (K$^+$).
Conclusions

The growth of M9-T337 seedlings was inhibited by salt and alkali stresses, resulting in ion toxicity, osmotic stress, and oxidative damage. However, application of exogenous MT reversed these phenomena and improved the salt-base tolerance of M9-T337 seedlings. In particular, exogenous MT treatment improved osmoregulation, facilitated reactive oxygen species (ROS) clearance, and improved photosynthetic capacity and ionic balance, thereby improving the salt-alkaline tolerance of M9-T337 seedlings. Furthermore, exogenous MT indirectly improved seedling saline-alkali tolerance through synergistic effects with other compounds such as ZT, IAA, GA$_3$, and ABA.

Declarations

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Authors’ contributions

Xulin Xian and Yanxiu Wang designed the research. Xulin Xian, Zhongxing Zhang, Jiao Cheng and Shuangcheng Wang performed the experiments. Yanlong Gao, Naiying Ma and Cailong Li performed the data analysis and interpretation. Xulin Xian and Yanxiu Wang prepared the figures and tables. Xulin Xian wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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Availability of data and materials

All the data is present inside the manuscript. There is no supplementary file.

Ethics approval and consent to participate

We all declare that manuscript reporting studies do not involve any human participants, human data, or human tissue. So, it is not applicable. Experimental research and field studies on plants (either cultivated or wild), including the collection of plant material, must comply with relevant institutional, national, and international guidelines and legislation. No permission is required. Plants material was purchased.

Consent for publication

Not applicable
Competing interests

The authors declare no competing interests

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**Figures**
Figure 1

Effects of exogenous MT on leaves and roots of M9-T337 seedlings under salt-alkali stress. CK represents watering with a 1/2 concentration of Hoagland nutrient solution only; SA represents the application of 100 mmol·L$^{-1}$ composite salt-alkali solution based on the control; MT represents the spraying of 200 μmol·L$^{-1}$ exogenous MT based on the control; MS represents the spraying of 200 μmol·L$^{-1}$ exogenous MT based on SA; HS represents the spraying of 100 μmol·L$^{-1}$ p-CPA on the basis of MS. Vertical bars represent the standard errors of the means (three replicates. Data show the mean ± SE (n = 3). Different lowercase letters indicate significant differences between treatments with P < 0.05. The same below.
Figure 2

Effects of exogenous MT on growth parameters and biomass accumulation parameters of M9-T337 seedlings under saline-alkali stress
Figure 3

Effects of exogenous MT on photosynthetic pigment in leaves of M9-T337 seedlings under saline-alkali stress
Figure 4

Effects of exogenous MT on photosynthetic gas parameters in leaves of M9-T337 seedlings under saline-alkali stress
Figure 5

Effects of exogenous MT on membrane lipid peroxidation degree in leaves of M9-T337 seedlings under saline-alkali stress.
Figure 6

Effects of exogenous MT on ASA-GSH cycle of M9-T337 seedlings under saline-alkali stress.
Figure 7

Effects of exogenous MT on Osmotic adjustment of M9-T337 seedlings under saline-alkali stress
Figure 8

Effects of exogenous MT on endogenous hormone content in leaves of M9-T337 seedlings under saline-alkali stress
Figure 9

Effects of exogenous melatonin on Na⁺, K⁺ and Ca²⁺ contents in leaves of M9-T337 seedlings under saline-alkali stress
Figure 10

Correlation and principal component analysis of various indexes of M9-T337 seedlings under different treatments

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