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Exogenous Melatonin Increases Alfalfa (*Medicago sativa* L.) Tolerance to Salt Stress

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Abstract: Salinity affects the growth and development of plants, including alfalfa (*Medicago sativa* L.). Melatonin (MT) is an important plant defense component. This study aimed to elucidate the effects of MT on alfalfa under salt stress conditions. Two types of alfalfa with different salt tolerances under normal growth (0 mmol·L⁻¹ NaCl) and salt stress (150 mmol·L⁻¹ NaCl) conditions were treated with 0, 50, 100, 150, and 200 μmol·L⁻¹ MT. MT treatment increased plant height, leaf area, and root morphology and repaired chloroplast damage. Osmoregulatory substances such as proline, soluble sugars, and soluble proteins were significantly elevated by NaCl treatment and continued to increase after MT treatment. MT inhibited lipid peroxidation, cellular membrane defects, and reactive oxygen species accumulation induced by NaCl stress. The NaCl stress decreased the K⁺ concentration and increased the Na⁺ concentration. However, MT treatment reversed these levels and increased antioxidant enzyme activities and antioxidant nonenzymatic substances to scavenge excess ROS, inhibited the degradation of leaf SPAD values, and promoted photosynthetic gas exchange and chlorophyll fluorescence parameters. The most effective treatment for inhibiting salt stress was 150 μmol·L⁻¹ MT. These results indicate that salt-tolerant and salt-sensitive alfalfa can be sprayed with MT to improve NaCl tolerance.

Keywords: Alfalfa; NaCl stress; Growth parameters; Physiology; Biochemistry; Cellular ultrastructure

1. Introduction

Alfalfa (*Medicago sativa* L.) is a perennial high-quality legume forage grass with high nutritional value that is grown worldwide. Alfalfa is referred to as the “king of forage grasses” because of its high protein content, nutritional value, and resistance to adversity^[1]. Furthermore, it is also a key grass species used to promote agricultural production and economic development in northern China's arid and semi-arid regions ^[2]. In recent years, with the continuous changes in climate and gradual intensification of irrational irrigation, the area of saline-alkali land in the main planting area of alfalfa in China is increasing year by year^[3]. Although alfalfa leaves have a salt-exclusion mechanism and are more resistant and tolerant than other forage crops, the yield and growth of alfalfa are severely constrained when salt levels exceed a given threshold. This indicates that alfalfa is not completely immune to the negative effects of salt stress. Consequently, the cultivation and use of alfalfa in saline soils have become primary concerns for alfalfa growers and researchers ^[4].

Salt stress is a significant abiotic stressor that affects plant growth and development globally. Subsequently, it threatens global food security and ecological sustainability by limiting plant yields and quality, diminishing the use of arable land, and the further exploitation of plants ^[5]. NaCl is the predominant salt in nature and an important contributor to salt stress; thus, Na⁺ and Cl⁻ are the primary salt ions toxic to plants ^[6]. Under salt stress, plant growth and development processes are affected to different degrees; salt stress usually results in slow development, inhibition of growth, and differentiation of tissues and organs, reducing the nutrient and flowering periods. The inhibitory effect of salt stress on plants is closely related to salt composition and concentration, salt exposure duration, and season ^[7]. The land area classified as saline has been increasing annually because of the deteriorating ecological environment. High salt stress leads to osmotic stress, ionic toxicity, ROS production, and cellular ultrastructure damage; thus, improving plant salt tolerance is crucial for sustainable agriculture.

Hormone regulation has been recognized as one of the most influential and environmentally friendly methods to achieve this in recent years; this is partly because it is less costly and more profitable than traditional saline land improvement methods [4].

Melatonin (MT) is a tryptophan indole derivative commonly found in the reproductive organs of plants [8]. The physiological functions of MT are similar to those of other hormones. MT regulates seed germination, root conformation, circadian rhythms, photoperiods, and biotic and abiotic stresses. As a pleiotropic and nontoxic signaling molecule, MT plays a role in plant stress responses [9]. As an antioxidant, MT scavenges multiple ROS and protects plants from oxidative stress [10]. MT treatment improves salt tolerance in a variety of crops, such as oilseed rape (*Brassica napus* L.) [11], oatmeal (*Avena sativa* L.) [12], banana (*Musa nana* Lour.) [13], grape (*Vitis vinifera* L.) [14], and cotton (*Gossypium* Linn.) [15]. Exogenous MT effectively alleviates salt stress. However, the responses of plants to salt stress and MT vary according to species type, genotype, and growth stage [16]. This study used salt-sensitive and salt-tolerant alfalfa to evaluate the mitigation effect of exogenous MT on seedlings under salt-stress conditions. This study aimed to determine appropriate MT levels for selected alfalfa varieties and to elucidate the effects and physiological response mechanisms towards MT in different seedling types. The results of this study will provide new ideas for salt-resistant alfalfa cultivation and help identify germplasm materials for the genetic excavation of salt-tolerant varieties.

2. Material methods

2.1. Test Site and Materials

This experiment was conducted at the Key Laboratory of Grassland Resources and Ecology of the Ministry of Education, Xinjiang Key Laboratory of Grassland Resources and Ecology, Xinjiang Agricultural University (43°81'08"N, 87°56'74"E), a western arid desert region, from October 2023 to May 2024. The salt-tolerant alfalfa (*Medicago sativa* L.) variety Zhongmu No. 3 and the salt-sensitive variety Gongnong

No. 1 were used as the test materials. These two improved varieties are widely planted in China. MT with a purity of 99% was purchased from Shandong Yusuo Chemical Technology Co. and stored away from the light at -20°C .

2.2. Experimental Design

Seeds of uniform size were selected for each alfalfa variety, lightly sandpapered, and sterilized following the International Seed Inspection Protocol. Sterilized seeds were placed in Petri dishes (100 per dish) lined with double-layered filter paper, which was moistened with distilled water, and then incubated in an artificial climatic chamber (constant temperature of 25°C and 80% humidity) until germination, with a photoperiod of 12 h/12 h (day/night). Ten-day-old seedlings were fixed in foam board holes by sponge wrapping and transferred to a hydroponic culture in a hydroponic box ($290 \times 130 \times 110$ mm) containing 1/2 Hoagland's nutrient solution. The culture solution was changed every 3 d. After four weeks, alfalfa plants with uniform growth were selected for stress treatment. The experiment included 10 treatments (Table 1): CK group was irrigated to the roots with $150 \text{ mmol} \cdot \text{L}^{-1}$ Hoagland's nutrient, and 50 mL of distilled water was sprayed on the front and back of the alfalfa leaves every 2 d; M_1 , M_2 , M_3 , and M_4 groups were irrigated to the roots with $150 \text{ mmol} \cdot \text{L}^{-1}$ Hoagland's nutrient, which were sprayed with 50, 100, 150, and $200 \mu\text{mol} \cdot \text{L}^{-1}$ MT, respectively and 50 mL of MT solution was sprayed on the front and back of the alfalfa leaves every 2 d; N group was irrigated to the roots with $150 \text{ mmol} \cdot \text{L}^{-1}$ NaCl solution every 2 days; and the NM groups for which the NaCl solution was pretreated with 50, 100, 150, and $200 \mu\text{mol} \cdot \text{L}^{-1}$ MT, respectively, and referenced as groups NM_1 , NM_2 , NM_3 , and NM_4 , respectively. Before the formal pressure, MT was pretreated with MT for 48 h, and then 50 mL of MT solution was sprayed on both sides of the alfalfa leaves every 2 days. The above treatment duration was 7 days, and each time was uniformly sprayed in the evening, a total of 3 times. At the end of the experiment, 5 seedlings were randomly selected from each group to measure the growth parameters, and the rest of the seedlings were frozen in liquid nitrogen and stored at -80°C for physiological measurements.

Table 1 Treatment components and concentrations

Treatment	Hoagland's (g·L⁻¹)	NaCl (mmol·L⁻¹)	MT (μmol·L⁻¹)
CK	1.96	0	0
N	1.96	150	0
M₁	1.96	0	50
M₂	1.96	0	100
M₃	1.96	0	150
M₄	1.96	0	200
NM₁	1.96	150	50
NM₂	1.96	150	100
NM₃	1.96	150	150
NM₄	1.96	150	200

2.3. Observations and Measurements

2.3.1. Growth Parameters

The growth parameters included plant height (PH) and the height between the ground and the top of the plant, which were measured using a scale and replicated three times for each treatment; leaf area (LA), which was determined by scanning the 2nd to 4th middle leaves from the tip to the base with a leaf area meter; total root length (L), which was measured using a scale, and each treatment was replicated three times; the root systems were arranged in transparent square boxes, and root images were analyzed using the professional version of the Root Analysis System software (WinRHIZO 2003) after scanning with a desktop scanner (Epson Expression 1200XL, Shanghai, China) to obtain the root surface area (SA), root volume (V), and average root diameter (T), with three replications for each treatment.

The mesophyll cell ultrastructures were also observed. At the end of the experiment, the leaf tissue of the middle 2nd to 4th leaves from the apical to the basal areas of the plants was obtained, and the leaf samples were cut into 1 × 3 mm pieces, with three replicates for each treatment. Immediately after sampling, the samples were fixed in glutaraldehyde solution with a mass fraction of 5% and then stored at 4°C in a refrigerator. After 24 h, the samples were removed from the glutaraldehyde and rinsed before being washed 3 times with 100 mmol/L phosphate buffer at pH 7.4. The samples were fixed in 1% buffered osmium tetroxide for 1 h (performed in a fume hood) and cleaned three times, 10 min each time. After cleaning, the samples were dehydrated in ethanol at different concentrations (30%, 45%, 60%, and 75%). Finally, the samples were treated with anhydrous ethanol and pure acetone for 30 min. After dehydration, the samples were immersed in Spurr epoxy resin (Sigma–Aldrich, St. Louis, MO, USA) for 12 h and stored in a dark environment. The samples were cut into 50 nm sections. After staining, the samples were observed and photographed under a transmission electron microscope (JEM 2100 High Resolution Transmission Electron Microscope, Tokyo, Japan). This was repeated three times, and three fields of view were observed randomly.

2.3.2. Osmotic Adjustment Substances

To determine soluble protein (SP), leaves (0.3–0.5 g) were placed in a frozen mortar with extraction solution (3 mL), and a small amount of quartz sand was added.

The leaves were quickly ground into a homogenate in an ice bath. The homogenate was then washed from the mortar into a centrifuge tube using 5 mL of extraction solution (divided into two times). The leaves were ground with 10 mL of deionized water and then boiled for 20 min at 4°C, and the resulting supernatant was the soluble protein extract. The supernatant (0.1 mL) was transferred to a test tube, and Tris buffer (0.9 mL) was added to each tube. For the blank control, 1 mL of Tris buffer was added. Caulmers Brilliant Blue Staining Solution (5 mL) was added to each tube, and the absorbance (A) value at 595 nm after shaking well was obtained.

To determine the soluble sugar content (SS), the sample (1 g) was ground into homogenate, collected into a 10 mL centrifuge tube, and 80% ethanol (6–7 mL) was added. Fresh leaves (0.3 g) were ground with 10 mL deionized water and then boiled for 20 min. After cooling to room temperature, the mixture was filtered and made up to 100 mL with deionized water. The extraction (1.0 mL) was added to 4 mL anthrone, and the mixture was then boiled at 100 °C for 10 min. After cooling to room temperature, absorbance was measured at 620 nm.

To determine the proline content (Pro), the sample (0.5 g) was placed in a test tube, and 3% sulfosalicylic acid solution (5 mL) was added and extracted in a boiling water bath for 10 min (during the extraction process, the sample was shaken frequently). The sample was then cooled and filtered in a clean test tube; the obtained filtrate was the proline extract. The extract (2 mL) was aspirated in a glass test tube. Glacial acetic acid (2 mL) and acid ninhydrin (2 mL) reagent were added and the resulting solution was heated in a boiling water bath for 30 min. The resulting solution was red. After cooling, toluene (4 mL) was added, shaken for 30 s, and left to stand. The upper layer of the liquid was transferred to a 10 mL centrifuge tube and centrifuged at 3000 × g for 5 min. The upper layer of the proline red toluene solution was gently moved to a colorimetric cup using a pipette. The toluene solution was used as a blank control in the spectrophotometer, and the samples were analyzed at 520 nm to determine the optical density. The proline content in 2 mL of the assay solution (X µg/2 mL) was determined according to the regression equation, and the percentage of proline content in the sample was determined as follows:

$$\text{Proline content (\%)} = \frac{X \times \frac{2}{5} \times 100}{\text{similar} \times 10^6} \quad (1)$$

2.3.3. Antioxidant system

The superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), glutathione S-transferase (GST), and glutathione reductase (GR) activity levels were determined. The absorbent enzyme solution (2 mL) was transferred to a test tube, and pH 6.0 buffer (5 mL) and 10% sucrose solution (1 mL) were added and maintained at 37 °C in a water bath for 0.5 h. The reaction solution (2 mL) was aspirated, and 3,5-dinitrosalicylic acid reagent (1.5 mL) was added, kept for 5 min in a boiling water bath, and then cooled. Distilled water was added to obtain a final volume of 20 mL. The tube containing the enzyme was boiled for 10 min to passivate the enzyme as a control, and the OD value at 540 nm was determined as previously described. The concentration of reducing sugar in the enzyme reaction solution was determined from the standard curve.

To determine the reduced ascorbic acid (ASA) and reduced glutathione (GSH), UV-absorption was measured at 412 nm using an enzyme labeling instrument (Thermo FC).

2.3.4. Oxidative Damage and Cell Membrane Stability

To determine the malondialdehyde content, the enzyme extract (2 mL) was mixed with 0.5% thiobarbituric acid solution (3 mL) (after dissolving 0.5 g of thiobarbituric acid in a small amount of the 1 mol·L⁻¹NaOH solution, 10% TCA was allowed to be fixed to 10 mL, which is the desired solution). The sample was placed in a 100 °C water bath for 10 min, cooled, and centrifuged (4000 × g for 10 min), and the absorbance was recorded at 450, 532, and 600 nm.

To determine the relative conductivity, fresh leaves (0.1 g) were added to distilled water (20 mL) in a centrifuge tube, which was shaken for 30 min and left to stand for 20 min. The initial conductivity (R1) was determined using a conductivity meter. The tube was sealed in a boiling water bath for 20 min, cooled, shaken for 30 min, and left to stand for 20 min. A conductivity meter (STARTER3100C) was then used to determine the conductivity (R2).

An ultraviolet absorption method was used to determine the hydrogen peroxide (H_2O_2) and hydroxyl radical ($-\text{OH}$) contents and the hydroxyl radical scavenging rate. The enzyme labeling instrument measured absorbance at 415, 532, and 510 nm.

2.3.5. Determination of K^+ and Na^+ Ion Contents

The dried samples were ground into powder, and 0.1 g was burned in a crucible and transferred to a muffle furnace (550°C) for 3 h. The ash was dissolved in concentrated HNO_3 , filtered, and then diluted to 25 mL using deionized water to determine the K^+ and Na^+ contents via a plasma emission spectrometer (IPC, PE3300DV, PerkinElmer, Germany).

2.3.6. Measurement of photosynthetic properties

The leaf net photosynthetic rate (Pn), stomatal conductance (Gs), intercellular CO_2 concentration (Ci), and transpiration rate (Tr) were determined. A leaf chamber in a CIRAS-3 photosynthesizer was used to clip the middle leaves of the alfalfa seedlings from the tip to the base, counting the 2nd to 4th leaves from 11:00 am to 16:00 pm of the day after the test was completed. The leaves of the middle part of the 10 treated alfalfa plants were measured separately. Each treatment was repeated three times, and after the data stabilized (1–3 min), the four indexes were recorded.

The potential photochemical activity of PSII (Fv/Fo), maximum photochemical efficiency (Fv/Fm), unit reflective center (ETo/Rc), and relative variable fluorescence at point J (Vj) were determined. The leaf chamber of the FluorPen FP110 hand-held chlorophyll fluorometer was used to clamp the alfalfa seedlings from the tip to the base, counting from the 2nd to the 4th leaf from 11:00 am to 16:00 pm the day after the test was completed. The leaves of the middle part of the 10 treated alfalfa plants were measured separately, and each treatment was repeated three times. After the obtained data were stabilized, the four indexes were recorded.

2.4. Statistical Analysis

Each indicator was measured three times. Analysis of variance (ANOVA) was conducted using SPSS 20 software, and $P < 0.05$ indicated a significant difference. The figure was created using Origin2024 pro software.

3. Results

3.1. Alfalfa seedling growth parameters

The results confirmed that exogenous MT was beneficial to the growth and development of alfalfa (Fig. 1A-F) and enhanced the response of alfalfa to various abiotic stresses. Most of the growth parameters increased after exogenous MT treatment under CK conditions. NaCl treatments had an inhibitory effect on the above indexes (Fig. 1A-F); however, MT spraying significantly improved these results, indicating that it could alleviate the growth inhibition of seedlings induced by salt stress. Interestingly, the average growth of 'Zhongmu No.3' alfalfa was greater than that of CK, and it had a significantly ($P < 0.05$) lower mean root diameter after MT spraying compared with that of the CK (Figs. 1F).

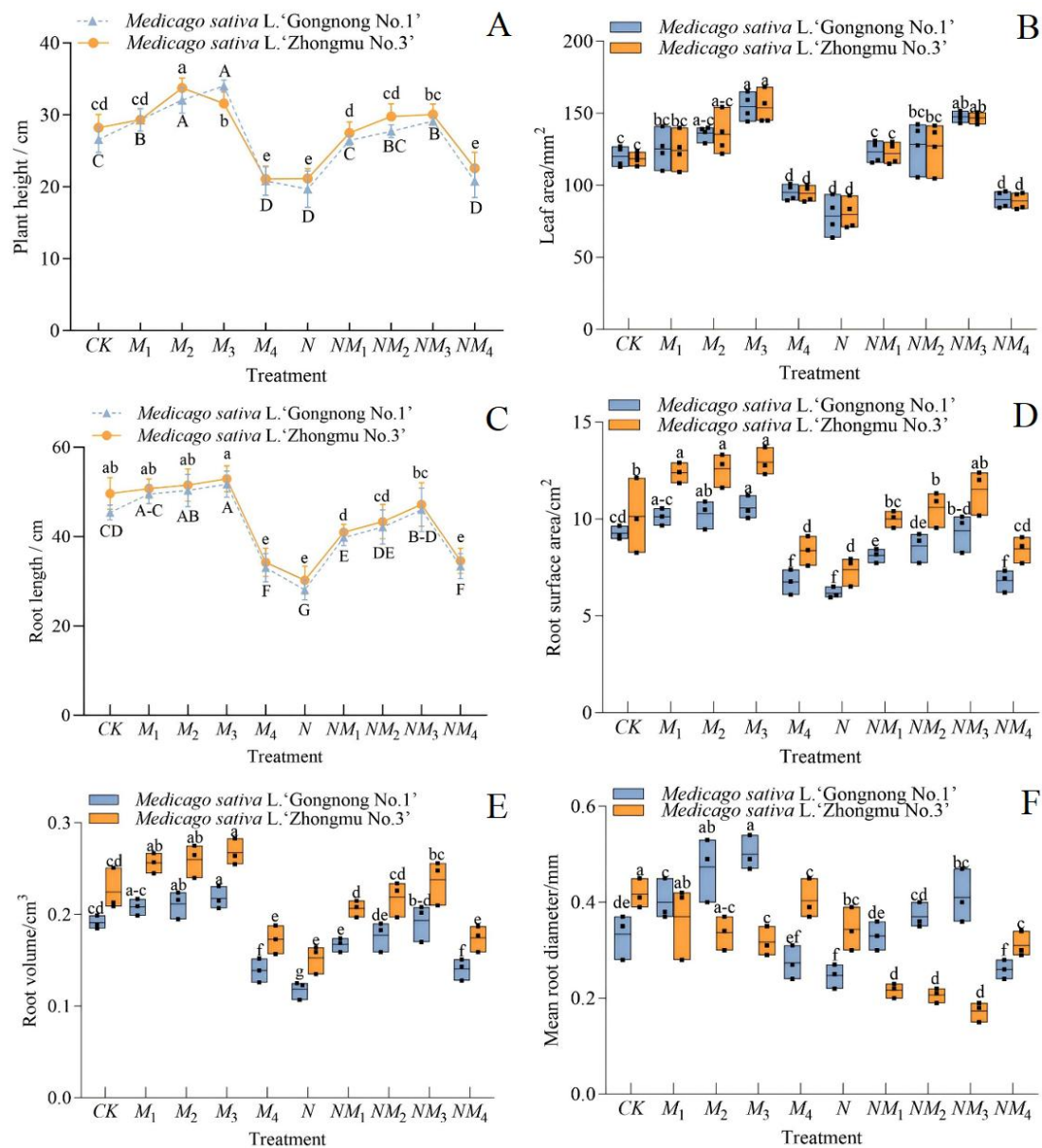


Fig.1. Growth status of alfalfa seedlings under different treatments. A: alfalfa seedling plant height; B: alfalfa seedling leaf area; C: alfalfa seedling total root length; D: alfalfa seedling root surface area; E: alfalfa seedling root volume; F: alfalfa seedling average root diameter; According to ANOVA, different capital letters in Figures A and C represent significant ($P < 0.05$) differences among different treatments of 'Gongnong No. 1', and different lowercase letters represent significant ($P < 0.05$) differences among different treatments of 'Zhongmu No. 3'. Other histograms with different lowercase letters represent significant ($P < 0.05$) differences among different treatments of the same variety.

Chloroplasts in the CK groups for both species of alfalfa (Figs. 2A-B, 1-1, 1-2, and 1-3) had intact cell walls and clear membrane boundaries, well-developed thylakoid systems, compact and precise grana lamellae arrangements, and thread granules that were ellipsoidal and distributed adjacent to the chloroplasts. When sprayed with $50 \mu\text{mol} \cdot \text{L}^{-1}$ MT (Figs. 2A-B, 2-1, 2-2, 2-3), the internal structures of the 'Gongnong No.

1' chloroplasts were intact, and the stroma-thylakoid were neatly stacked and visible; the internal 'Zhongmu No. 3' chloroplast structures were also intact. However, slight swelling occurred, and the number of basidiomycetes was lower in 'Gongnong No. 1' than in the CK. When sprayed with $100 \mu\text{mol}\cdot\text{L}^{-1}$ (Figs. 2A-B, 3-1, 3-2, and 3-3) and $150 \mu\text{mol}\cdot\text{L}^{-1}$ MT(Figs. 2A-B, 4-1, 4-2, and 4-3), the chloroplasts of both alfalfa varieties were similar to those of the CKs, with the inner stromal lamellae visible and the mitochondria ellipsoidal. When $200 \mu\text{mol}\cdot\text{L}^{-1}$ MT was sprayed (Figs. 2A-B, 5-1, 5-2, and 5-3), the cell membranes of the two varieties ranged from tightly adhering to the cell wall to undergoing wall separation, and the chloroplasts were damaged and deformed externally, as they had transformed from elongated ellipsoids to ellipsoids or almost round. The laminar structure had a loose and disorganized arrangement. The stromal lamellae of 'Gongnong No. 1' were fuzzy and disordered, and the interlamellar gaps were larger; the matrix lamellae in some chloroplasts of 'Zhongmu No.3' were disintegrated, and the shape was unrecognizable.

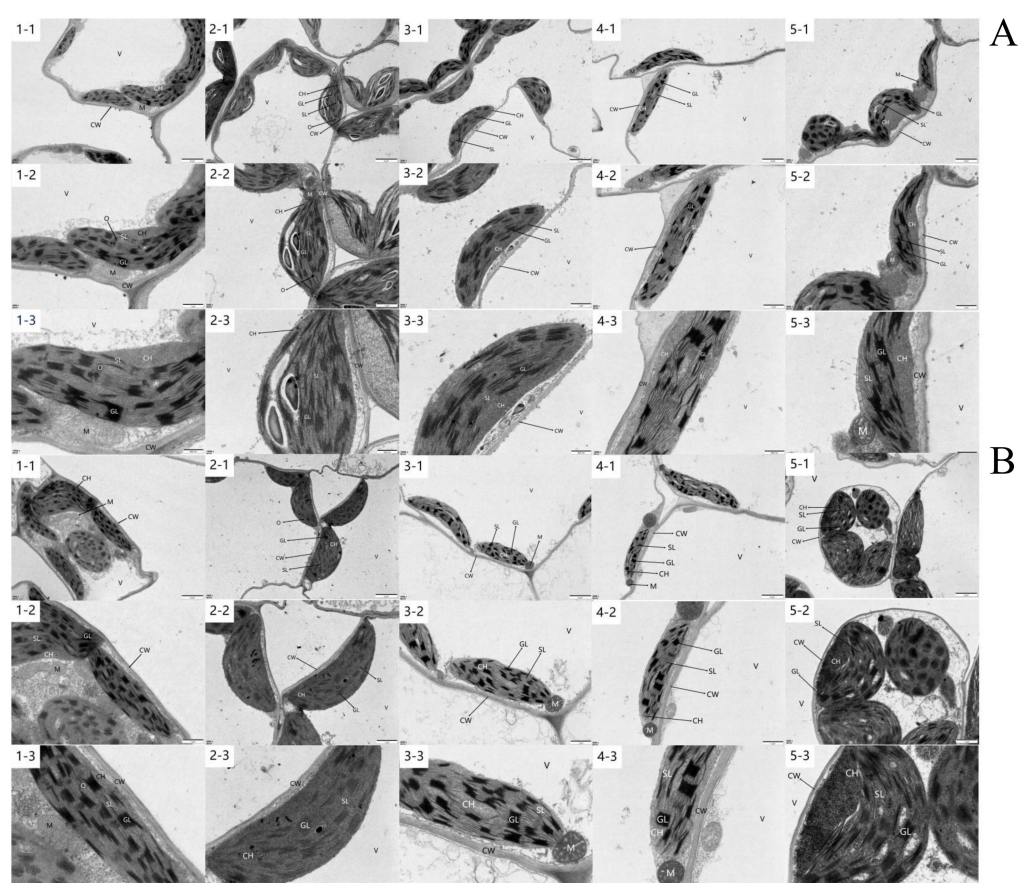


Fig.2 Effect of exogenous melatonin on the cellular ultrastructure of alfalfa seedling leaves

Note: Figures A and B show 'Gongnong No.1' and 'Zhongmu No.3', respectively. 1-1, 1-2, 1-3:CK,Ultrastructure of mesophyll cells under spray water (The scale bar is $2\mu\text{m}$, $1\mu\text{m}$, 500nm); 2-1, 2-2, 2-3: M₁, Spray $50\mu\text{mol}\cdot\text{L}^{-1}$ (The

scale bar is 2 μ m,1 μ m,500nm); 3-1, 3-2, 3-3: M₂, Spray 100 μ mol $\cdot$ L⁻¹(The scale bar is 2 μ m,1 μ m,500nm); 4-1, 4-2, 4-3: M₃, Spray 150 μ mol $\cdot$ L⁻¹(The scale bar is 2 μ m,1 μ m,500nm); 5-1, 5-2, 5-3: M₄, Spray 200 μ mol $\cdot$ L⁻¹(The scale bar is 2 μ m,1 μ m,500nm); CW: cell wall; CH: chloroplasts; GL: basal lamellae; SL: stromal lamellae; O: osmiophilic granules; M : mitochondria; V: vesicles.

Chloroplasts in the CK groups for the two alfalfa varieties (Figs. 3A-B, 1-1, 1-2, and 1-3) were ellipsoidal, neatly arranged, and tightly attached to the cell wall, with the membrane boundary visible, the stromal lamellae were structurally intact and orderly, and the mitochondria were ellipsoidal and distributed adjacent to the chloroplasts. With 150 mmol $\cdot$ L⁻¹ salt stress (Figs. 3A-B, 2-1, 2-2, 2-3), the periplasmic boundaries of the 'Gongnong No.1' leaf cells were disrupted, and chloroplasts were deformed, with a decrease in length, an increase in width, and a decrease in the aspect ratio. The number of chloroplast basal granules was reduced, the lamellar structure was disordered, the mitochondria were irregularly shaped, and decomposition occurred. However, the salt-tolerant alfalfa 'Zhongmu 3' had clear periplasmic boundaries, clearly visible stromal lamellae, and intact chloroplast structures, but plasma wall separation was observed.

When sprayed with 50 μ mol $\cdot$ L⁻¹MT (Figs. 3A-B, 3-1, 3-2, 3-3), 'Gongnong No. 1' chloroplasts were reduced in size and the plasmolysis phenomenon appeared compared with N. However, the internal structure was more complete with M1, and the granum-thylakoids were neatly stacked. The 'Zhongmu No. 3 ' chloroplasts underwent swelling, the internal structure was complete, and the number of basidiomycetes was higher than with the 150 mmol $\cdot$ L⁻¹ NaCl (N). When sprayed with 100 μ mol $\cdot$ L⁻¹MT (Figs. 3A-B, 4-1, 4-2, and 4-3), part of the chloroplast structure of 'Gongnong No. 1' was swollen, the chloroplast membranes were slightly disrupted, the internal stroma-thylakoid were visible, and the mitochondria were ellipsoid in shape. When 150 μ mol $\cdot$ L⁻¹ MT was sprayed (Figs. 3A-B, 5-1, 5-2, and 5-3), chloroplasts of the two varieties were deformed compared with those of the CKs, but the periplasmic boundaries were visible. The internal structures were more intact without the wall separation phenomenon than the 150 mmol $\cdot$ L⁻¹ NaCl(N). The chloroplasts of the two varieties were deformed compared with their CK. When 200 μ mol $\cdot$ L⁻¹MT was sprayed (Figs. 3A-B, 6-1, 6-2, 6-3), the chloroplasts of 'Gongnong No. 1' were irregularly shaped, with increased internal structural voids, slight wall separation phenomenon, and loose basal lamellae; the chloroplast shape of 'Zhongmu

No.3' was close to that of the CK, the internal structure was complete, the mitochondrial shape was regular, but the envelope boundary was destroyed.

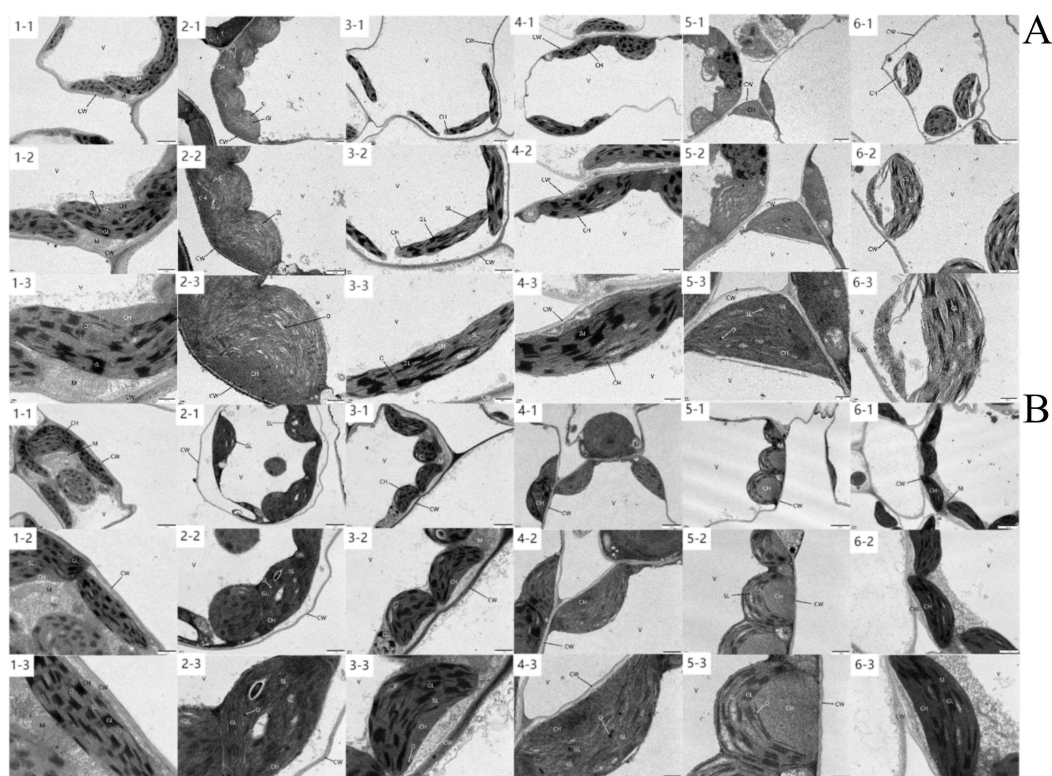


Fig. 3 Effect of exogenous melatonin on the cellular ultrastructure of alfalfa seedling leaves under salt stress

Note: Figures A and B show 'Gongnong No.1' and 'Zhongmu No.3', respectively. 1-1、1-2、1-3: CK, Ultrastructure of mesophyll cells under spray water (The scale bar is 2 μ m, 1 μ m, 500nm); 2-1、2-2、2-3: N, Ultrastructure of Leaf Succulent Cells under Root Irrigation with 150mmol·L⁻¹NaCl (The scale bar is 2 μ m, 1 μ m, 500nm); 3-1、3-2、3-3: NM₁, Spray 50 μ mol·L⁻¹MT (The scale bar is 2 μ m, 1 μ m, 500nm); 4-1、4-2、4-3: NM₂, ultrastructure of 100 μ mol·L⁻¹ MT (The scale bar is 2 μ m, 1 μ m, 500nm); 5-1、5-2、5-3: NM₃, ultrastructure of 150 μ mol·L⁻¹ MT (The scale bar is 2 μ m, 1 μ m, 500nm); 6-1、6-2、6-3: NM₄, ultrastructure of 200 μ mol·L⁻¹ MT (The scale bar is 2 μ m, 1 μ m, 500nm); CW: cell wall; CH: chloroplast; GL: grana lamella; SL: stromal lamellae; O: osmiophilic granules; M: mitochondria; V: vacuole.

3.2. Osmoregulatory Substances

Under the CK condition of spraying only distilled water, the free proline, soluble protein, and soluble sugar contents of the 'Gongnong No. 1' and 'Zhongmu No. 3' alfalfa leaves were highest under the 150 μ mol·L⁻¹ MT(M₃) treatment. In contrast, the

levels following the $200 \mu\text{mol}\cdot\text{L}^{-1}$ MT treatment were lower than those following the other MT treatments (Figs. 4A-C). After the $150 \text{ mmol}\cdot\text{L}^{-1}$ salt stress treatment, the osmoregulatory substance content in the leaves of both alfalfa varieties was increased compared with that of the CK. After spraying exogenous MT, the free proline and soluble protein contents were both highest with $150 \mu\text{mol}\cdot\text{L}^{-1}$ of the NM_3 treatment (Figs. 4A-B) and the soluble sugar content was highest with $100 \mu\text{mol}\cdot\text{L}^{-1}$ of the NM_2 treatment (Fig. 4C). Osmoregulatory substances in the 'Zhongmu 3' alfalfa leaves were highest with $150 \mu\text{mol}\cdot\text{L}^{-1}$ of NM_3 (Figs. 4A-C).

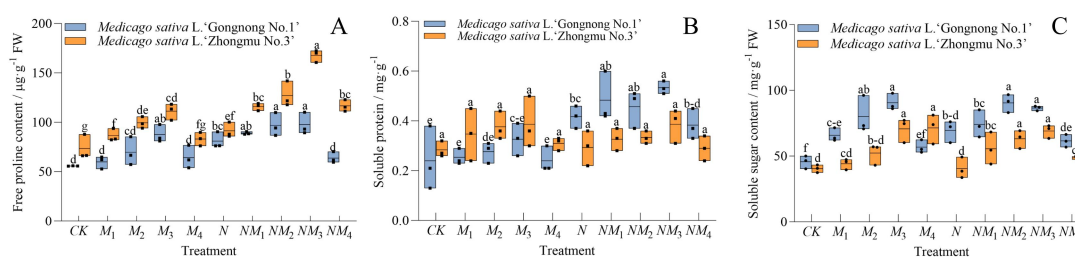


Fig. 4. Effect of melatonin on osmoregulatory substance content of alfalfa seedlings under salt stress

A: free proline content; B: soluble protein content; C: soluble sugar content; according to the analysis of variance (ANOVA), different letters in the graphs denote the differences between different treatments of the same species ($P < 0.05$), the same as below.

3.3. Antioxidant Capacity

MT application significantly improved the activities of alfalfa superoxide dismutase, catalase, peroxidase, glutathione-S-transferase, and glutathione reductase (Figs. 5A-E). The activity levels in the alfalfa leaves of both varieties tended to increase and then decrease with MT concentration, except for the activity of glutathione reductase, which increased with the MT concentration. After applying $150 \text{ mmol}\cdot\text{L}^{-1}$ salt stress treatment, the antioxidant enzyme activities in the leaves of the two alfalfa species were increased compared with the CK; the superoxide dismutase, catalase, glutathione-S-transferase, glutathione reductase activities of the leaves of both alfalfa varieties and the peroxidase activity in the leaves of the 'Zhongmu 3' alfalfa reached their highest levels with the NM_3 treatment after the MT treatment. The peroxidase activities of the leaves of the 'Gongnong 1' alfalfa reached their highest level with the NM_4 treatment. Peroxidase activity in 'Zhongmu 3' leaves was highest after the NM_3 treatment, whereas the peroxidase activity in the 'Gongnong No. 1' leaves was highest after the NM_4 treatment.

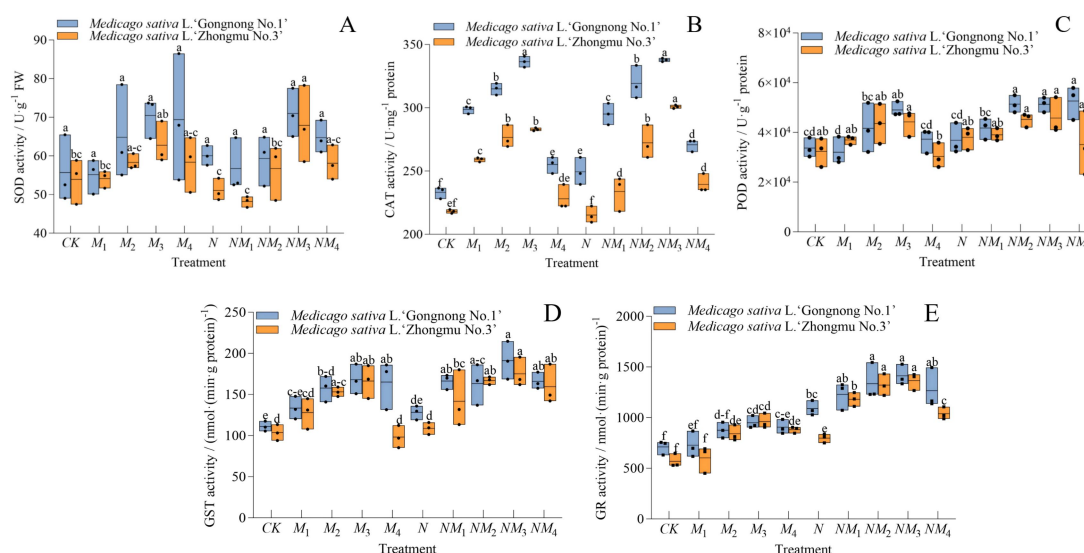


Fig. 5. Effect of melatonin on antioxidant enzyme activities of alfalfa seedlings under salt stress

A: superoxide dismutase activity; B: catalase activity; C: peroxidase activity; D: glutathione S-transferase activity; E: glutathione reductase activity.

The physiological responses of the alfalfa leaves to the antioxidant content are shown in Figure 6A-B. The leaf-reduced ascorbic acid and reduced glutathione contents of the two alfalfa species were increased after spraying the MT treatment under CK conditions; after applying NaCl stress, the reduced ascorbic acid contents of the two alfalfa species were increased compared with those of the CK, and the reduced glutathione content decreased significantly compared with that of the CK. The results showed that the M treatment could significantly alleviate salt damage. The ascorbic acid levels in both types of alfalfa were highest under the NM₃ treatment; the content of ascorbic acid in two alfalfa varieties was the highest under NM₃ treatment, and the reduced glutathione content of 'Zhongmu No. 3' was the highest under NM₁ treatment.

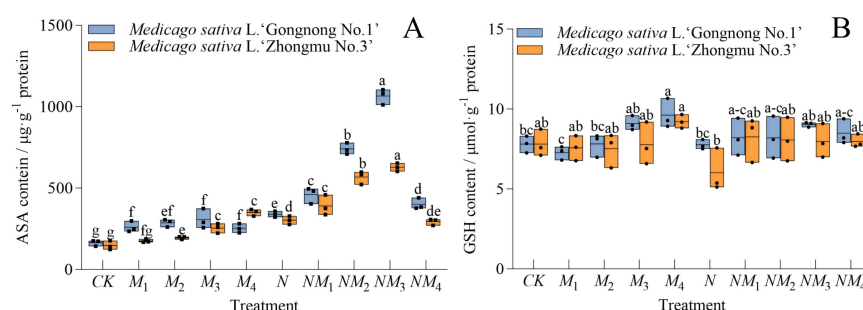


Fig. 6. Effect of melatonin on antioxidant content of alfalfa seedlings under salt stress

A: reduced ascorbic acid content; B: reduced glutathione content.

3.4. Ionic Homeostasis

The effects of MT on the ionic homeostasis of alfalfa seedlings under salt stress are shown in Figure 7A-C. Under CK conditions sprayed with MT, the K^+ content and K^+/Na^+ ratio of the two alfalfa varieties were significantly increased, while the Na^+ content was significantly decreased. Under NaCl stress, the K^+ content and K^+/Na^+ ratio of the two alfalfa varieties were significantly lower than those of the CK ($P < 0.05$). The Na^+ content was significantly higher than that of the CK ($P < 0.05$). The K^+ content and K^+/Na^+ ratio of the two alfalfa varieties increased, and the Na^+ content decreased after the exogenous MT treatments and decreased to their lowest with the NM_2 treatment.

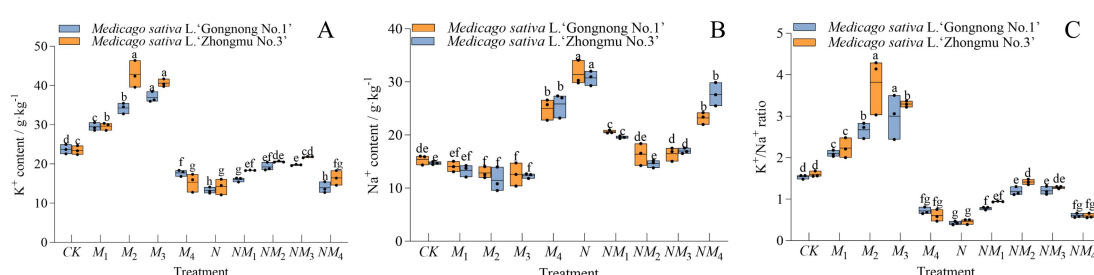


Fig. 7. effect of melatonin on ionic homeostasis of alfalfa seedlings under salt stress.

A: K^+ content; B: Na^+ content; C: K^+/Na^+ ion ratio.

3.5. Oxidative Damage and Cell Membrane Stability

Spraying MT alone without the salt stress conditions resulted in no significant changes in the hydroxyl radical content, hydroxyl radical scavenging rate, malondialdehyde, and hydrogen peroxide content in the two alfalfa varieties compared with their respective CKs (Figs. 8, A-E). After NaCl stress, the above indexes for both alfalfa varieties were increased under the N treatment compared with the CK; these changes were significantly reversed after exogenous MT treatment and were most alleviated by NM_3 .

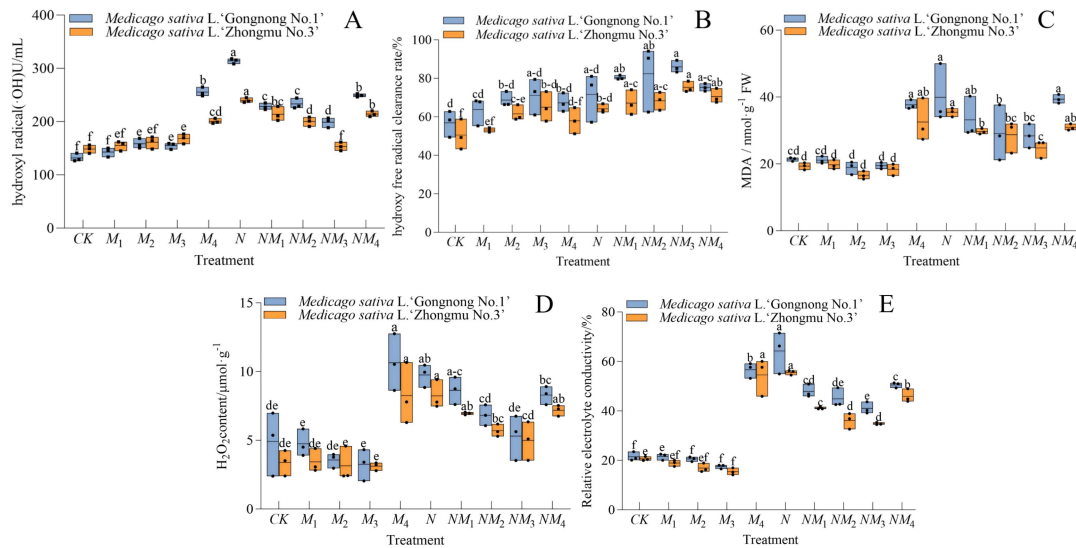


Fig. 8. Effect of melatonin on oxidative damage and cell membrane stability in alfalfa seedlings under salt stress. A: hydroxyl radical content; B: hydroxyl radical scavenging rate; C: malondialdehyde content; D: hydrogen peroxide content; E: relative conductivity.

3.6. Photosynthetic Capacity

The effects of MT on the SPAD and chlorophyll fluorescence parameters of alfalfa seedlings under salt stress are shown in Figures 9A-E. Without salt stress, the SPAD values, potential photochemical activity, maximum photochemical efficiency, and unit reflective center of the leaves of both alfalfa varieties were highest under the M₃ treatment. The opposite was true for relative variable fluorescence. After administering SPAD and chlorophyll fluorescence parameters, the leaves of the two alfalfa varieties were significantly decreased compared with the CK after exposure to 150 mmol · L⁻¹ NaCl stress. The sprayed MT treatments could significantly alleviate the photosynthetic damage caused by salt stress, and the effect for both varieties was greatest with the NM₃ treatment.

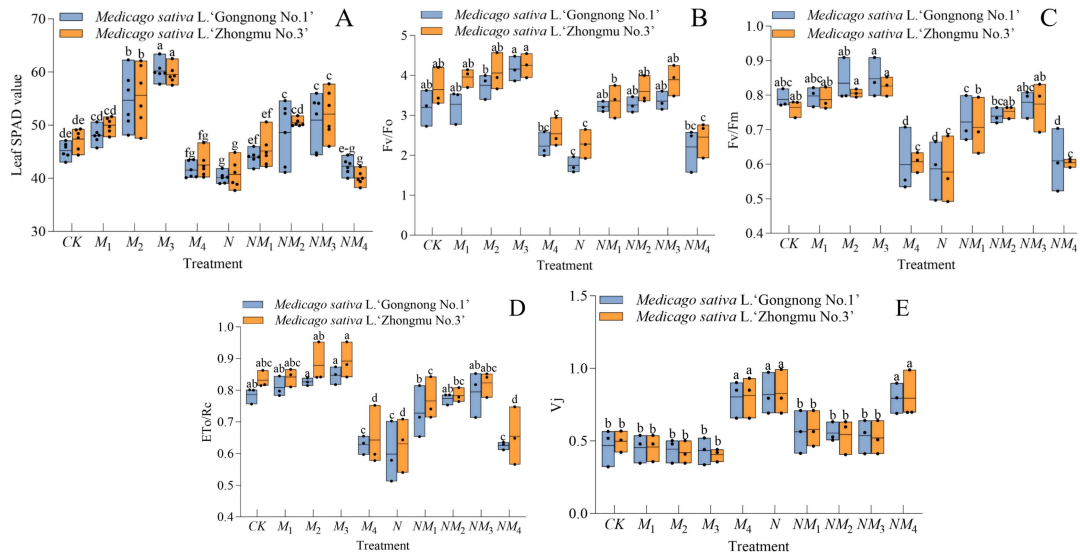


Fig. 9. Effect of melatonin on SPAD and chlorophyll fluorescence parameters of alfalfa seedlings under salt stress. A: leaf SPAD value; B: potential photochemical activity of PSII; C: maximum photochemical efficiency; D: unit reflecting center; E: relative variable fluorescence at J point.

The effects of MT on the photosynthetic gas exchange parameters of alfalfa seedlings under salt stress are shown in Figures 10A-D. When MT was sprayed under CK conditions, the net photosynthetic rate and transpiration rate of the leaves of the two alfalfa varieties were highest under the M₃ treatment, the stomatal conductance was highest under the M₂ treatment, and the inter-cellular CO₂ concentration was highest under the M₄ treatment. After NaCl stress alone, the leaf net photosynthetic rate, transpiration rate, and stomatal conductance were significantly lower than those of the CK ($P < 0.05$), and the inter-cellular CO₂ concentration was significantly higher than that of the CK ($P < 0.05$). After spraying MT under NaCl condition, the content of the above indexes was significantly different from that of NaCl treatment alone ($P < 0.05$).

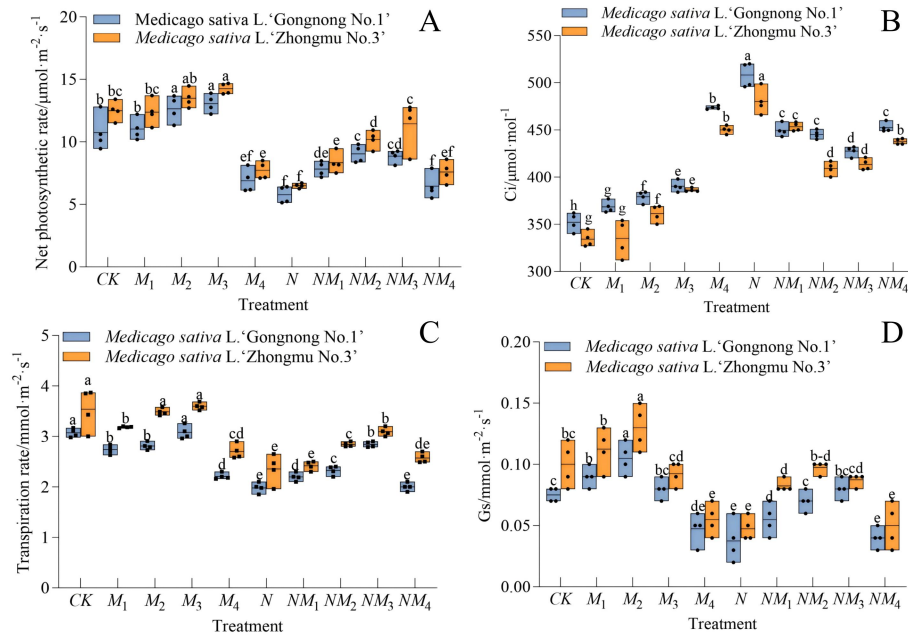


Fig. 10. Effect of melatonin on photosynthetic gas exchange parameters of alfalfa seedlings under salt stress. A: net photosynthetic rate; B: intercellular CO₂ concentration; C: leaf transpiration rate; D: stomatal conductance.

3.7. Correlation Between Measured Parameters in Alfalfa Plants Under Salt Stress

According to the results of the correlation analysis in Figure 11, there is a correlation between the various indicators. Among them, CO₂, MDA, RC, H₂O₂, ·OH, Na⁺, Vj were strongly negatively correlated with PH, LA, RL, RAD, RV, RSA, Pn, Gs, Tr, SPAD ($p \leq 0.001$). ETo/Rc, Fv/Fm, Fv/Fo, K⁺/Na⁺, Na⁺, CAT, SPAD, Tr and Gs were significantly positively correlated with PH, LA, RL, RAD, RV, RSA and PN ($p \leq 0.001$). SS was significantly positively correlated with PH, LA, RAD and POD ($p \leq 0.01$), and CAT was significantly positively correlated with TR ($p \leq 0.05$).

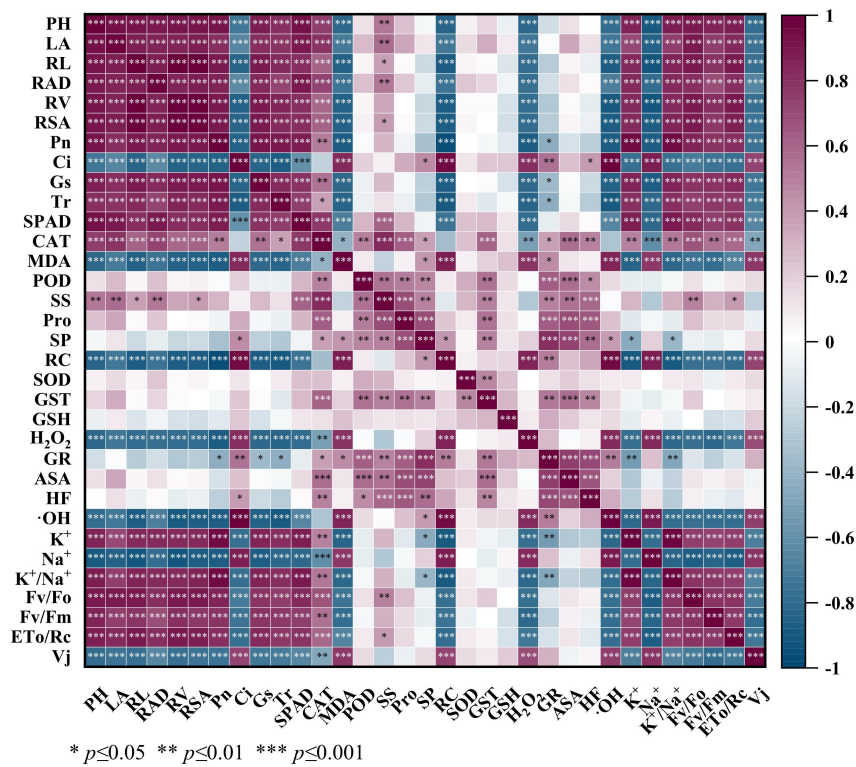


Fig. 11. Correlation analysis between measured parameters of 'Gongnong No.1' alfalfa plant under salt stress.

Note: The PH is plant height, LA is the leaf area, RL is the root length, RAD is the mean root diameter, Where RV is the root volume, RSA is the root surface area, Pn is the net photosynthetic rate, Ci is the intercellular CO₂ concentration, Gs is for the stomatal conductance, Tr is the leaf transpiration rate, SPAD is the relative chlorophyll content, CAT for catalase activity, MDA for malondialdehyde content, POD for peroxidase activity, SS is the soluble sugar content, Pro is for the proline content, SP is the soluble protein content, RC is the relative conductivity, SOD for the superoxide dismutase activity, GST is a glutathione S-transferase, GSH reduced glutathione content, H₂O₂ is the hydrogen peroxide content, GR for the glutathione reductase activity, ASA reduced the ascorbic acid content, HF for the hydroxyl radical clearance rate, ·OH for the hydroxyl radical content, K⁺ is the potassium ion content, Na⁺ is the sodium ion content, K⁺/Na⁺ as K⁺ and Na⁺ ion ratio, Fv/Fo is for the potential photochemical activity of PSII, Fv/Fm for the maximum photochemical efficiency, ETo/Rc is the unit reflection center, Vj is the variable fluorescence at point J, the same below.

There is some correlation between the various indicators, as shown in Figure 12. CO₂, MDA, RC, H₂O₂, ·OH, Na⁺, Vj were significantly negatively correlated with PH, LA, RL, RV, RSA, Pn, Gs, Tr, SPAD ($p \leq 0.001$). PH, LA, RL, RV, RSA, Pn, Gs, Tr, SPAD were significantly positively correlated with ETo/Rc, Fv/Fm, Fv/Fo, K⁺/Na⁺, Na⁺ ($p \leq 0.001$). POD and SP were significantly positively correlated with RL and SPAD ($p \leq 0.01$). K⁺/Na⁺, Fv/Fm and Na⁺ were positively correlated with SP ($p \leq 0.05$).

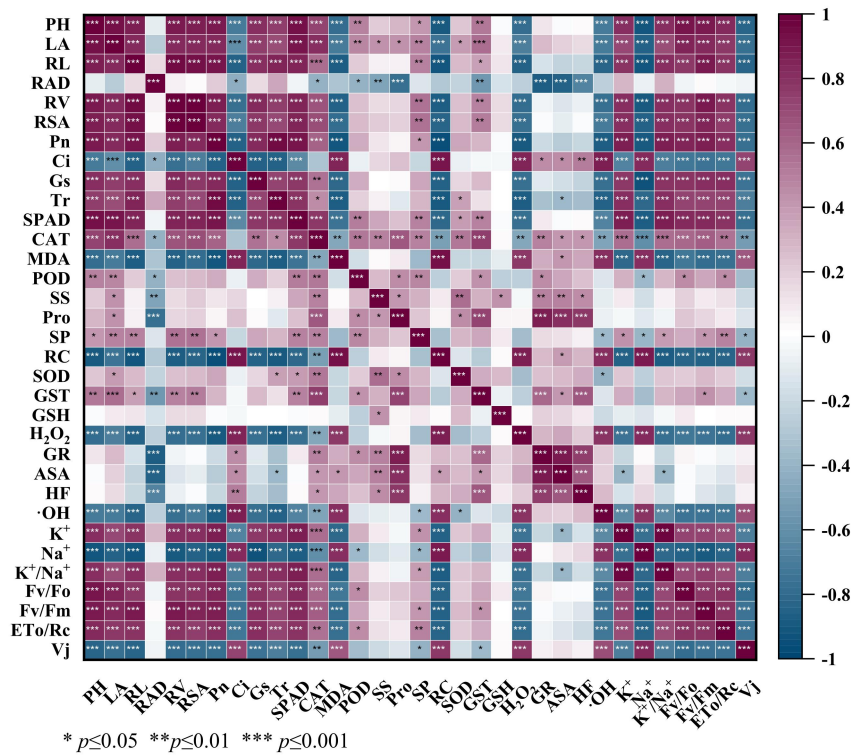


Fig. 12. Correlation analysis between measured parameters of 'Zhongmu No.3' alfalfa plants under salt stress.

3.8. Principal Component Analysis of the Measured Alfalfa Parameters Under Salt Stress

The principal component analysis of salt tolerance of 'Gongnong No.1' alfalfa seedlings after spraying MT was carried out (Table 2 and Figure 13). According to the principle of eigenvalue greater than 1, three principal component factors were determined. The cumulative contribution rate of the first three principal components was 82.147 %. The first principal component represented the net photosynthetic rate, with a contribution rate of 57.342 %, which was the most important principal component. The representative index of the second principal component was the content of reduced ascorbic acid, and the contribution rate was 19.932 %, which was the second important principal component. The representative index of the third principal component was superoxide dismutase activity, with a contribution rate of 4.873%. These three principal components can be used as important indicators to reflect the salt tolerance of 'Gongnong No.1'.

Table 2 Principal component loading matrix and variance contribution rate of 'Gongnong No.1' alfalfa

Indicator	PC1	PC2	PC3	PC4	PC5
PH	0.220	0.080	-0.024	0.034	0.096

LA	0.204	0.144	0.008	-0.143	-0.005
L	0.221	0.015	-0.028	0.004	-0.122
T	0.205	0.087	0.108	0.178	-0.021
V	0.221	0.006	-0.040	-0.012	-0.126
SA	0.220	0.013	-0.031	0.051	-0.110
Pn	0.222	-0.042	0.059	0.044	0.094
Ci	-0.200	0.147	0.073	0.163	0.210
Gs	0.214	-0.024	-0.016	-0.015	-0.029
Tr	0.206	-0.059	0.042	-0.207	-0.079
SPAD	0.204	0.114	0.181	0.077	0.177
CAT	0.141	0.282	0.026	0.110	0.112
MDA	-0.204	0.067	0.100	-0.125	-0.041
POD	0.008	0.282	0.145	0.094	-0.337
SS	0.084	0.308	0.012	0.318	0.212
Pro	0.010	0.329	-0.172	0.091	0.241
SP	-0.054	0.305	-0.248	-0.135	-0.098
RC	-0.213	0.107	-0.014	0.046	0.055
SOD	0.011	0.130	0.584	0.133	-0.380
GST	0.006	0.293	0.262	-0.051	-0.134
GSH	-0.026	0.114	0.423	-0.638	0.465
H ₂ O ₂	-0.202	0.011	0.055	0.072	-0.058
GR	-0.078	0.327	-0.108	-0.189	-0.060
ASA	-0.001	0.333	-0.158	-0.254	-0.225
HF	-0.030	0.301	-0.191	0.128	0.105
·OH	-0.208	0.114	0.044	0.204	0.161
K ⁺	0.209	-0.069	0.168	0.199	0.199
Na ⁺	-0.216	-0.012	0.030	0.180	0.084
K ⁺ /Na ⁺	0.212	-0.049	0.168	0.153	0.184
Fv/Fo	0.206	0.082	0.005	-0.050	0.068
Fv/Fm	0.199	0.011	-0.170	-0.071	0.085
Eto/Rc	0.203	0.044	-0.069	-0.038	-0.192
Vj	-0.187	0.017	0.246	0.068	-0.096
Eigenvalue	18.923	6.578	1.608	0.967	0.819
Variance	57.342	19.932	4.873	2.929	2.482

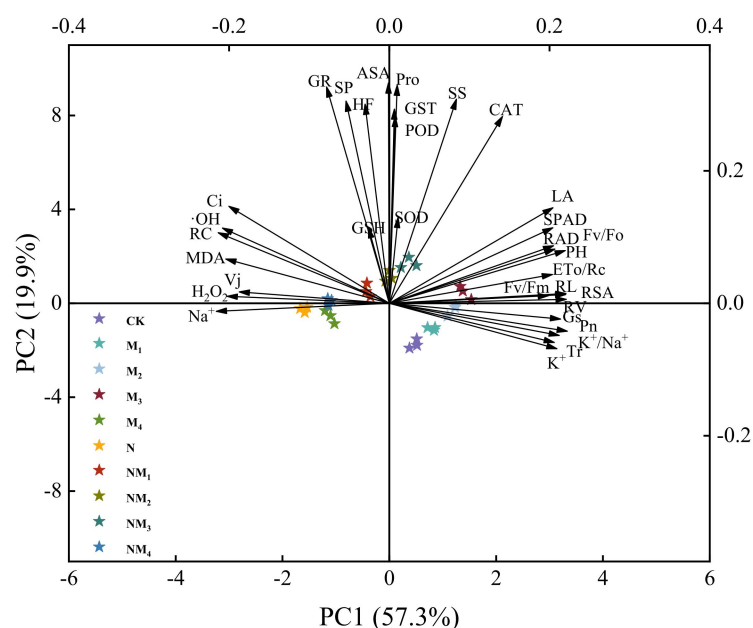


Fig. 13. Principal component analysis between measured parameters of 'Gongnong No.1' alfalfa plants under salt stress

The principal component analysis of salt tolerance of 'Zhongmu No.3' alfalfa seedlings after spraying MT was carried out (Table 3 and Figure 14). According to the principle of eigenvalue greater than 1, four principal component factors are determined. The cumulative variance contribution rate of the first four principal components was 83.635%. The first principal component represented the net photosynthetic rate, with a contribution rate of 55.482 %, which was the most important principal component. The index represented by the second principal component was glutathione reductase activity, with a contribution rate of 19.655 %, which was the second important principal component. The representative index of the third principal component was the content of reduced glutathione, with a contribution rate of 5.065 %. The representative index of the fourth principal component was superoxide dismutase activity, with a contribution rate of 3.433%. These four principal components can be used as important indicators to reflect the salt tolerance of 'Zhongmu No.3'.

Table 3 Principal component loading matrix and variance contribution rate of 'Zhongmu No.3' alfalfa

Indicator	PC1	PC2	PC3	PC4	PC5
PH	0.222	0.067	0.116	0.086	0.027
LA	0.208	0.140	0.007	0.079	0.016
L	0.223	-0.033	0.049	0.069	0.086
T	0.024	-0.352	0.039	0.246	0.083

V	0.220	0.013	0.031	0.058	0.137
SA	0.214	0.035	0.031	0.007	0.145
Pn	0.226	-0.044	0.024	0.063	0.000
Ci	-0.196	0.169	0.030	0.208	0.006
Gs	0.208	-0.040	0.059	0.173	0.063
Tr	0.205	-0.106	0.111	0.111	0.006
SPAD	0.218	0.075	0.033	0.150	0.046
CAT	0.161	0.236	0.015	0.146	0.093
MDA	-0.208	0.105	0.061	0.005	0.061
POD	0.089	0.194	0.326	0.133	0.542
SS	0.039	0.247	0.384	0.205	0.227
Pro	0.032	0.357	0.059	0.066	0.179
SP	0.114	0.107	0.021	0.279	0.422
RC	-0.220	0.095	0.074	0.061	0.083
SOD	0.076	0.167	0.408	0.414	0.027
GST	0.097	0.271	0.199	0.101	0.374
GSH	0.011	0.059	0.641	0.280	0.007
H ₂ O ₂	-0.206	0.074	0.029	0.075	0.022
GR	-0.019	0.369	0.040	0.164	0.011
ASA	-0.040	0.349	0.036	0.239	0.119
HF	-0.031	0.326	0.032	0.140	0.345
·OH	-0.197	0.057	0.229	0.110	0.029
K ⁺	0.204	-0.056	0.078	0.300	0.082
Na ⁺	-0.222	0.001	0.014	0.171	0.050
K ⁺ /Na ⁺	0.205	-0.060	0.055	0.250	0.087
Fv/Fo	0.205	0.040	0.038	0.197	0.070
Fv/Fm	0.211	0.017	0.044	0.119	0.050
Eto/Rc	0.204	0.004	0.059	0.123	0.253
Vj	-0.198	-0.008	0.067	0.085	0.028
Eigenvalue	18.309	6.486	1.672	1.133	0.974
Variance	55.482	19.655	5.065	3.433	2.951

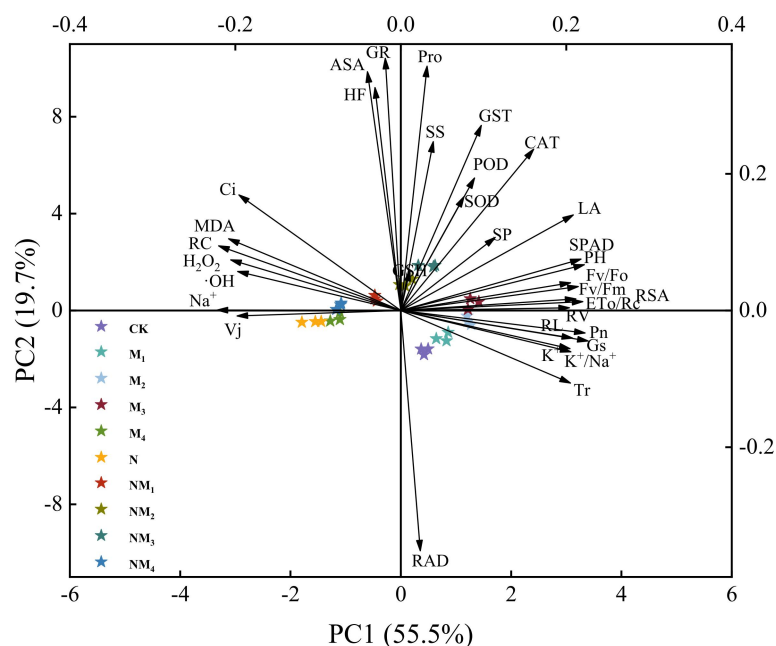


Fig. 14. Principal component analysis between measured parameters of 'Zhongmu No.3' alfalfa plants under salt stress

3.9. Comprehensive Evaluation of Salt Tolerance of Alfalfa Under Salt Stress by MT

The membership function was used to comprehensively evaluate 33 indexes such as morphology and physiology of 'Gongnong No.1' and 'Zhongmu No.3' alfalfa under 10 treatments. As shown in Figures 15 (A) and 15 (B). The average membership degree of the two varieties was the highest under the treatment of $150 \mu\text{mol}\cdot\text{L}^{-1}$ MT under the conditions of no salt stress and salt stress. They were 0.866 and 0.747, respectively. 0.840 and 0.731, respectively. The average membership degree of 33 indexes such as morphology and physiology of two kinds of alfalfa with different salt tolerance under 10 treatments was compared. As shown in Fig. 15 (C), after spraying MT under no salt stress. The average membership degree of salt-tolerant alfalfa 'Zhongmu No.3' at $50 \mu\text{mol}\cdot\text{L}^{-1}$, $100 \mu\text{mol}\cdot\text{L}^{-1}$ and $200 \mu\text{mol}\cdot\text{L}^{-1}$ was higher than that of salt-sensitive alfalfa 'Gongnong No.1'. After spraying MT under salt stress, the average membership degree of 'Gongnong No.1' at 50 - $200 \mu\text{mol}\cdot\text{L}^{-1}$ concentration was higher than that of 'Zhongmu No.3'.

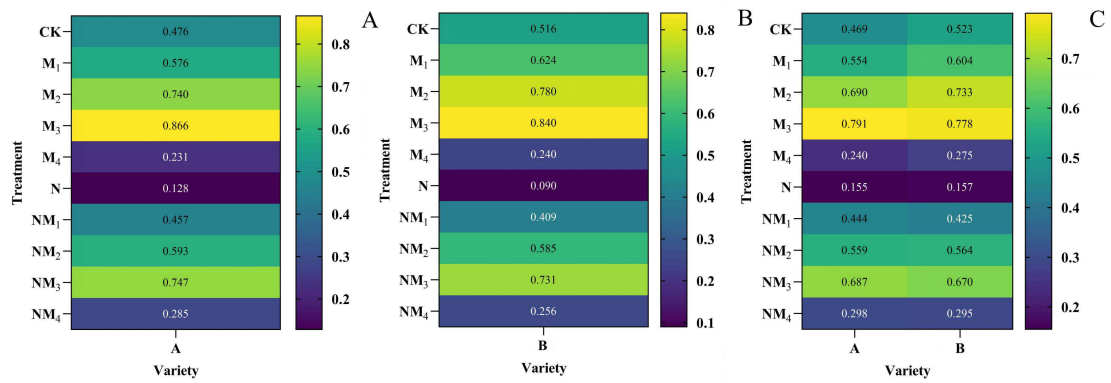


Fig. 15. Heatmap of comprehensive evaluation of exogenous MT on salt tolerance of alfalfa seedlings under salt stress.

Note : A is 'Gongnong No.1' alfalfa, B is 'Zhongmu No.3' alfalfa.

4. Discussion

Plants are exposed to adverse environments that can inhibit normal growth, development, and metabolism, known as adversity. Adversity is the main factor limiting plant growth [17]. MT is a novel plant growth regulator with multiple functions in biotic and abiotic stress regulation [9]. In this study, we evaluated the effects of MT on alfalfa phenotypic characteristics, osmoregulation, antioxidant systems, ionic toxicity, oxidative damage, cell membrane stability, and photosynthetic properties. The results show that exogenous MT can ameliorate the adverse effects of salt stress on alfalfa seedlings and cellular ultrastructures. Furthermore, it was also shown that high levels of MT can inhibit alfalfa growth and development.

4.1. Effect of exogenous MT on the phenotype of alfalfa seedlings

Under salt stress, the most obvious manifestations of plant stress are slow growth and disruptions in development.[18]. In this study, appropriate MT treatment under normal growth conditions promoted alfalfa's growth and development characteristics, including plant height, leaf area, and root morphology. NaCl treatment significantly inhibited the phenotypes of alfalfa seedlings, resulting in reduced seedling leaf area, root length, root volume, root surface area, and average root diameter. MT significantly alleviated the damage caused by salt

stress. This indicates that MT promotes plant growth under normal conditions and significantly induces salt tolerance/resistance in plants. The structures and functions of plant cells are closely related. They work together to form highly coordinated and complementary systems that enable cells to fulfill various physiological functions ^[19]. Among plant organelles, chloroplasts are an important source of ROS production ^[20]. Therefore, it is crucial to understand the changes in the ultrastructures of leaf pulp cells under salt stress. In this study, electron microscopy showed that the membranes of alfalfa cysts in salt-sensitive alfalfa 'Gongnong No. 1' were expanded, chloroplasts were swollen, and the number of stacks of stroma lamellae was greatly reduced in salt stress conditions. The chloroplast damage was lower in the salt-tolerant alfalfa 'Zhongmu No.3' than in 'Gongnong No. 1'. The damage caused by salt stress could be alleviated with 50–150 $\mu\text{mol}\cdot\text{L}^{-1}$ MT treatment. However, the 200 $\mu\text{mol}\cdot\text{L}^{-1}$ MT treatment and salt stress distorted and may disintegrate the organelles in the leaf pulp. The chloroplast and mitochondrial structures were seriously damaged, the membrane structure was incomplete, the amount of substrate efflux was increased, the shape of the chloroplasts was irregular or nearly round, and the internal structures of the chloroplast vesicles were altered.

4.2. Effects of Exogenous MT on Osmotic Homeostasis in Alfalfa Seedlings

Osmotic adjustment substances are synthesized by plants through physiological and metabolic activities to improve stress adaptations. When plants are subjected to salt stress, the osmotic balance of the cells is disrupted ^[21]. To alleviate this phenomenon, plants self-regulate by producing osmoregulatory substances such as Pro, SP, and SS to achieve an osmotic balance at the cellular level ^[22-23]. Pro in plant cells enhances some antioxidant enzyme activities, photosystem activities, and plant growth ^[24]. In addition, Pro maintains the balance of cellular osmotic potential and protects the structures of biomolecules, which helps plants resist external stresses ^[25].

Pro is a major organic osmoregulator in alfalfa seedlings under NaCl stress [26]. In this study, the MT treatment under normal growth conditions significantly increased the Pro, SP, and SS contents of alfalfa, which indicated that MT could promote the production of osmoregulatory substances in plants. When salt stress treatment was applied, the Pro, SP, and SS also increased significantly compared with normal growth conditions. This indicates that plants produce osmoregulatory substances through self-regulation to resist adversity

damage. Pro, SP, and SS contents continued to increase after MT treatment, attributed to MT acting as a scavenger of reactive oxygen species (ROS) to reduce excessive intracellular ROS and balance the osmotic homeostasis under salt stress.

4.3. Effects of Exogenous MT on the Antioxidant System of Alfalfa Seedlings

Plants activate their stress mediation mechanisms in response to salt stress and produce large amounts of ROS [27]; excessive accumulation of ROS disrupts the dynamic balance of the reactive oxygen species scavenging mechanism in the cell, leading to the formation of oxidative damage [28]. Under adversity stress, the plant body has evolved a systematically sound antioxidant system to resist external stress damage [5,29]. This system consists primarily of enzymatic systems (such as SOD, POD, CAT, GST, and GR) and non-enzymatic antioxidant systems (such as ASA and GSH). ASA is an important reductive antioxidant in the non-enzymatic scavenging system, which is abundant in photosynthetic tissues and can catalyze the formation of monodehydroascorbic acid by APX followed by the direct scavenging of ROS to maintain the stability of cell membranes. GSH, an -SH-containing reductive antioxidant prevalent in plants, plays a central role in the antioxidant system [30]. In this study, after salt stress treatment, the SOD, POD, CAT, GST, and GR activities of the two alfalfa varieties were enhanced. ASA and GSH contents increased compared with normal growth because a large accumulation of ROS in the plant prompted the antioxidant system to produce many antioxidant enzymes and antioxidants to scavenge ROS. When MT was

sprayed, the activities of these antioxidant enzymes and the content of the non-enzymatic substances continued to increase, indicating that the MT can stimulate the antioxidant enzyme system to respond to salt stress, reduce reactive oxygen groups, and decrease oxidative damage.

4.4. Effects of Exogenous MT on Ionic Homeostasis in Alfalfa Seedlings

Over-enrichment of Na^+ ions in plants under salt-stressed environments inhibits the uptake of K^+ ions and disrupts the K^+/Na^+ balance, leading to an imbalance in intracellular ionic homeostasis, resulting in ion toxicity [31]. Moreover, Na^+ competes for ionic sites originally on the Ca^{2+} plasma membrane and intracellular membrane, destroying the integrity of the membrane structure while preventing some membrane functions from exerting their effects [32]. For plants, K^+ is the major cellular cation and an essential nutrient. To adapt to the salt environment, plants reduce the Na^+ concentration in the cytoplasm via Na^+ efflux or compartmentalization to maintain a high K^+/Na^+ ratio and thus establish a new ionic homeostasis [33]. In this study, it was found that under salt stress conditions, the K^+ ion content was significantly decreased compared with the CK, while the Na^+ ion was significantly increased, leading to a lower K^+/Na^+ ratio, and the ionic homeostasis was affected; the ionic homeostasis was significantly reversed after MT spraying.

4.5. Effects of Exogenous MT on Oxidative Damage and Cell Membrane Stability in Alfalfa Seedlings

When faced with the threat of salt stress, plants take up large amounts of salt, and when the plant's salt concentration is elevated, ROS groups accumulate in large quantities [34]. ROS include $\cdot\text{OH}$, H_2O_2 , and membrane lipid peroxidation products [35]. When the accumulation of ROS exceeds the threshold, it causes lipid membrane peroxidation. This leads to an increase in its product, MDA [36], which ultimately increases the permeability of the cytoplasmic membrane, preventing the normal transport of substances, transporters, and information transfer, and the structure of the cellular membrane is damaged [37]. MDA is an important indicator that reflects plant

cell responses to stress and the damage status of cell membranes. RC is an important indicator that reflects the state of plant cell membranes [38]. When plants are stressed, the cell membranes are damaged, leading to the outflow of cytosol and increased RC. The hydroxyl radical scavenging rate is a self-protection measure evolved by plants to protect themselves from injury [39], when plants are under stress, they rapidly remove ROS produced in the body to increase their defense ability [40].

In this study, the MDA, $\cdot\text{OH}$, and H_2O_2 contents, RC, and hydroxyl radical scavenging rate of the two alfalfa varieties were elevated after the salt stress treatment. At the same time, other indexes were significantly reduced after MT treatment, except for the hydroxyl radical scavenging rate, which continued to increase. This indicated that the hydroxyl radical scavenging rate was not proportional to hydroxyl radical content. With the increase of MT concentration, MDA, $\cdot\text{OH}$, H_2O_2 , and RC of both alfalfa species showed a tendency to increase and then decrease, indicating that the ability of MT to alleviate membrane lipid peroxidation is limited.

4.6. Effect of Exogenous MT on Photosynthetic Capacity of Alfalfa Seedlings

4.6.1. Effect of Exogenous MT on Photosynthesis in Alfalfa Seedlings Under Salt Stress

Photosynthesis is the basis of plant life [41], and the intermediate products of its activities can help plants obtain the required substances and energy to ensure normal plant growth, which is important for plant life activities [42]. However, the normal functioning of photosynthesis is affected by adverse environmental conditions, such as salt stress, which impacts osmotic stress, ionic toxicity, and photosynthesis products [43]. In response to salt stress, plants can close their stomata to mitigate the damage, which leads to a decrease in gas exchange [44]. Photosynthesis is affected by salt stress for two main reasons: stomatal and non-stomatal factors [45-46]. In this study, P_n , T_r , and G_s decreased under salt stress treatment. In contrast, C_i increased, indicating that salt stress reduced the photosynthetic rate, leading to a

subsequent increase in C_i , directly affecting photosynthesis and ultimately closing the stomata. This indicates that the influence of alfalfa photosynthesis is primarily due to non-stomatal limitations. After adding the appropriate concentration of MT, the C_i of the two alfalfa species decreased, and the P_n , T_r , and G_s were promoted. When the MT concentration was too high, the C_i increased, and the P_n , T_r , and G_s decreased to different degrees. The overall trend of the changes in the photosynthetic parameters was reduced, which indicates that the appropriate concentration of MT promotes the photosynthesis capacity of the plants under salt stress. However, when the concentration is too high, photosynthesis will be weakened.

4.6.2. Effects of Exogenous MT on Chlorophyll SPAD and Chlorophyll Fluorescence Parameters

Chlorophyll content in plants can reflect photosynthetic intensity and conversion accumulation. This indicates that chlorophyll is an important plant pigment used to absorb light energy for photosynthesis and an important indicator of the plant's ability to withstand adversity ^[47]. The SPAD chlorophyll tester can obtain SPAD values by comparing optical density differences on the leaf, which enables direct measurement of the chlorophyll content ^[48]. In this study, salt stress reduced leaf SPAD values, however, these values were significantly improved by MT application. Chlorophyll fluorescence parameters can accurately and instantly reflect the status of the leaf photosystem in terms of the conversion absorption, transmission, and distribution of light energy ^[49], and serve as a potential indicator for the study of the immediate situation of the plant, as it can reflect the environmental influence on the plant^[50]. Chlorophyll fluorescence parameters can internally show the effect of adversity stress on the plant's photosynthetic process and photosynthetic system so that the plant's photosynthetic pigments absorb light energy and emit fluorescence ^[51]. The maximum photochemical efficiency (F_v/F_m) can visualize the efficiency of plants for light energy utilization ^[52], and the maximum light energy conversion potential (F_v/F_o) can reflect the potential activity of PSII ^[53]. In this study, both F_v/F_m and F_v/F_o ratios decreased significantly due to salt stress; at 200 $\mu\text{mol-L}^{-1}$ MT, F_v/F_m and

Fv/Fo ratios decreased similarly compared with that of salt stress, suggesting that salt stress and high concentration of MT affected the capture of light energy by the PSII system. The Fv/Fm and Fv/Fo ratios were significantly increased after spraying MT treatments under normal growth and salt stress, indicating that MT could improve the efficiency of PSII system for light energy utilization and enhance the potential photosynthetic capacity of alfalfa. ETo/RC represents the electron transfer capacity of the leaf unit reaction center ^[54]. In this study, salt stress decreased ETo/RC, and due to the limitation of alfalfa's regulatory capacity, the decrease of ETo/RC was larger, the electron transfer capacity of PSII reaction centers was weakened, and ETo/RC was significantly increased after spraying MT.

These results indicate that MT can enhance the electron transfer capacity of PSII reaction centers, thus improving PSII vigor and alfalfa's tolerance to salt stress. The relative variable fluorescence intensity (Vj) at point J in the OJIP curve is a key indicator of the degree of stress damage to plant leaves. The Vj of alfalfa was elevated and significantly greater than normal levels after being subjected to salt stress. This suggests that the donor and acceptor sides of PSII were disrupted, the function was impaired, and the activity of the reaction centers was reduced^[55]. Foliar spraying of MT resulted in a significant reduction of Vj, indicating that exogenous MT can enhance the photosynthetic performance of PSII in alfalfa and restore the activity of its donor and acceptor sides. In conclusion, all fluorescence parameters were significantly restored after the application of MT, and the effect of 150 $\mu\text{mol}\cdot\text{L}^{-1}$ treatment was the most obvious, indicating that the appropriate concentration of MT treatment is conducive to the enhancement of the ability of alfalfa seedlings to capture and utilize light energy under salt stress.

5. Conclusion

Based on the results of this study, we developed a simplified model to describe the positive effects of exogenous MT treatment on alfalfa seedlings under salt stress. MT foliar spraying significantly mitigated the damage caused by salt stress to alfalfa, irrespective of salt tolerance (Fig.5-1). This

mitigating effect was attributed to the promotion of the synthesis of osmoregulatory substances, enhancement of antioxidant enzyme activity, increase in antioxidant content, enhancement of photosynthetic capacity, and reduction in Na^+ accumulation to re-establish ionic homeostasis and scavenging of harmful ROS and free radicals. In summary, alfalfa evolved osmoregulatory, antioxidant, ion transport, reactive oxygen species scavenging, and photosynthetic capacity enhancement mechanisms to adapt to salt stress. These five mechanisms interacted and were synergistically regulated to enable alfalfa to survive in saline environments. These positive changes in alfalfa's physiological and biochemical parameters increased its morphological characteristics and repaired and improved the broken ultrastructure of the leaf pulp cells. Of the MT concentrations analyzed in this study, $150\ \mu\text{mol}\cdot\text{L}^{-1}$ was optimal for increasing the tolerance of alfalfa to salt stress

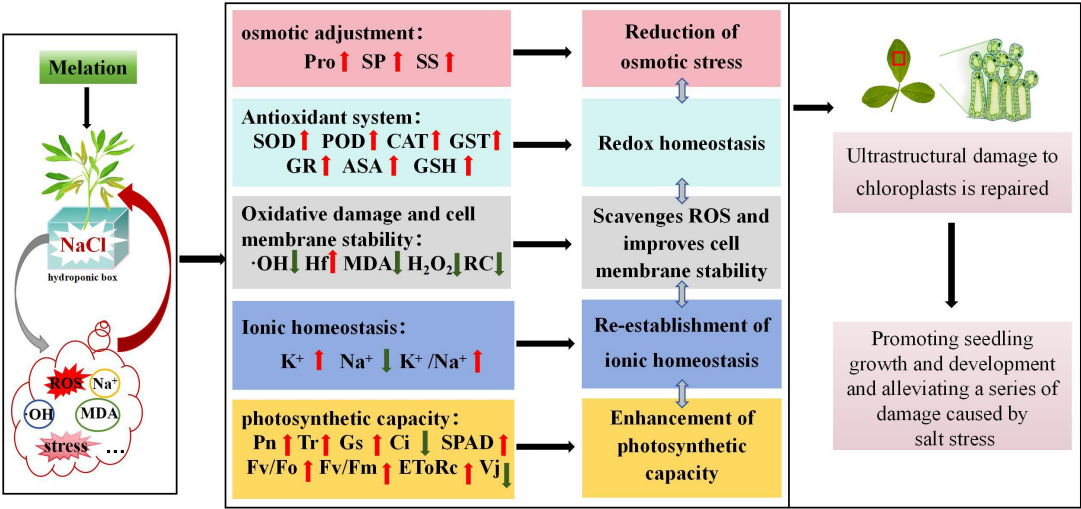


Fig. 5-1. Positive effects of melatonin on alfalfa seedlings under salt stress. Red and green arrows indicate increases and decreases, respectively.

Abbreviations

All abbreviations are defined at first use in the text; a list of abbreviations is provided in 2.3. Observations and Measurements.

Ethics approval and consent to participate

Not applicable.

Consent for publication

For manuscripts containing any individual person's data in any form written informed consent for publication was obtained from the participant.

Availability of data and materials

All data generated or analysed during this study are included in this published article and its supplementary information files.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Yixin Liu: Writing - review & editing, Writing - original draft, Data curation.

Xinyao Wang: Writing - review & editing, Writing - original draft, Data curation.

Xiaoqing Sui: Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Jing Qi:** Writing - review & editing, Writing-original draft.

Yifan Zhou: Data curation. **Amanula·Yimingniyazi:** Methodology, Writing - review & editing.

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