PROTECTIVE EFFECTS OF MELATONIN LIPOSOME NANOPARTICLES ON *MEDICAGO SATIVA* L. SEEDLINGS UNDER CADMIUM STRESS

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Abstract. Cadmium (Cd) is one of the main heavy metals causing global environmental pollution, and it is also the heavy metal with the strongest toxic effect on growth and development of crops and other plants. In this study, we established six treatment groups, including normal culture group (CK), exogenous melatonin treatment group (MT), melatonin liposome nanoparticles treatment group (MT-SLNs), cadmium chloride stress group (Cd), compound treatment with cadmium chloride stress and exogenous melatonin group (Cd+MT), compound treatment with cadmium chloride stress and melatonin liposome nanoparticles group (Cd+MT-SLNs). We found that $CdCl_2$ stress inhibited the growth of alfalfa seedlings and decreased cell viability, resulting in significant cadmium stress damages by measuring and analyzing plant phenotype, cell viability, stress damage, reactive oxygen species scavenging system, and Cd accumulation of alfalfa seedlings under six conditions. At the same time of cadmium stress, spraying MT or MT-SLNs significantly increased the cell viability of alfalfa seedling leaves, and significantly induced antioxidant synthesis and antioxidant enzyme activity. The levels of ROS (Reactive oxygen species) and free Cd accumulation in tissues decreased, indicating that exogenous MT or MT-SLNs can effectively alleviate the inhibitory effect of $CdCl_2$ stress on alfalfa by activating the reactive oxygen species scavenging system and promoting Cd efflux, and spraying MT-SLNs has a more significant protective effects on alfalfa seedling cadmium stress. In addition, spraying MT or MT-SLNs under normal growth conditions is also beneficial for the growth and development of plant seedlings.

Keywords: *Medicago sativa L., cadmium chloride stress, melatonin, liposome nanoparticles, reactive oxygen species scavenging system*

Introduction

Recently, with the rapid development of industrialization, heavy metal pollution has become increasingly serious, which has been widely concerned by researchers all over the world (Antoniadis et al., 2017; Geng et al., 2019; Palansooriya et al., 2020; Taoufik et al., 2022; Lu et al., 2023). Cadmium (Cd) is recognized as one of the most toxic heavy metals in the world (Rai et al., 2019). Previous studies have displayed that cadmium pollution has the characteristics of wide pollution range, long duration, strong concealment, and irreversibility (Huang et al., 2022). Cadmium pollution has strong genetic toxicity to organisms, thus posing a great threat to plant growth. Studies have shown that cadmium pollution can reduce plant height and root length, causing protein denaturation, DNA damage, chromosomal structural changes, and inducing oxidative stress. Therefore, long-term excessive accumulation of cadmium in plants can ultimately lead to plant death (Li et al., 2015; Rizwan et al., 2017; Chen et al., 2022; Luo et al., 2022).

Alfalfa (*Medicago sativa* L.) is a high-quality perennial economic crop in the legume family, characterized by fast growth rate, short cultivation period, high dry matter yield, and rich nutritional value (Shi et al., 2017). Alfalfa can absorb and enrich Cd, Cu, Ni, Zn,

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Se and other heavy metal elements from soil (Peralta-Videa et al., 2004). It is a precious grass species with great development prospects in reducing the concentration of heavy metals in soil and improve the ecological environment (Jing et al., 2018).

Melatonin (MT) is a type of indolehormone, widely distributes in various organisms in nature (Rüdiger, 2016; Wang et al., 2021). It can not only regulate the growth and metabolic balance of organisms, but also help organisms to resist various biotic and abiotic stressors. Natural melatonin was firstly discovered in the animal pineal gland. Therefore, it is also known as pinealin (Lerner et al., 1960). The latest studies have indicated that plant melatonin have various functions such as delaying leaf aging, scavenging reactive oxygen species (ROS), defensing biotic and abiotic stress, and controlling plant growth and organ development (Ma, 2019; Sun et al., 2020). Melatonin is also recognized as an endogenous free radical scavenger that can regulate oxidative stress responses caused by environmental stress (Dubbels et al., 1995; Sharif et al., 2018; Moustafa-Farag et al., 2019). However, the internal secretion of natural melatonin has strict rhythmicity and photosensitivity, leading to its unstable structure, easy oxidation and decomposition when exposed to light, which seriously restricts its promotion and application in agricultural production practice (Feng et al., 2021).

Melatonin liposome nanoparticles (MT-SLNs) can effectively improve the sustained release efficiency of melatonin by encapsulating and preserving its activity, avoiding its limitations of easily decomposition due to structural instability. Moreover, the nanoparticles have good biocompatibility, small particle size, and can quickly enter biological cells to achieve efficient absorption and utilization of melatonin (Ding et al., 2014; Wang et al., 2019).

The primary aim of this study will enhance the protective effects of melatonin on cadmium stressed plants through improving the absorption and utilization efficiency of melatonin in plants. Melatonin acted as cadmium stressed alfalfa seedlings in the form of nanoparticle aqueous solution. The protective effects and molecular mechanism of melatonin liposome nanoparticles treatment on cadmium stressed alfalfa were evaluated in detail by analysis of seedling phenotype, cell viability, cadmium stress damages, ROS scavenging system and Cd accumulation. The results found that MT-SLNs can effectively activate plant antioxidant metabolism, promote Cd efflux and the binding of free Cd with chelating peptides, in order to enhance cadmium tolerance of alfalfa seedings. Moreover, MT-SLNs had showed more significant protective effects on plants under cadmium stress.

Materials and methods

Plant material and growth conditions

Mature alfalfa seed (*Medicago sativa* L., variety 'Golden Queen') was selected as biological materials. Mature alfalfa seeds were inoculated into nutrient soil-vermiculite at a ratio of 3:1. In the stress condition, 100 μmol/L cadmium chloride solution ($CdCl_2$) was evenly applied into the soil, and in the normal culture group, water was applied. Germinated for 2 days under dark conditions at 25℃-28℃, and then transferred to an incubator (GXZ-310C, Ningbo Yanghui Instrument Co., Ltd.) with a temperature of 23±2℃, a relative humidity of 70%, a photoperiod of light/dark 16 h/8 h, and a light intensity of 10000 Lux for 15 days. MT or MT-SLNs were sprayed on plant seedlings at the 5th, 7th, 10th and 13th days of culture, and the 15-day-old materials were taken and analyzed.

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Methods

Screening of cadmium stress critical concentration

50 seeds with the same size were selected and inoculated into dishes covered with two layers of gauze. Germinating seeds were stressed with 0, 50, 100, 150, 200, 250 and 300 μmol/L $CdCl_2$ solution for two hours per day respectively, and continuously for three days. The elongation of hypocotyls was measured by using WinRHIZO REG 2007 software.

Alfalfa cultivation and stress treatment

After the germination of alfalfa seeds, the seeds with the same germination rate were cultured under different conditions. We established six treatment groups (*Table 1*), including normal culture group (CK), exogenous melatonin treatment group (MT), melatonin liposome nanoparticles treatment group (MT-SLNs), cadmium chloride stress group (Cd), compound treatment with cadmium chloride stress and exogenous melatonin group (Cd+MT), compound treatment with cadmium chloride stress and melatonin liposome nanoparticles group (Cd+MT-SLNs). According to our previous experimental results, the application concentration of exogenous MT and MT-SLNs was 200 μmol/L.

Table 1. *Treatment groups*

Abbreviation	The whole name of treatment groups
CK	normal culture group
MT	exogenous melatonin treatment group
MT-SLNs	melatonin liposome nanoparticles treatment group
Cd	cadmium chloride stress group
Cd+MT	compound treatment with cadmium chloride stress and exogenous melatonin group
Cd+MT-SLNs	compound treatment with cadmium chloride stress and melatonin liposome nanoparticles group

Phenotypic and growth parameter detection of alfalfa seedlings

The phenotypes of 15-day-old alfalfa seedlings in different groups were observed and photographed with camera (Cannon, 5D4, Japan). The height of alfalfa seedlings was measured by using ruler. The fresh weight of each seedling leaf was weighed by using analytical balance (M5 Satorious, Germany). About 0.1 g leaves were transfered 10 mL centrifuge tube containing 5 mL anhydrous ethanol and placed at dark condition for 48 hours. The absorbance of chlorophyll extract at 665 nm and 649 nm was determined by ultraviolet spectrophotometer (Hitachi U-2900, Japan).

Analysis of physilogical damage and cell activity

The seedling cotyledons, the first true leaf, and the second true leaf were soaked in 0.25% (W/V) Evans blue solution for 24 hours, then decolorized in boiling decolorization solution (anhydrous ethanol:glycerol=9:1) for 30 minutes. Leaves of seedlings were soaked in trypan blue solution preheated at 65℃ for 20 minutes, then decolorized in anhydrous ethanol at 95℃. The electrical conductivity and malondialdehyde content were determined according to refer to Wassie's method (Wassie et al., 2020).

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Determination of melatonin content

The melatonin content of alfalfa seedlings in different groups were determined by using the Plant Melatonin Quantitative Detection Kit (ELISA) (purchased from Quanzhou Ruixin Biotechnology Co., Ltd.) and followed the instructions for specific steps.

Reactive oxygen species detection

The seedling cotyledons, the first true leaf, and the second true leaf were soaked in nitrogen blue tetrazolium solution (2 mM) and placed at room temperature for 5.5 hours under dark condition, then decolorized in anhydrous ethanol at 95℃. Leaves of seedlings were soaked in a diaminobenzidine solution (1 mg/mL, pH 3.8), and placed at 25℃ light condition for 8 hours, then the leaves were decolorized by anhydrous ethanol at 85℃.

$O_2^{\cdot-}$ content was measured by using superoxide anion content detection kit, and H_2O_2 content was measured by using hydrogen peroxide content detection kit. Both of them were purchased from Abbkine Scientific Co., Ltd. and the specific steps were carried out according to the instructions.

Reactive oxygen species scavenging system

The content of ascorbic acid (AsA) and glutathione (GSH) were measured according to our previous experimental method (Xi, 2022). About 0.2 g leaves from different treatments were grinded into homogenate in pre-cooled and sterilized the mortar with 2 mL PBS buffer (50 mM, pH 7.0), then centrifuged at 12000g 4℃ for 10 minutes. The supernatant was the antioxidant enzyme extract. SOD (superoxide dismutase) activity was measured according to Giannopolites method (Giannopolites and Ries, 1977); POD (guaiacol peroxidase) activity was measured according to Dias and Costa's method (Dias and Costa, 1983); CAT (catalase) activity was measured according to Rao's method (Rao et al., 1996); APX (ascorbate peroxidase) activity was measured according to Nakano and Asada's method (Nakano and Asada, 1980).

Extract RNA from the leaves of alfalfa seedlings and reverse transcribe it into cDNA. Then the expression of antioxidant isozyme gene was determined by using the Perfect Start Green qPCR Super Mix kit (purchased from Beijing Quanshijin Biotechnology Co., Ltd.). The primer sequences of antioxidant isozyme gene and internal reference gene *Actin* in alfalfa are shown in the table below (*Table 2*).

Analysis of cadmium accumulation and cadmium stress-related proteins

Cd content in the leaves of alfalfa seedlings were determined according to Li Hang's method (Li et al., 2022; Li, 2022) which has made slight modifications. The content of chelating peptides in different treatment groups were indirectly determined by detecting the content of reduced glutathione (GSH) and non-protein thiol (NPT). Both GSH and NPT detection kits were purchased from Nanjing Jiancheng Biotechnology Research Institute, and the specific steps were carried out according to the instructions. The calculation formula for PCs was as follows (*Equation 1*).

$$\mathrm{PCs}(\mu\mathrm{mol/L\ FW}) = \mathrm{NPT}(\mu\mathrm{mol/L\ FW}) - \mathrm{GSH}(\mu\mathrm{mol/L\ FW}) \quad \text{(Eq.1)}$$

The content of ABC transporter in different groups were determined by the plant adenosine triphosphate binding cassette transporter enzyme-linked immunosorbent assay kit, which was purchased from Shanghai Enzyme-linked Biotechnology Co., Ltd. The

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analysis method of *MsABCG1* gene was the same as the determination of antioxidant isozyme gene expression mentioned above. The primer sequence of *MsABCG1* gene was shown in *Table 2*.

Table 2. *Primer information*

Gene Name	Forward Primer (5'-3')	Reverse Primer (5'-3')
Actin	AAAAGGATGCCTATGTTGGTG	AAGTGGAGCCTCAGTTAGAAGTA
Cu/Zn-SOD	TAATTGCTGATGCCAACG	ACCACAGGCTAATCTTCCAC
Fe-SOD	GAACCATCATCATCATCCACTCCTTACC	CCTCCTCTTTCTCCCTCTCCGCAAT
Mn-SOD	TGTCATCAGCGGCGTAATCAT	GGGCTTCCTTTGGTGGTTCA
POD1A	TCAATCGTACGTGGTGTGCT	TGCACTTTGCTCGCTCACTA
POD1B	AGCTGCATTTGCTGCTCAAG	TTGGTAAGGTTCGTGCCAGG
POD1C	CCTCGCATGCTTGCTAGTCT	GGACCTTGTGCCAGAACAGA
POD2	TCCTGCTACCCTTCGTCTCT	TTCTGCACTGTGGAACAGCA
CAT	CCTATTTGATGATGTGGGTGTCC	GTCTTGAGTAGCATGGCTGTGGT
APX	TTTCGGAACCATCAAGCAC	GCAACAACACCAGCCAACT
MsABCG1	TGAATGGAGGAGAAGATAAGGG	GGCTTTGCATAACCAGTCAGA

Data analysis

The significant difference was analyzed by SPSS25.0 software. Data were presented with means ± SD. Differences among treatments were analyzed by one-way ANOVA, taking $P < 0.05$ as significant according to Duncan's multiple range test.

Results and analysis

Screening of cadmium stress critical concentration

By measuring the hypocotyls of alfalfa under seven different concentrations of cadmium chloride stress, we found that cadmium chloride stress inhibited the elongation of seedling hypocotyls, and with the concentration of cadmium chloride stress increased, the length of hypocotyls became shorter and shorter (*Fig. 1*). When under 100 μmol/L $CdCl_2$ stress, compared with 0 μmol/L $CdCl_2$, the length of plant hypocotyls decreased by 56.91%. Therefore, 100 μmol/L cadmium chloride solution was selected as the critical stress concentration.

Phenotypic observation and growth parameters determination of alfalfa seedlings

The phenotypic observation and growth parameters determination of alfalfa seedlings under different conditions showed that spraying exogenous MT or MT-SLNs under normal conditions further promoted plant growth and development (*Fig. 2*). After Cd stress, the phenomenon of plant dwarfism is obvious, with some leaves wilting and their color changing to light green or grayish yellow. Spraying MT or MT-SLNs under Cd stress can significantly improve plant phenotype abnormalities and dwarfism, increase leaf biomass, and alleviate the above effects of Cd stress. Moreover, spraying exogenous MT-SLNs showed better alleviation effects on Cd stress-seedlings.

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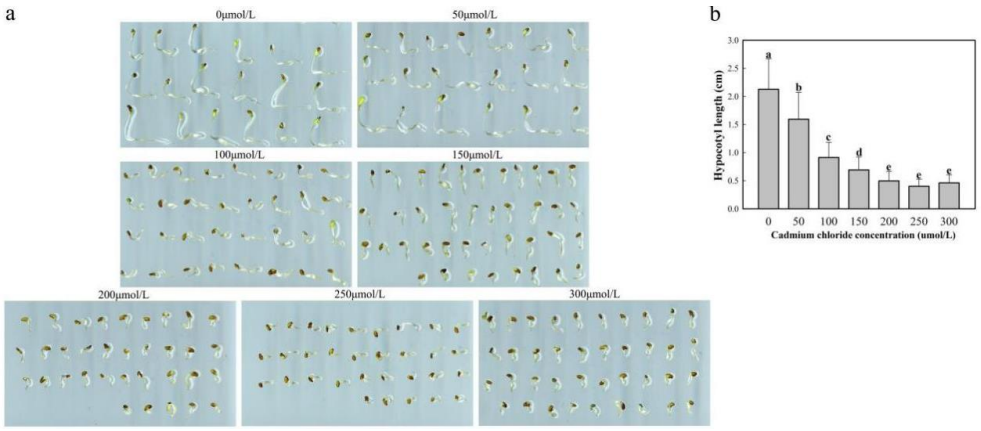

Figure 1. *Analysis of hypocotyl elongation of alfalfa. (a) Hypocotyl phenotype; (b) Hypocotyl length. *Different letters indicated significant differences among different treatment groups (P<0.05)*

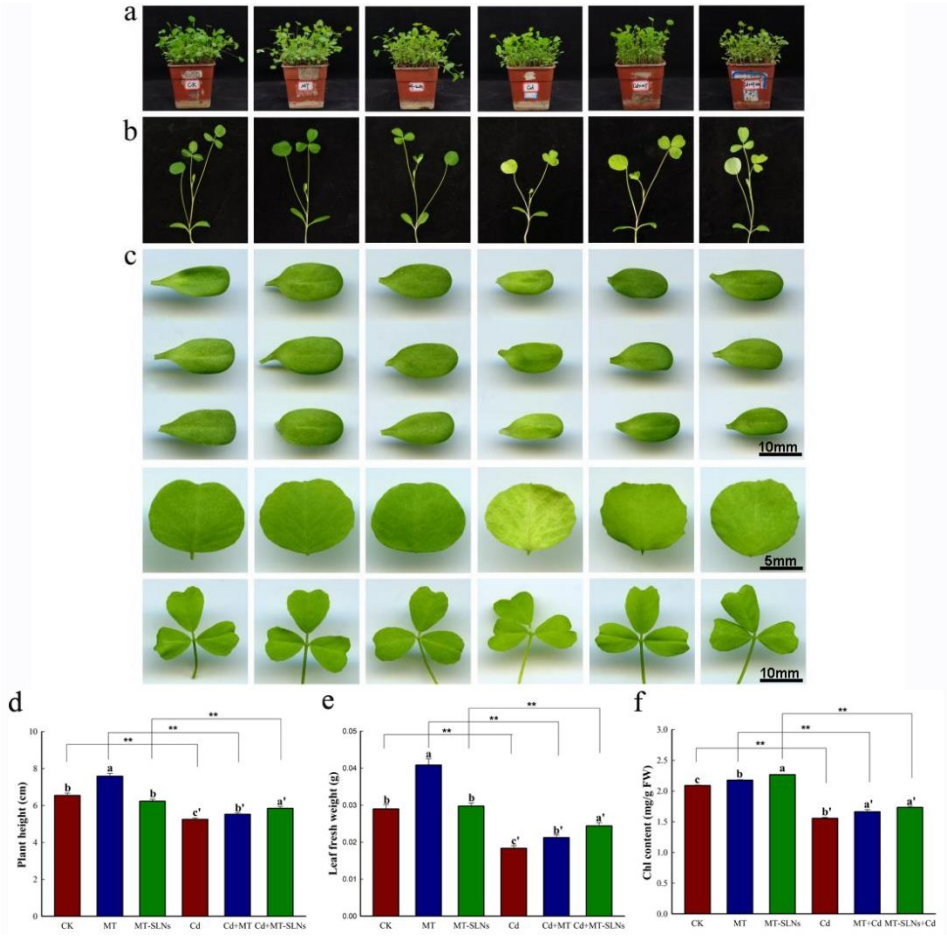

Figure 2. *Phenotype and growth parameters of alfalfa seedlings under different treatments. (a) Alfalfa phenotype; (b) Single seedling phenotype; (c) Leaf phenotypic; (d) Plant height of alfalfa; (e) Fresh weight of alfalfa leaves; (f) Chlorophyll content of alfalfa. *a, b, c showed significant differences among the three negative control groups (P<0.05); a', b', c' indicated significant differences among the three treatment groups under cadmium stress (P<0.05); **(P<0.01) means significant differences among the same color histogram*

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Analysis of physilogical damage and cell activity of alfalfa seedlings

Evans blue staining showed that Cd stress caused serious damages to seedling leaves, and spraying exogenous MT or MT-SLNs can effectively alleviate these damages (*Fig. 3*). Further determination data on ion leakage rate and MDA content in leaves were consistent with Evans blue staining. We also detected leaf cell activity through trypan blue staining, indicating that Cd stress significantly inhibited the cell viability of seedling leaves. After spraying exogenous MT or MT-SLNs, cell viability increased again, and the protective effects of MT-SLNs on cadmium stress damage and cell viability was more significant.

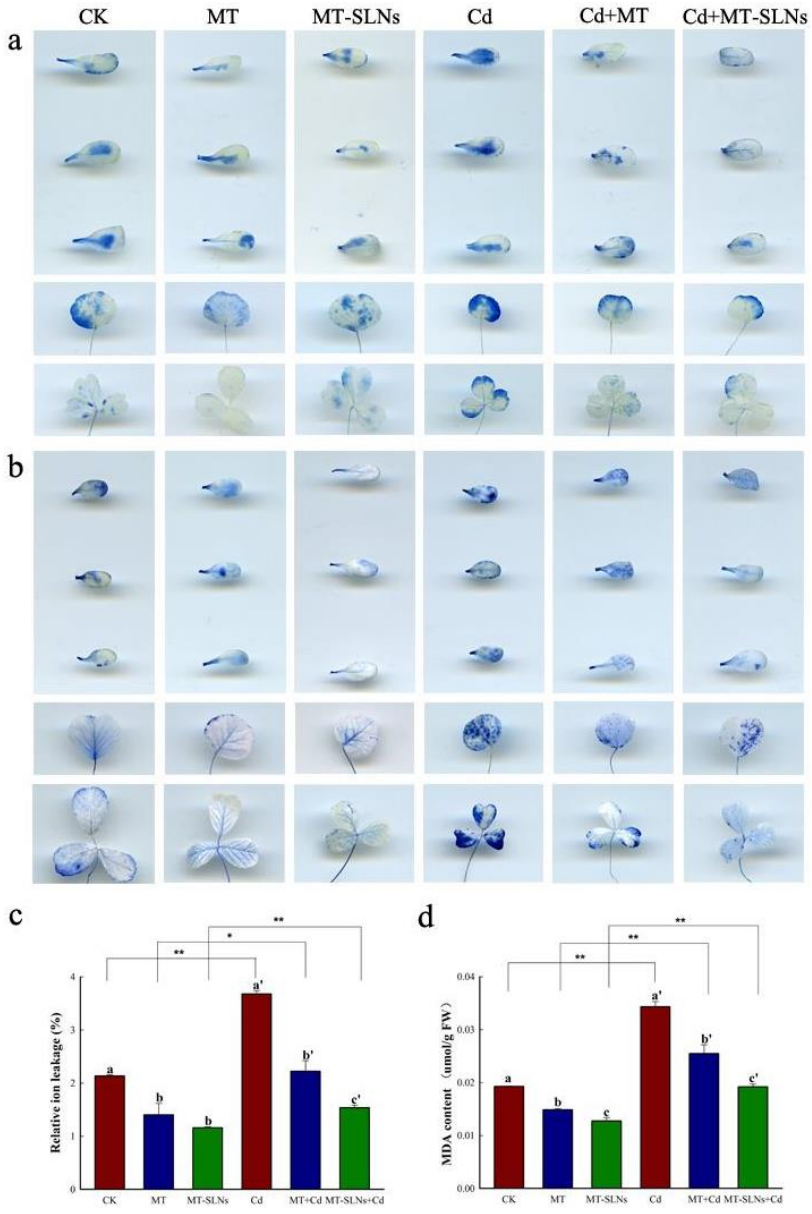

Figure 3. *Analysis of physilogical damage and cell activity of alfalfa seedlings. (a) Evans blue staining; (b) Trypan blue staining; (c) Relative ion leakage; (d) MDA content. *a, b, c showed significant differences among the three negative control groups (P<0.05); a', b', c' indicated significant differences among the three treatment groups under cadmium stress (P<0.05); *(P<0.05) and **(P<0.01) means significant differences among the same color histogram*

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Determination of melatonin content in alfalfa seedlings

According to the results in *Fig. 4*, Cd stress induced the synthesis of melatonin in alfalfa leaves, and the content of melatonin was further induced after spraying MT or MT-SLNs (*Fig. 4*). In addition, under normal conditions, MT or MT-SLNs was also beneficial for the accumulation of melatonin. Moreover, the induction effect of MT-SLNs on melatonin synthesis was more obvious.

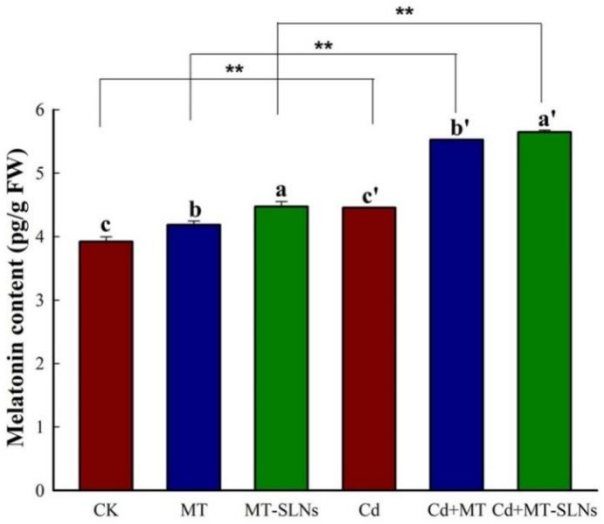

Figure 4. *Melatonin content of alfalfa seedlings. *a, b, c showed significant differences among the three negative control groups (P<0.05); a', b', c' indicated significant differences among the three treatment groups under cadmium stress (P<0.05); **(P<0.01) means significant differences among the same color histogram*

Analysis of reactive oxygen species accumulation in alfalfa seedlings

Through NBT staining and DAB staining (*Fig. 5*), it was found that Cd stress led to a significant increase in $O_2^{\cdot-}$ and H_2O_2 content in the leaves of alfalfa seedlings, indicating excessive accumulation of reactive oxygen species in cells. However, when spraying MT or MT-SLNs at the same time of Cd stress, the content of reactive oxygen species significantly decreased, indicating that the application of MT or MT-SLNs can effectively eliminate the excess ROS in cells. Under normal conditions, spraying MT or MT-SLNs can also reduce the content of ROS. The results of quantitative determination of $O_2^{\cdot-}$ and H_2O_2 in *Figure 5-c and 5-d* were consistent with the results of histological staining.

Analysis of reactive oxygen species scavenging system

Under Cd stress conditions, the ascorbic acid content and POD enzyme activity in alfalfa seedlings remarkably increased, while glutathione synthesis and the activity of the other three antioxidant enzymes (SOD, CAT, and APX) were inhibited (*Fig. 6*). Spraying MT or MT-SLNs at the same time of Cd stress further increased ascorbic acid content and POD enzyme activity, and the inhibitory effect of Cd stress on glutathione synthesis and other three antioxidant enzyme activities were significantly alleviated. Moreover, the effect of MT-SLNs was more pronounced. In addition, under normal conditions, MT or MT-SLNs can also activate the synthesis of two antioxidants and the catalytic activity of four antioxidant enzymes in certain extent. It can be seen that the antioxidant defense

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system (including ascorbic acid, AsA; glutathione, GSH; superoxide dismutase, SOD; guaiacol peroxidase, POD; catalase, CAT; ascorbate peroxidase, APX) plays an important role in promoting the resistance of alfalfa seedlings to cadmium stress by MT and MT-SLNs.

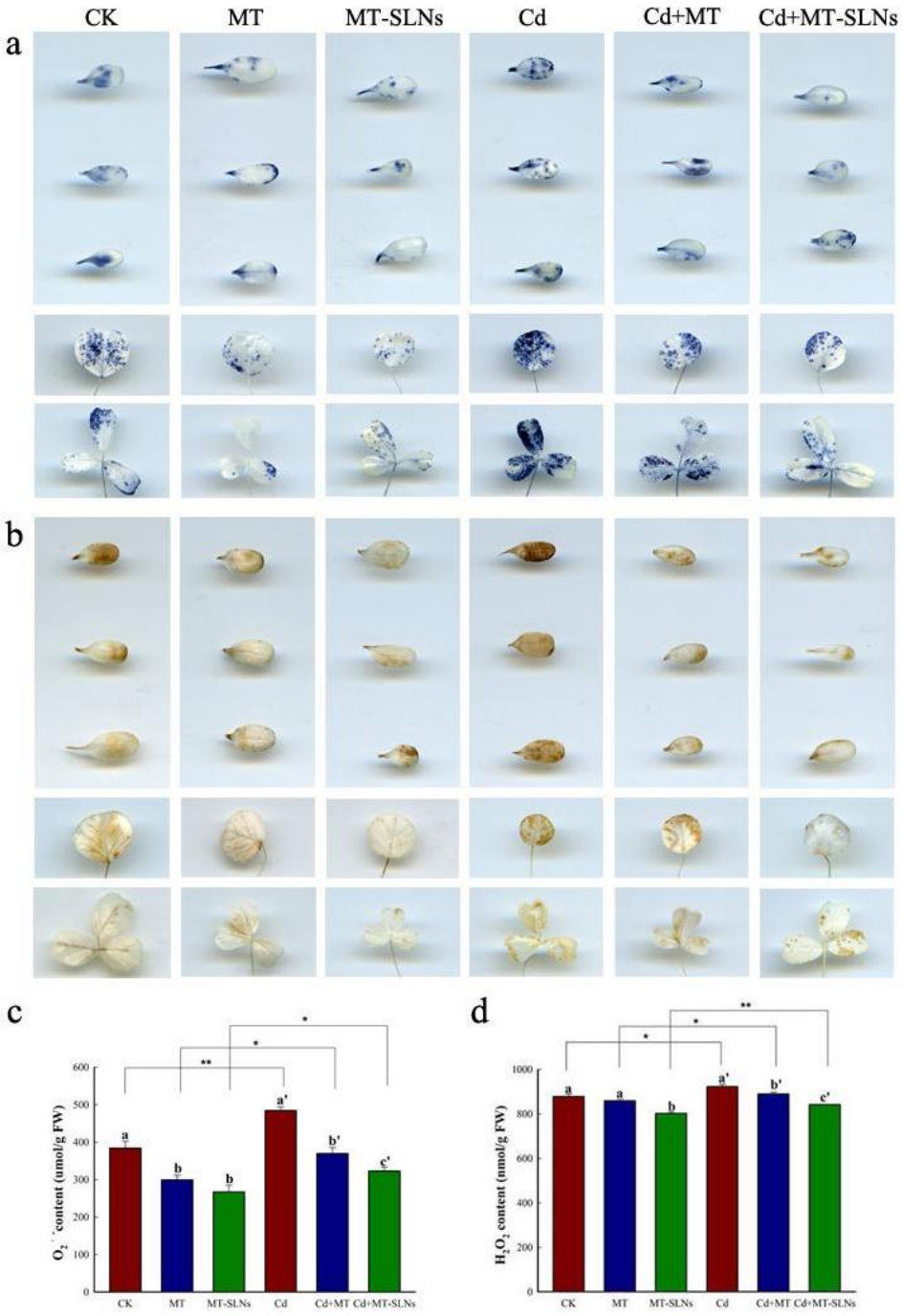

Figure 5. *Detection of reactive oxygen species in alfalfa leaves. (a) NBT staining; (b) DAB staining; (c) Superoxide anion content; (d) Hydrogen peroxide content. *a, b, c showed significant differences among the three negative control groups (P<0.05); a', b', c' indicated significant differences among the three treatment groups under cadmium stress (P<0.05); *(P<0.05) and **(P<0.01) means significant differences among the same color histogram*

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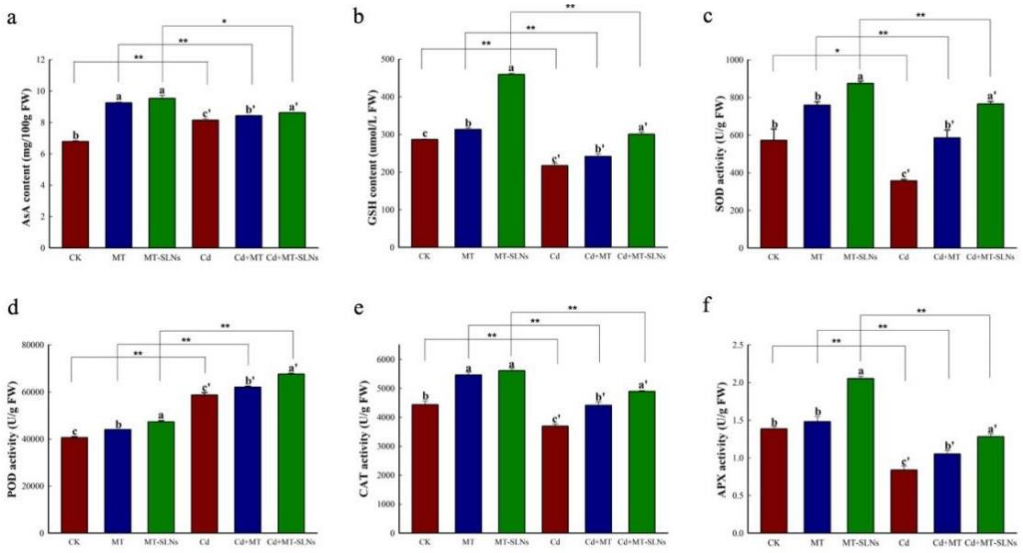

Figure 6. *Content of antioxidants and activities of antioxidant enzymes in alfalfa seedlings. (a) AsA content; (b) GSH content; (c) SOD activity; (d) POD activity; (e) CAT activity; (f) APX activity. *a, b, c showed significant differences among the three negative control groups (P< 0.05); a', b', c' indicated significant differences among the three treatment groups under cadmium stress (P<0.05); *(P<0.05) and **(P<0.01) means significant differences among the same color histogram*

We further detected the expression activities of nine antioxidant enzyme genes and found that Cd stress could upregulate the expression of *POD1A*, *POD1B*, *POD1C*, and *POD2* genes in the leaves of alfalfa seedlings, but downregulated *Cu/Zn-SOD*, *Fe-SOD*, *Mn-SOD*, *CAT*, and *APX* genes expression (*Fig. 7*). After spraying MT or MT-SLNs at the same time of cadmium stress, the expression of nine genes were all significantly upregulated, and MT-SLNs had better induction effects on gene expression. Under normal conditions, MT or MT-SLNs treatment can also activate the expression of these nine genes. So, the application of MT or MT-SLNs activated the expression of antioxidant enzyme genes, thus enhanced the catalytic activity of the four antioxidant enzymes.

Analysis of cadmium accumulation and cadmium stress-related proteins

Compared with control group, the content of free cadmium, chelating peptide, and ABC transporter in alfalfa seedlings remarkably increased and the expression of *MsABCG1* gene was greatly upregulated by Cd stress (*Fig. 8*). However, spraying MT or MT-SLNs simultaneously with Cd stress resulted in a decrease in cadmium and chelating peptide content, while ABC transporter content and *MsABCG1* gene expression further increased. Spraying MT or MT-LNs under normal conditions can also lead to a significant decrease in cadmium content and chelating peptide content, as well as an increase in ABC transporter content and *MsABCG1* gene expression. Therefore, we believed that MT or MT-SLNs treatment decreases Cd accumulation by accelerating the binding of chelating peptides with free Cd ions and mediates Cd efflux via inducing *MsABCG1* expression and biosynthesis of ABC transporter. Thereby the accumulation of free Cd in plant cells was reduced and the toxic effect of cadmium on plant cells was alleviated.

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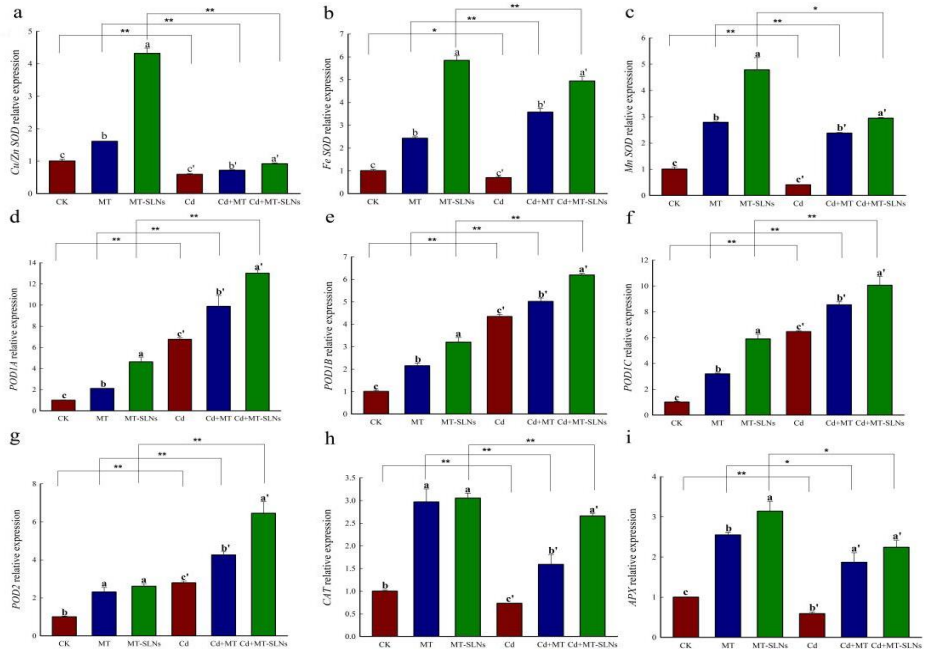

Figure 7. *Gene expression of antioxidant enzyme isozyme in alfalfa seedling leaves. (a) Relative expression of Cu/Zn-SOD gene; (b) Relative expression of Fe-SOD gene; (c) Relative expression of Mn-SOD gene; (d) Relative expression of POD1A gene; (e) Relative expression of POD1B gene; (f) Relative expression of POD1C gene; (g) Relative expression of POD2 gene; (h) Relative expression of CAT gene; (i) Relative expression of APX gene. *a, b, c showed significant differences among the three negative control groups (P<0.05); a', b', c' indicated significant differences among the three treatment groups under cadmium stress (P<0.05); *(P<0.05) and **(P<0.01) means significant differences among the same color histogram*

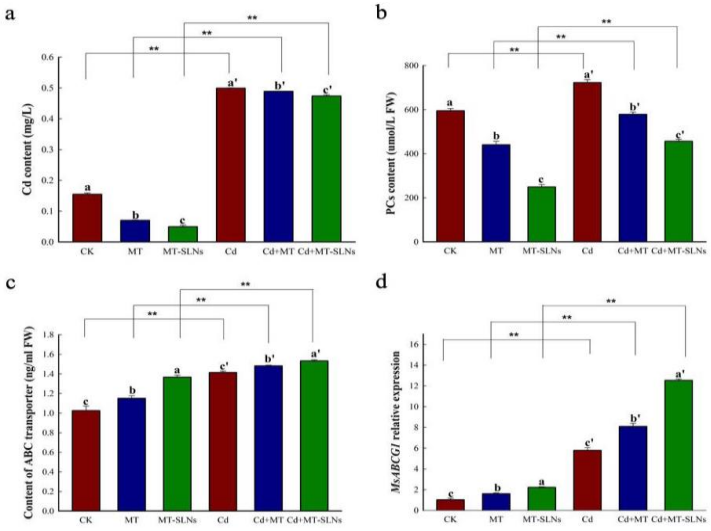

Figure 8. *Changes of cadmium metabolism in alfalfa seedlings. (a) Cd content; (b)PCs content; (c) Content of ABC transporter; (d) Relative expression of MsABCG1 gene. *a, b, c showed significant differences among the three negative control groups (P<0.05); a', b', c' indicated significant differences among the three treatment groups under cadmium stress (P<0.05); **(P<0.01) means significant differences among the same color histogram*

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Discussion

Cadmium is one of the common heavy metals with strong toxicity to plants, and Cd^{2+} can enter the plant body through the transportation of other essential elements such as Ca^{2+} and Fe^{2+}, to cause toxic effects on plant cells. When cadmium accumulates to a certain extent in the plant, plant growth and development will be significantly inhibited or even killed (Hu et al., 2022; Qu et al., 2022; Zhang et al., 2015). In this study, the protective effect and possible mechanism of melatonin liposome nanoparticles treatment on cadmium stressed alfalfa were explored by selecting 100 μmol/L $CdCl_2$ solution as the critical stress concentration.

Cadmium stress induces the production of excessive reactive oxygen species in plants, leading to cell membrane lipid peroxidation, which seriously affects plant growth and development (Qu et al., 2018; Wang et al., 2023). Therefore, in order to resist the influence of adverse environmental factors, plants have evolved a series of completed antioxidant metabolic system to eliminate reactive oxygen species, mainly including enzymatic and non-enzymatic systems (Kang et al., 2021). In the enzymatic system, SOD mainly scavenges excessive $O_2^{\cdot-}$ in plants by converting superoxide anion ($O_2^{\cdot-}$) into H_2O_2, while POD, CAT, and APX are mainly responsible for scavenging H_2O_2 by generating water and oxygen. Thus, ROS levels in plant cells are reduced and the redox homeostasis are maintained (Zhou et al., 2020; Wang et al., 2021; Bela et al., 2022; Zhou and Xie, 2023). This study showed that $CdCl_2$ stress increased MDA content, ion leakage rate, and ROS levels in alfalfa seedlings, indicating that cadmium stress caused oxidative damage to plants. Under cadmium stress conditions, POD enzyme activity of alfalfa seedlings was enhanced, while the activities of SOD, CAT, and APX were inhibited, which was consistent with the research results of Yang (Yang, 2022). The application with MT or MT-SLNs at the same time of cadmium stress, four enzyme activities were all significantly induced, which effectively alleviated cadmium stress damages. In addition, after cadmium stress, the content of ascorbic acid in alfalfa seedlings increased, but the synthesis of glutathione was inhibited. We speculated that alfalfa mainly relied on ascorbic acid to scavenge ROS under cadmium stress, while the chelating ability of GSH was higher than its antioxidant capacity (Jana et al., 2021). These results indicated that the reactive oxygen species scavenging system played an important role in alleviating cadmium stress damages by MT or MT-SLNs, and the protective effect of MT-SLNs was more significant.

Another primary mechanism for plants to deal with heavy metal stress is to synthesize chelating peptides (PCs) for reducing their concentration, and thus the toxic effects on plant cells are alleviated (Quan et al., 2022). Many researches have shown (Quan et al., 2022; Zhang et al., 2023) that GSH, as a precursor for the synthesis of PCs, plays a crucial role in plant response to heavy metal stress. PCs can form complexes with Cd^{2+} in plants, and transport them to vacuoles through cadmium transporter, forming compartmentalization (Vangronsveld et al., 2012; Li et al., 2019; Foyer et al., 2001). Our study found that the content of plant chelating peptides and ABC transporter increased, and the expression of *MsABCG1* gene was upregulated when $CdCl_2$ stress. After spraying MT or MT-SLNs, the content of plant chelating peptides decreased. We speculated that MT or MT-SLNs might promote the binding of PCs with free cadmium, leading to a decrease in PCs. In addition, MT or MT-SLNs induced further increases in the content of ABC transporter and the expression of *MsABCG1* gene. These results suggested that ABC transporter and its coding genes played an important role in reducing cadmium accumulation in plant cells, and the increase of their expression greatly improved the

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efficiency of cadmium efflux. Moreover, spraying MT-SLNs showed a better protective effect on plant cadmium stress. Fu (2019) also reported in her research that *OsABCG36* participated in the Cd tolerance by efflux of Cd or Cd complexes from rice root cells, which was consistent with the results of our study. In summary, our research indicated that the application of MT or MT-SLNs can alleviate the inhibitory effect of cadmium stress on plant growth and development by activating the reactive oxygen species scavenging system and promoting the occurrence of cellular cadmium efflux.

Conclusion

In this study, it was found that $CdCl_2$ stress inhibited the growth of alfalfa seedlings and decreased cell viability, resulting in significant cadmium stress damages. Spraying MT or MT-SLNs at the same time of cadmium stress significantly increased leaf cell activity, induced antioxidant synthesis and antioxidant enzyme activity, and decreased ROS level and free Cd accumulation in tissue. It was indicated that exogenous MT or MT-SLNs could effectively alleviate the inhibitory effect of $CdCl_2$ stress on alfalfa, and the protective effect of spraying MT-SLNs on seedling cadmium stress was more significant. In addition, spraying MT or MT-SLNs under normal growth conditions was also beneficial to the growth and development of plant seedlings. At the same time, this study shows that alfalfa is an excellent plant for repairing soil pollution caused by heavy metals.

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