Physiological Response Mechanisms of Rice to Cadmium, Manganese, and Their Combined Stress

Zhenqing LIU[1], Dongli LIU[1], Chenzhong JIN[2], Jing ZHOU[1,2]*

1. College of Agriculture and Biotechnology, Hunan University of Humanities, Science and Technology, Loudi 417000, China; 2. Key Laboratory of Green Control of Crop Pests in Hunan Higher Education, Loudi 417000, China

Abstract [**Objectives**] This study was conducted to comprehensively elucidate the antioxidant response mechanisms of rice to cadmium (Cd) and manganese (Mn) stress and the interactive effects of their combined stress. [**Methods**] This study systematically investigated the effects of individual and combined Cd and Mn stress on oxidative damage indicators, activities of major antioxidant enzymes (SOD, POD, CAT, APX), expression of key antioxidant and detoxification genes (*e.g.*, SOD, POD, CAT, GSH1, GST), and contents of osmoregulatory substances (soluble sugars, soluble proteins, proline) in rice leaves. [**Results**] (1) Under single or combined stress of Cd and Mn, the accumulation of reactive oxygen species (ROS) in rice leaves exhibited significant dose-dependence and synergistic effects. (2) Under the Mn500 treatment, the activities of SOD and CAT increased by 7.0% and 19.8%, respectively. Under the Cd5 stress treatment, the activities of POD and APX increased by 22.1% and 55.0%, respectively. The combined stress with low-concentration Mn and Cd significantly increased the activities of SOD and CAT in rice leaves, rising by 18.9% and 45.1%, respectively, compared with single Cd stress. The expression levels of genes encoding CAT and SOD were also significantly upregulated. The combined stress with low-concentration Mn and Cd (Mn500 + Cd) significantly upregulated the expression level of glutathione S-transferase (GST). Conversely, under high-concentration Mn and Cd combined stress (Mn3000 + Cd20), the expression of glutathione synthesis-related genes was significantly inhibited. (3) Under Cd5 stress, the contents of soluble sugars, soluble proteins, and proline in rice leaves increased by 14.2%, 11.5%, and 90.0%, respectively. However, when stress intensity increased (*e.g.*, under Cd20), leaf soluble protein content decreased significantly. Therefore, it was concluded that high-concentration single stress of either Cd or Mn induced massive accumulation of reactive oxygen species (H_2O_2) and membrane lipid peroxidation (elevated MDA), thereby damaging cell membrane integrity. Under combined stress, high-concentration Mn and Cd synergistically exacerbated oxidative damage, with H_2O_2 and MDA accumulation levels significantly higher than those under individual treatments. The response of the antioxidant system exhibited concentration-dependent characteristics. Specifically, low-concentration Mn alleviated Cd-induced oxidative stress by enhancing the activities of SOD and CAT. In contrast, the combined stress with high-concentration Mn and Cd inhibited the activities of antioxidant enzymes and the expression of their encoding genes (such as SOD, CAT, and the glutathione synthase GSH1), significantly compromising cellular defense capacity. [**Conclusions**] This study provides a reference for understanding the physiological responses and coping mechanisms of rice to combined stress from other heavy metals.

Key words Rice; Cadmium; Manganese; Stress induction

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Heavy metal-induced oxidative stress is a core link of phytotoxicity. Both cadmium (Cd) and manganese (Mn) can disrupt the intracellular redox balance in plant cells, leading to a burst of reactive oxygen species (ROS), which, in turn, triggers membrane lipid peroxidation, protein denaturation, and DNA damage. Plants have evolved a complex defense network, comprising enzymatic antioxidant systems and non-enzymatic antioxidant compounds, to counteract oxidative stress. However, the intrinsic regulatory mechanisms by which individual and combined stress of Cd and Mn affect the dynamic equilibrium of reactive oxygen species (ROS) metabolism, the activities of antioxidant enzymes and the expression of their encoding genes, as well as the accumulation of osmoregulatory substances in rice leaves, remain unclear. This study systematically investigated the effects of individual and combined Cd and Mn stress at multiple levels. The parameters examined included oxidative damage indicators, activities of major antioxidant enzymes (SOD, POD, CAT, APX), expression of key antioxidant and detoxification genes (*e.g.*, SOD, CAT, GSH1, GST), and contents of osmoregulatory substances (soluble sugars, soluble proteins, proline) in rice leaves. The aim was to comprehensively elucidate the antioxidant response mechanisms of rice to Cd and Mn stress and the interactive effects of their combined stress.

Materials and Methods

Plant materials

The rice (*Oryza sativa* L.) cultivar used in this experiment was Zhong'an 2, provided by Hunan Jinjian Seed Industry Technology Co., Ltd.

The soil used for the experiment was collected in 2024 from the 0 - 20 cm plow layer in typical double-cropping rice areas in Hunan Province. The soil was air-dried, sieved through a 2 mm mesh, and sterilized prior to use. The basic physicochemical properties of the soil were as follows: pH 6.01 ± 0.2, organic matter (34.49 ± 5.2) g/kg, cation exchange capacity (CEC) (21.32 ± 3.6) cmol(+)/kg, available N (142.38 ± 28.32) mg/kg, available P (9.83 ± 3.2) mg/kg, available K (116.35 ± 18.95) mg/kg, available Mn 39.87 mg/kg, and Cd content 0.21 mg/kg.

Experimental design

Rice seeds were surface-sterilized with a 1% sodium

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Zhenqing LIU (1995 -), male, P. R. China, master, devoted to research about resource utilization.

* Corresponding author. Jing ZHOU (1983 -), female, associate professor, devoted to research about physiological ecology of rice with high yield and good quality.

hypochlorite (NaClO) solution for 15 min, followed by soaking in distilled water for 12 h. The seeds were then germinated at 35 ℃ for 24 h and subsequently sown in a moistened mixture of perlite and vermiculite (1 ∶ 1, *v*/*v*). The seedlings were irrigated with half-strength Hoagland nutrient solution via drip irrigation. Upon reaching the three-leaf and one-heart stage, uniform and healthy seedlings (free from pests and diseases) were selected and transplanted into 8 L black plastic buckets. Each bucket contained 5 hills, with 3 seedlings per hill. Based on previous studies, 9 treatments were established in triplicate (Table 1). Soil added with heavy metals was thoroughly mixed in plastic pots and equilibrated for 2 weeks before rice seedling transplantation. During the stress period, soil moisture in the pots was maintained at approximately 75% of field capacity. Water loss was regularly replenished using the weighing method, and standard water and fertilizer management for rice cultivation were followed. After 2 weeks of stress treatment, the flag leaves of rice seedlings from each treatment were collected, immediately frozen in liquid nitrogen, and stored at −80 ℃ for subsequent analysis.

Determination methods for indicators

Determination of oxidative stress indicators Malondialdehyde (MDA) content was determined according to the method described by Huang *et al.*[1]. Hydrogen peroxide (H_2O_2) content was measured following the method of Yang *et al.*[2].

Determination of antioxidant enzyme activities The activities of SOD, POD, CAT, and APX were assayed following the method described by Li *et al.*[3].

Determination of osmoregulatory substance contents The contents of soluble sugars, soluble proteins, and free proline were determined according to the method described by Zhang *et al.*[4].

Real-time quantitative PCR (qRT-PCR) analysis of antioxidant-related gene expression Total RNA was extracted using the RNAprep Pure Plant Plus Kit (Tiangen, China) following the manufacturer's instructions. RNA purity was assessed using a NanoDrop 2000 spectrophotometer, with an A_{260}/A_{280} ratio between 1.9 and 2.1. RNA integrity was verified with an Agilent 2100 Bioanalyzer, ensuring an RNA Integrity Number (RIN) ⩾ 7.5. Subsequently, 1 μg of RNA was reverse transcribed into cDNA using HiScript III RT SuperMix (Vazyme) under the following conditions: 42 ℃ for 2 min for genomic DNA removal, followed by 37 ℃ for 15 min for cDNA synthesis.

The qPCR primers were designed using Primer-BLAST, spanning intron regions. Primer specificity was validated by analyzing the melting curve and performing agarose gel electrophoresis, confirming the presence of a single band. Amplification was performed using the ChamQ Universal SYBR qPCR Master Mix (Vazyme) on a Bio-Rad CFX96 system. The thermal cycling protocol consisted of an initial denaturation at 95 ℃ for 30 s, followed by 40 cycles (95 ℃ for 10 s and 60 ℃ for 30 s). The UBQ5 gene was used as the internal reference for normalizing relative expression levels. Specific primer sequences are listed in Table 1. The relative gene expression was calculated using the $^{\Delta\Delta}$Ct method.

Table 1 Primers used for gene expression analysis

Gene ID	Forward primer sequences (5'→3')	Reverse primer sequences (5'→3')
Ubq5	ACCACTTCGACCGCCACTACT	ACGCCTAAGCCTGCTGGTT
LOC_Os07g46990(*OsSOD1*)	CAGCAACCTCGTCCTCAACT	TCCACCACCTTCTCCATGCT
LOC_Os01g73170(*OsPOD*)	GGCTTCGATGCTGTGAAGAT	CAGCCAAACCGGTAGACCTT
LOC_Os02g02400(*OsCAT*)	TGCGGATGTTGTGGAGAAGT	AATGCCAGGTCCGGTTAATC
LOC_Os05g49940(*OsGSH1*)	GCTGGTGCTGTCCTTCATGT	TGGGCAAAGAGGTGAGTGAG
LOC_Os01g72160(*OsGST*)	CCTGCCTTCACCATCCTCTT	GCAGGAAGGCTTCTGCTTCT
LOC_Os03g49510(*OsGR*)	GCAACTGGGACACATCCTCA	ACCGTCGTTGCTGTAGAACG
LOC_Os04g46930(*OsGPX*)	GAGCAAGGAGGAGGAGGTGA	TGTCGTCGAGGTTCACGATA

Data processing

Data were analyzed using GraphPad Prism statistical software (version 11.0). One-way analysis of variance (ANOVA) was conducted to compare differences among treatment groups, followed by the Least Significant Difference (LSD) significance test. Results in all bar graphs are presented as mean ± standard deviation.

Results and Analysis

Effects of Cd, Mn, and their combined stress on ROS accumulation in rice leaves

The hydrogen peroxide (H_2O_2) content in rice leaves was measured (Fig. 1). The results indicated that Mn500 stress did not significantly affect leaf H_2O_2 content. In contrast, Mn3000 stress increased leaf H_2O_2 content by 3.75 times compared with the control (CK). Meanwhile, Cd5 and Cd20 stress treatments increased leaf H_2O_2 content by 1.79 and 5.41 times, respectively, compared with the CK. Furthermore, Mn500 and Cd5 co-treatment resulted in a 13.5% decrease ($P<0.05$) in leaf H_2O_2 content compared with the Cd5 treatment alone. However, the co-treatment with high concentrations of Mn and Cd further increased H_2O_2 accumulation. Specifically, under Mn3000 + Cd20 co-stress, leaf H_2O_2 content increased by 2.41 and 1.69 times compared with the Mn3000 and Cd20 single treatments, respectively. These results demonstrated that high concentrations of Mn and Cd stress individually increased ROS accumulation in rice leaves, and their combined exposure further exacerbated ROS accumulation.

Effects of Cd, Mn and their combined stress on membrane lipid peroxidation in rice leaves

The degree of membrane lipid peroxidation in rice leaves under both individual stress and combined stress of Cd and Mn was

further evaluated (Fig. 2). The results showed that Mn500 stress increased the relative electrical conductivity in leaves by only 12.9%, while it had no significant effect on MDA content, indicating that membrane integrity was maintained under Mn500 treatment. In contrast, Mn3000 stress increased leaf MDA content and relative electrical conductivity by 5.36 and 2.76 times, respectively, compared with the CK. Both Cd5 and Cd20 stress treatments significantly increased leaf MDA content and relative electrical conductivity. Furthermore, combined Cd and Mn stress further elevated both leaf MDA content and relative electrical conductivity. Specifically, under Mn3000 + Cd20 co-stress, leaf MDA content increased by 2.34 and 2.82 times compared with the Mn3000 and Cd20 individual treatments, respectively, and leaf relative electrical conductivity increased by 1.50 and 135 times compared with the Mn3000 and Cd20 individual treatments, respectively.

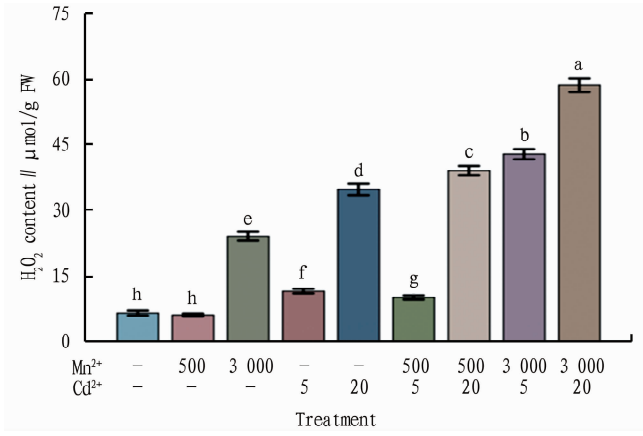

Fig. 1 Effects of cadmium, manganese and their co-contamination stress on H_2O_2 content in rice leaves

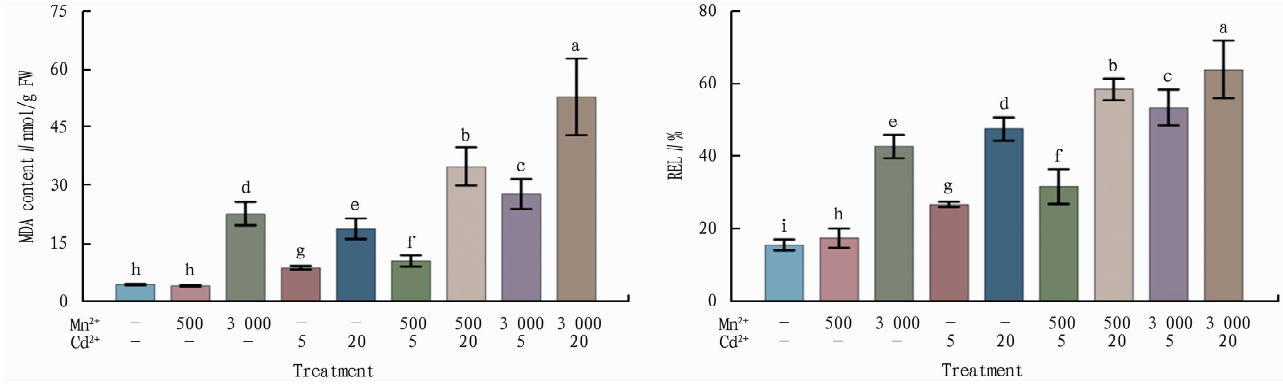

Fig. 2 Effects of Cd, Mn and their co-contamination stress on MDA content and relative electrical conductivity in rice leaves

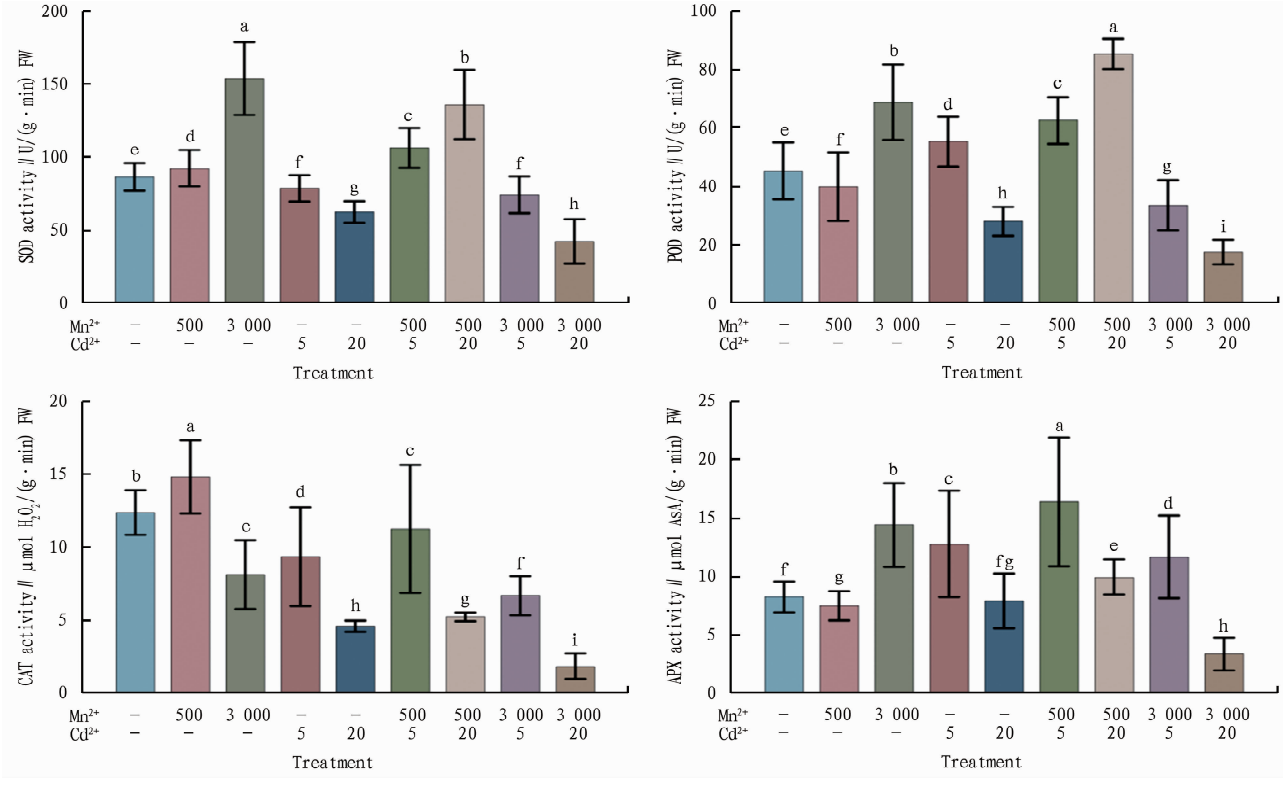

Fig. 3 Effects of cadmium, manganese and their co-contamination stress on antioxidant enzymes activity in rice leaves

Effects of Cd, Mn, and their combined stress on antioxidant enzyme activities in rice leaves

Under environmental stress, plants can scavenge excess reactive oxygen species (ROS) through their antioxidant enzyme systems. Therefore, changes in the activities of several major antioxidant enzymes in rice leaves under individual and combined Cd and Mn stress were further determined. As shown in Fig. 3, the effects of Mn stress treatments on antioxidant enzyme activities in rice leaves exhibited some variations. Specifically, Mn500 treatment increased leaf SOD and CAT activities by 7.0% and 19.8%, respectively, compared with the CK ($P < 0.05$), while it decreased leaf POD and APX activities by 12.2% and 9.1%, respectively. In contrast, Mn3000 treatment increased leaf SOD, POD, and APX activities by 78.3%, 51.9%, and 75.1%, respectively, compared with the CK, but decreased leaf CAT activity by 34.2%. The Cd5 stress treatment decreased leaf SOD and CAT activities by 9.0% and 24.3%, respectively, compared with the CK, while it increased leaf POD and APX activities by 22.1% and 55.0%, respectively. In contrast, Cd20 stress significantly inhibited the activities of leaf SOD, POD, and CAT. The combined stress of Mn500 and Cd increased leaf SOD, POD, CAT, and APX activities by 18.9% – 35.4%, 13.1% – 19.6%, 20.0% – 45.1%, and 28.4% – 47.5%, respectively, compared with Cd stress alone. The combined stress of Mn3000 and Cd5 increased leaf SOD and POD activities by 72.9% and 54.3%, respectively, compared with Cd5 stress alone. However, under the combined stress of Mn3000 and Cd20, leaf SOD, POD, and CAT activities were all significantly lower than those under Cd20 stress alone.

Effects of Cd, Mn, and their combined stress on the contents of osmoregulatory substances in rice leaves

Under stress conditions, soluble sugars, soluble proteins, and free proline effectively maintain cellular osmotic pressure and protect enzymatic proteins. Therefore, changes in the contents of these osmoregulatory substances in rice leaves under individual and combined Cd and Mn stress were determined. As shown in Fig. 4, Mn500 stress treatment only increased the soluble protein content in rice leaves by 23.8% compared with the CK. In contrast, the Mn3000 stress treatment increased leaf soluble sugar and free proline contents by 34.6% and 134.2%, respectively, compared with the CK, while it decreased soluble protein content by 28.7%. The Cd5 stress treatment significantly increased the contents of soluble sugars, soluble proteins, and free proline in rice leaves, rising by 14.2%, 11.5%, and 90%, respectively, compared with the CK. In contrast, the Cd20 stress treatment only increased free proline content by 228.9% compared with the CK, while it decreased soluble sugar and soluble protein contents by 10.0% and 38.8%, respectively.

The effects of combined Mn and Cd stress on the contents of osmoregulatory substances in rice leaves varied considerably. The combined stress of Mn500 and Cd5 increased leaf soluble protein content by 7.5% compared with Cd5 stress alone ($P < 0.05$). The combined stress of Mn500 and Cd20 decreased leaf soluble sugar and free proline contents by 12.3% and 19.7%, respectively, compared with Cd20 stress alone, but it increased soluble protein content by 22.4% compared with the Cd20 treatment. The combined stress with high concentrations of Mn and Cd could significantly increase soluble sugar content in rice leaves while significantly decreasing soluble protein content. However, the combined stress of Mn3000 and Cd5 increased leaf free proline content by 55.1% compared with Cd5 stress alone. In contrast, the combined stress of Mn3000 and Cd20 decreased leaf free proline content by 5.95 times compared with Cd20 stress alone.

Effects of Cd, Mn, and their combined stress on the expression of POD, CAT, and SOD genes in rice leaves

As shown in Fig. 5, Mn, Cd, and their combined stress significantly affected the expression of genes encoding antioxidant enzymes in rice leaves. Under Mn stress alone, the Mn500 treatment increased the expression levels of genes encoding SOD and CAT in rice leaves by 18.8% and 23.8%, respectively, compared with the CK, but decreased the expression of the POD-encoding gene by 8.8%. In contrast, Mn3000 treatment increased the expression levels of genes encoding SOD and POD by 109.5% and 66.3%, respectively, compared with the CK, but decreased the expression of the CAT-encoding gene by 16.3%. Under Cd stress alone, only the Cd5 treatment increased the expression level of the gene encoding POD in rice leaves by 41.3% compared with the CK. Under all other Cd treatments, the expression levels of genes encoding SOD, POD, and CAT were significantly lower than the CK. Low-concentration Mn stress effectively alleviated the inhibitory effect of Cd stress on the expression of genes encoding SOD, POD, and CAT. Specifically, after the combined stress of Mn500 and Cd5, the expression levels of genes encoding SOD, POD, and CAT increased by 68.6%, 17.7%, and 29.8%, respectively, compared with Cd5 stress alone. Similarly, after the combined stress of Mn500 and Cd20, the expression levels of these genes increased by 45.6%, 22.2%, and 34.1%, respectively, compared with Cd20 stress alone. High-concentration Mn stress effectively alleviated the inhibitory effect of low-concentration Cd stress on the expression of genes encoding SOD and POD. Specifically, after Mn3000 + Cd5 combined stress, the expression levels of genes encoding SOD and POD in rice leaves increased by 117.3% and 44.2%, respectively, compared with Cd5 stress alone. However, high-concentration Mn stress exacerbated the inhibitory effect of high-concentration Cd stress on the expression of genes encoding SOD, POD, and CAT. For example, after the combined stress of Mn3000 and Cd20, the expression levels of genes encoding SOD, POD, and CAT in rice leaves decreased by 35.4%, 36.9%, and 44.0%, respectively, compared with Cd20 stress alone.

Effects of Cd, Mn, and their combined stress on the expression of glutathione and antioxidant-related genes in rice leaves

Glutathione (GSH) is a crucial antioxidant molecule involved in heavy metal detoxification. Key enzymes in this process include glutathione synthetase (GSH1), glutathione S-transferase (GST), glutathione reductase (GR), and glutathione peroxidase

(GPX). Therefore, this study further analyzed the effects of Mn, Cd, and their combined stress on the expression levels of these glutathione-related antioxidant and detoxification genes, such as GSH1, GST, GR, and GPX. As shown in Fig. 3 – Fig. 6, under single Mn stress, the Mn500 treatment increased the expression levels of GSH1, GST, GR, and GPX in rice leaves by 44.1%, 29.5%, 19.5%, and 8.7%, respectively, compared with the CK. In contrast, Mn3000 treatment increased the expression levels of GST and GPX by 109.5% and 58.8%, respectively, but decreased the expression of GSH1 and GR by 23.6% and 33.8%, compared with the CK. Under single Cd stress, the Cd5 treatment significantly increased the expression levels of GSH1, GST, GR, and GPX compared with the CK. In contrast, the Cd20 treatment significantly decreased the expression levels of all four genes (GSH1, GST, GR, and GPX) compared with the CK.

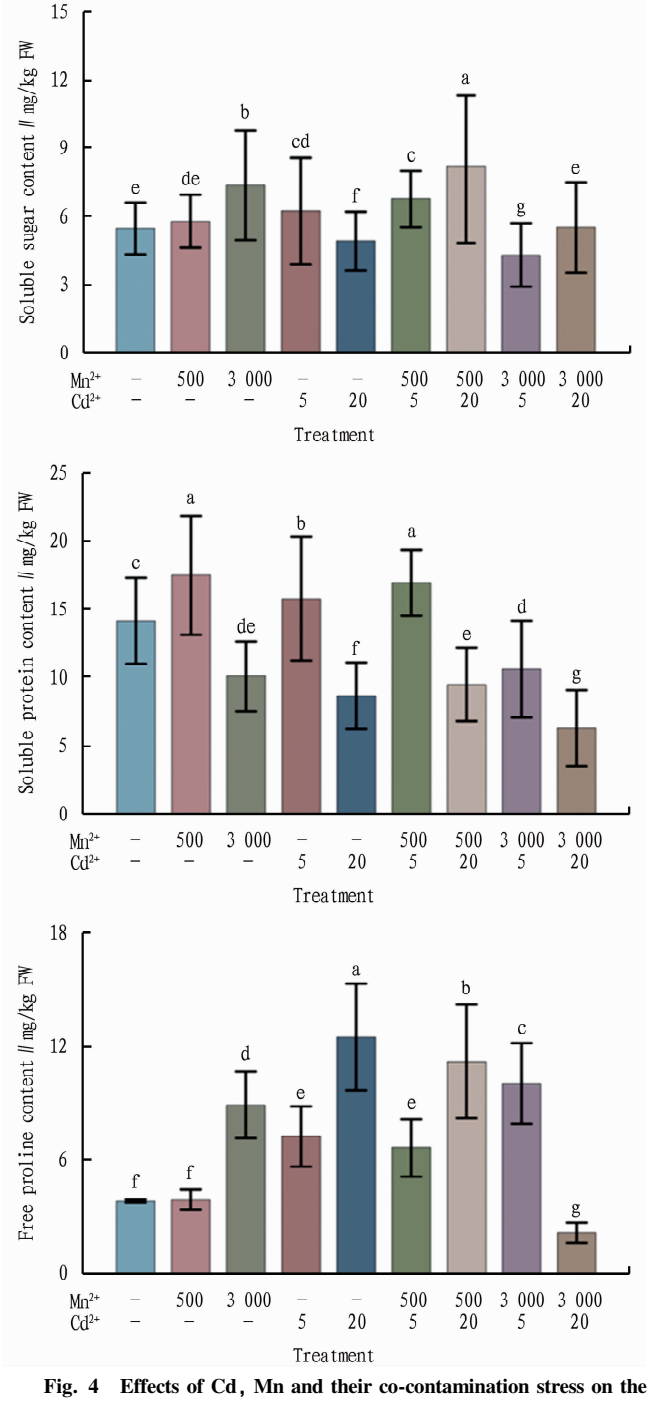

Fig. 4 Effects of Cd, Mn and their co-contamination stress on the contents of osmoregulatory substances in rice leaves

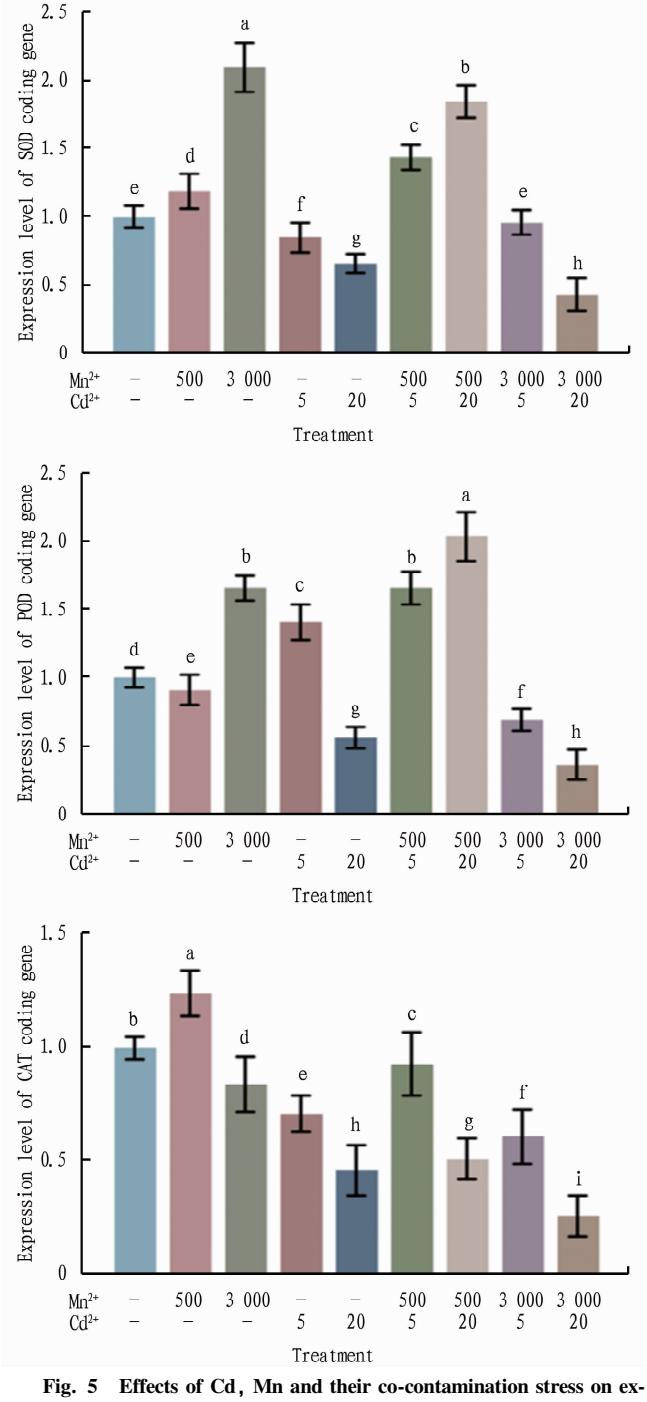

Fig. 5 Effects of Cd, Mn and their co-contamination stress on expression levels of SOD, POD, and CAT coding genes in rice leaves

After the combined stress of Mn500 and Cd5, the expression levels of GSH1, GST, GR, and GPX in rice leaves increased by 11.8%, 19.0%, 15.4%, and 20.1%, respectively, compared with Cd5 stress alone. In contrast, after the combined stress of Mn500 and Cd20, only the GST expression level increased by 32.4% compared with Cd20 stress alone, while the expression levels of GSH1, GR, and GPX were significantly lower than the Cd20 treatment. Furthermore, under the combined stress of high-concentration Mn and Cd, except for the treatment of Mn3000 and Cd5, which increased the expression levels of GST and GPX by 35.2% and 26.8%, respectively, compared with Cd5 stress alone, all other combined stress treatments significantly reduced the expression levels of GSH1, GST, GR, and GPX compared with the corresponding single Cd stress treatments.

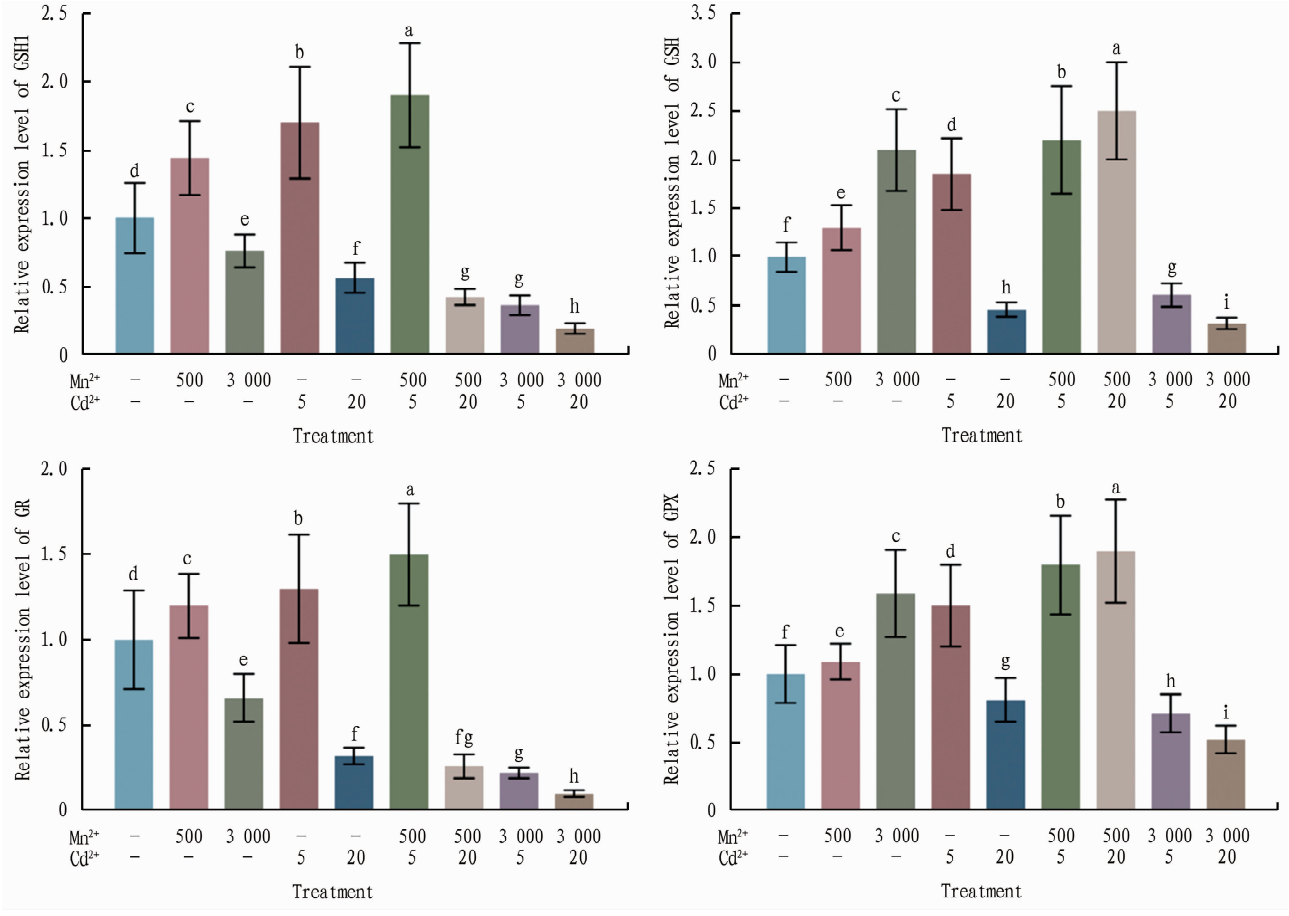

Fig. 6 Effects of Cd, Mn and their co-contamination stress on expression levels of glutathione antioxidant and reduction-related genes in rice leaves

Conclusions and Discussion

Oxidative damage and membrane system damage induced by Cd and Mn stress

In the physiological and biochemical responses of plants to heavy metal stress, the dynamic balance between oxidative stress and the antioxidant defense system is a key determinant of plant survival and adaptability[5-7]. The results of this study demonstrated that under single or combined stress of Cd and Mn, the accumulation of reactive oxygen species (ROS) in rice leaves exhibited a significant dose-dependent and synergistic effect. High-concentration single stress treatments (*e.g.*, Mn3000 and Cd20) significantly increased the hydrogen peroxide (H_2O_2) content in rice leaves. Under combined stress of the two heavy metals, H_2O_2 accumulation was further enhanced. Specifically, under the combined stress of Mn3000 and Cd20, H_2O_2 content increased by 2.41 and 1.69 times compared with the corresponding single Cd (Cd20) and single Mn (Mn3000) stress treatments, respectively. This phenomenon might stem from two mechanisms. On the one hand, the coexistence of Cd^{2+} and Mn^{2+} may interfere with electron transfer in both the mitochondrial electron transport chain and the photosynthetic electron transport chain, leading to increased leakage of superoxide anions ($O_2 \cdot^-$)[8-9], which are subsequently converted to H_2O_2 via SOD catalysis. On the other hand, high concentrations of metal ions may generate the more destructive hydroxyl radical ($\cdot OH$) through a Fenton-like reaction ($Fe^{2+} + H_2O_2 \rightarrow \cdot OH + OH^-$)[10]. Under specific conditions, Mn^{2+} can partially substitute for Fe^{2+} in participating in this reaction[11]. Furthermore, the results of this study revealed that low-concentration Mn exhibited a certain mitigating effect on Cd toxicity. Under the combined stress of Mn500 and Cd5, leaf H_2O_2

content decreased by 13.5% compared with single Cd stress (Cd5). This finding suggests that an appropriate amount of Mn may bind to free Cd^{2+} [12] through chelation, thus reducing its active sites involved in redox reaction, and further reducing the production of ROS[13].

The massive accumulation of reactive oxygen species (ROS) not only disrupts cellular homeostasis, but also directly targets membrane systems, triggering lipid peroxidation and ultimately compromising cellular integrity[14-15]. MDA, a symbolic product of lipid peroxidation, serves as an indicator of oxidative damage to cell membrane system, with its content change reflecting the extent of membrane system impairment[16]. The results of this study demonstrated that under the combined stress of Mn3000 and Cd20, the MDA content in rice leaves increased by 2.82 and 2.34 times compared with the corresponding single Cd (Cd20) and single Mn (Mn3000) stress treatments, respectively, accompanied by a significant rise in leaf relative electrolyte leakage. It indicates that high-concentration heavy metal combined stress oxidizes polyunsaturated fatty acids in membrane phospholipids, disrupting the structural stability of the plasma membrane and organelle membranes, thereby leading to intracellular ion leakage and cellular dysfunction[17]. In contrast, low-concentration Mn (Mn500) stress had a relatively minor impact on the membrane system of rice leaves. This finding aligns with studies by Wang *et al.* [18] and Zhao *et al.* [19] on rice and wheat, which suggests that low-concentration Mn has the potential to mitigate heavy metal toxicity under specific conditions.

The coordinated response mechanism of the antioxidant defense network

To cope with oxidative pressure caused by ROS accumulation, plants have evolved a complex antioxidant system, in which antioxidant enzymes play a crucial role in scavenging ROS and maintaining redox homeostasis[20-21]. The effects of Mn and Cd stress treatments at different concentrations on the antioxidant enzyme system in rice leaves exhibited significant concentration dependence and regulatory complexity. The results showed that under the low-concentration Mn500 treatment, the activities of SOD and CAT increased by 7.0% and 19.8%, respectively. It might be attributed to Mn acting as a cofactor for Mn-SOD, directly promoting the disproportionation of superoxide anions, thereby effectively reducing subsequent H_2O_2 production[22-23]. Under the Cd5 stress treatment, the activities of peroxidase (POD) and ascorbate peroxidase (APX) increased by 22.1% and 55.0%, respectively. This is consistent with the typical response pattern in which plants activate the ascorbate-glutathione (ASA-GSH) cycle to scavenge H_2O_2 under Cd stress[24-25]. However, under combined Cd and Mn stress, the regulation of the antioxidant enzyme system exhibited a more complex pattern. The combined stress with low-concentration Mn and Cd significantly increased the activities of SOD and CAT in rice leaves, rising by 18.9% and 45.1%, respectively, compared with the corresponding single Cd stress treatment. The expression levels of genes encoding CAT and SOD were also significantly upregulated. This suggests that an appropriate amount of Mn may alleviate Cd-induced inhibition of key antioxidant enzyme-encoding genes at the transcriptional level, thereby enhancing the plant's antioxidant capacity[26]. In contrast, under high-concentration combined stress (Mn3000 + Cd20), the activities of both SOD and CAT were significantly inhibited, which might be related to the structural damage caused by Cd to enzyme proteins. Studies have shown that Cd^{2+} can oxidize the thiol (−SH) groups at the catalytic centers of enzyme proteins[27-28], while Mn^{2+} may compete for enzyme protein binding sites[29], further exacerbating the decline in enzyme activity.

Furthermore, the role of glutathione (GSH) in the response to heavy metal stress was also investigated in this study. The results showed that the combined stress with low-concentration Mn and Cd (Mn500 + Cd) significantly upregulated the expression level of glutathione S-transferase (GST), indicating that the GSH pathway may play an active role in heavy metal detoxification. As an important non-enzymatic antioxidant within cells, GSH can not only directly scavenge reactive oxygen species (ROS)[30], but may also reduce heavy metal toxicity by participating in the chelation and transport processes of heavy metal ions[31-32]. However, under high-concentration combined stress (Mn3000 + Cd20), the expression of glutathione synthesis-related genes (such as GSH1) was significantly inhibited, which might impede GSH synthesis, and further affect the overall function of the cellular antioxidant system[33].

Metabolism of osmoregulatory substances and homeostasis imbalance

In response to heavy metal stress, plants also maintain intracellular environmental stability by regulating the metabolism of osmoregulatory substances[34-35]. The results of this study showed that under Cd5 stress, the contents of soluble sugars, soluble proteins, and proline in rice leaves increased by 14.2%, 11.5%, and 90.0%, respectively. Among these, proline serves not only as a crucial osmoregulatory substance, helping to maintain cell turgor pressure, but also plays a role in scavenging hydroxyl radicals[36-37]. Soluble proteins contribute to stabilizing membrane structures and preventing protein denaturation. This regulatory mechanism reflects the adaptive response of plants under stress conditions to some extent. However, when stress intensity increased (*e.g.*, under Cd20), leaf soluble protein content decreased significantly, which might be related to the inhibition of ribosomal function by heavy metals, leading to the obstruction of protein synthesis[38]. Under such conditions, plants may prioritize allocating limited energy resources to repair oxidative damage rather than maintaining protein synthesis. Under the combined stress of Mn3000 and Cd20, the proline content in rice leaves decreased by 5.95 times compared with single Cd treatment, indicating that the synergistic toxicity of the heavy metals exceeded the plant's metabolic regulatory capacity, leading to the collapse of osmotic protection mechanism. Under such conditions, plants may enter a "survival mode", in which limited resources are reallocated to

sustain core metabolic processes, such as respiration, to ensure the continuation of essential life activities.

Moreover, in this study, it was found that low-concentration Mn alleviated the osmotic metabolic stress caused by Cd stress to some extent. For example, the combined stress of Mn500 and Cd5 significantly increased the proline content in rice leaves. It suggests that an appropriate amount of Mn may enhance plant stress tolerance by upregulating the expression of proline synthesis genes, thereby promoting the synthesis of osmoregulatory substances. However, under the combined stress of Mn3000 and Cd20, although proline content increased by 55.1% compared with single Cd stress, soluble sugar content decreased by 12.3%, which might be because plants may reduce sugar metabolism to maintain the nitrogen-intensive osmotic protection mechanism under limited resource availability. This finding reflects the physiological adaptation and resource optimization strategies of plants under adverse conditions to some extent.

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(Continued from page 7)

mechanisms of HC is not yet fully understood. The research group is committed to continuing investigations to explore the effects of HC-NPs on a broader range of cancers and to uncover the underlying mechanisms of its anti-cancer activity.

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