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Hydrogen sulfide promotes copper nanoparticles tolerance in rice by maintaining oxidative metabolism, cell morphology and gene expression

Quanxiang Tian¹, Mengyuan Dong¹, Yuchen Ping¹, Shuang Li¹, Yongbo Li¹, Tongyuan Yu¹, Jue Lu¹, Yunxia Fang¹, Xiaoqin Zhang¹, Zhiquan Liu², Xiaoguang Chen^{1*} and Dawei Xue^{1*}

Abstract

Background Copper oxide nanoparticles (nCuO) are widely used in electronics, energy storage, biomedicine, and various other fields. However, few studies have investigated their impact on the environment and crop growth.

Results Here, we employs rice as a model organism to investigate the effects of nCuO on rice growth at the physiological, biochemical, cellular, and molecular levels, and CuSO₄ used as the control. The results indicated that treatment with nCuO and CuSO₄ significantly decreased rice plant height, fresh weight, and cell length. Furthermore, transcriptome analysis, RT-qPCR, and the external application of sodium hydrosulfide (NaHS) demonstrated that hydrogen sulfide (H₂S) enhanced the antioxidant capacity of rice by regulating the expressions of heavy metal ion transporter *OsIRT2*, metallothionein *OsMT2a*, and other related genes, and reduced the accumulation of Cu ions in plant, thus, improving the resistance of rice to nCuO and CuSO₄.

Conclusion This study revealed that nCuO caused damage to plants similar to free copper ions and exogenous H₂S could improve rice resistance to nanomaterial and heavy metal stress at the physiological and molecular levels, offering a theoretical basis and reference for improving rice stress resistance and quality.

Keywords Copper oxide nanoparticles, Copper sulfate, Hydrogen sulfide, Rice

Introduction

Copper oxide nanoparticles (nCuO) are widely utilized engineered nanoparticles due to their unique physico-chemical properties, finding applications across various fields such as water treatment, cosmetics, medical treatments, vehicle catalysts, coatings, electronics, and agriculture [1, 2]. However, the extensive production and application of nCuO have raised concerns regarding their environmental impact, particularly their toxicity to plants, which are foundational to the food chain and cannot escape exposure. When plants are exposed to nCuO, they undergo stress that results in the overproduction of

*Correspondence:

Xiaoguang Chen
xg.chen@hznu.edu.cn
Dawei Xue
dwxue@hznu.edu.cn

¹College of Life and Environmental Sciences, Hangzhou Normal University, Hangzhou 311121, China

²College of Engineering, Hangzhou Normal University, Hangzhou 310018, China



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reactive oxygen species (ROS), thereby hindering normal growth and development [3].

The toxicity of nCuO is related to size, nanoparticles with diameters ranging from 1 to 100 nm can produce varying toxic effects. Larger particles tend to adsorb onto the surface of plant roots, while smaller particles can penetrate plant cells, releasing Cu^{2+} that induce ionic toxicity [4–6]. CuSO_4 is a soluble copper salt that can provide free copper ions, which is used to simulate the influence of dissolved copper in nCuO. In recent years, there has been a growing focus on the environmental implications of the increasing presence of engineered nanoparticles (ENPs) in agricultural soils [7]. Copper, which exists in the forms of Cu^+ and Cu^{2+} , is essential for various plant functions, including photosynthesis, oxidative stress defense, and hormone signaling. However, excessive copper can lead to non-target effects, resulting in cellular damage [8–10].

Hydrogen sulfide (H_2S) is a gaseous signaling molecule in plants that plays a regulatory role in various growth and developmental pathways, including seed germination and stomatal closure [11]. Research has shown that the application of H_2S can enhance drought resistance in *Arabidopsis* by modulating the expression of genes related to drought tolerance [12]. Furthermore, H_2S alleviates zinc (Zn) ion stress by enhancing metallothionein activity, regulating the expression of antioxidant enzyme genes to chelate excess zinc, and reducing Zn-induced oxidative stress [13]. Additionally, H_2S promotes the expression of ZRT and IRT genes, which are involved in Zn uptake, while inhibiting the synthesis of ZIP family transporter proteins, thereby reducing Zn uptake in plants [14].

Moreover, H_2S can alleviate various types of stress in plants by modulating enzyme activities. For instance, Chen et al. found that the external application of (sodium hydrosulfide) NaHS significantly increased stress resistance, enhanced the antioxidant system, and reduced salt-induced oxidative damage [15]. Zhu et al. reported that exogenous H_2S decreased the content of pectin and hemicellulose in rice roots under aluminum stress, diminished the negative charge in the cell wall, and lowered aluminum ion concentration at the root tip, effectively alleviating aluminum stress [16]. H_2S donors have also been shown to inhibit lipoxygenase activity, decrease levels of superoxide anion, H_2O_2 , and malondialdehyde, and enhance antioxidant capacity during wheat seed germination under copper stress [17]. Additionally, H_2S plays a crucial role in regulating ion homeostasis, aiding plants in coping with stress. Sun et al. demonstrated that exogenous H_2S treatment in poplar reduced Cd stress by upregulating the plasma membrane H^+ pump and promoting the function of the root cytoplasmic membrane $\text{Cd}^{2+}/\text{H}^+$ reverse transport system, facilitating the reverse transmembrane transport of Cd^{2+} and H^+ [18].

Rice (*Oryza sativa* L.) is an important food crop and feeds more than half of the world population [19]. nCuO and CuSO_4 severely inhibit rice seed germination and growth, however, the molecular and physiological mechanisms by which nCuO/ CuSO_4 inhibit rice growth and development remain unclear. In this study, the effects of nCuO/ CuSO_4 on physiology, biochemistry, and cellular morphology of rice were first identified, and then, the mechanism of H_2S regulation in enhancing Cu stress tolerance in rice was revealed by determining physiological and biochemical indices, observing cellular morphology, and analyzing the expression of key gene under nCuO and H_2S treatment. This research provides a theoretical foundation and reference for the prevention and control of nanomaterial pollution, the improvement of rice stress resistance, and the enhancement of crop quality.

Materials and methods

Plant material and treatment

The *Oryza sativa* cv. Nipponbare was used in this study. Seeds were disinfected using a 2.5% sodium hypochlorite (v/v) solution, followed by incubation in darkness at 35 °C for 48 h to promote germination. After germination, the seeds were transferred to 96-well plate for hydroponic cultivation to two-leaf-one-heart stage. The seedlings were randomly assigned to six treatment groups: (1) Control group: treated with rice full nutrient solution for 5 days; (2) NaHS group: exposed to NaHS (50 $\mu\text{mol/L}$) for 5 days; (3) nCuO group: subjected to nCuO (250 mg/L) for 5 days; (4) NaHS + nCuO group: treated with nCuO (250 mg/L) in combination with NaHS (50 $\mu\text{mol/L}$) for 5 days; (5) CuSO_4 group: treated with CuSO_4 (40 mg/L) for 5 days; (6) NaHS + CuSO_4 group: exposed to CuSO_4 (40 mg/L) along with NaHS (50 $\mu\text{mol/L}$) for 5 days. nCuO were obtained from MACKLIN Company (CAS: 1317-38-0, spherical shape with a diameter of 40 nm, purity of 99.5%). Each treatment was replicated three times.

Growth parameters measurement

The treated seedlings are extracted from the 96-well plate, and remove excess water for measurements of plant height, root length, and fresh weight (FW). Plant height is measured from the base to the tip of the uppermost leaf, and root length is measured from the base to the tip of the longest root. Subsequently, the plants were dried at 80 °C for 15 min, and then further dried at 60 °C until a constant weight was achieved to determine the dry weight (DW). The length, width, and number of root cells in the different treatment groups were observed and counted using a Laser Scanning Confocal Microscope (Zeiss LSM 710) and Image J. The “cell number” means the number of cells present within a 0.01 square millimeter area.

Physiological and biochemical parameter determination

Malondialdehyde (MDA) content, superoxide dismutase (SOD) activity, and catalase (CAT) activity were measured using test kits from Suzhou Greatris Bio-technology Co., Ltd. DAB staining was utilized to assess hydrogen peroxide (H_2O_2) levels in cells, following the protocol established by Ouyuan et al. [20].

RNA extraction, cDNA library construction, and illumina sequencing

Total RNA was extracted from each sample using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) in according to the manufacturer's instructions. The quality of RNA was assessed by the Agilent® 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Library construction was carried out according to the Illumina Stranded mRNA Prep kit (Illumina, San Diego, CA, USA) manufacturer's instructions. The RNA samples were sequenced using the Illumina NovaSeq 6000 platform by Parsortix Biological Technology Co., Ltd., Shanghai, China. Trimomatic and Cutadapt were employed to trim low-quality reads and remove adapters, respectively, in order to obtain clean reads for further analysis. The clean reads were then mapped to the reference genome of *Oryza sativa* GCF_001433935.1_IRGSP-1.0 using HISAT2 v2.2.1. Stringtie was utilized for the quantification of each sample, while edgeR was applied to identify differentially expressed genes. Three biological replicates were performed.

Differential expression genes (DEGs) enrichment analysis

Differential gene expression was assessed using DESeq. The criteria for identifying DEGs were as follows: an expression fold change $|\log_2\text{FoldChange}| > 1$ and a significance P -value < 0.05 . Subsequently, the topGO and the Kyoto Encyclopedia of Genes and Genomes (KEGG) database were utilized for enrichment analysis of Gene Ontology (GO) functions and KEGG pathways associated with the DEGs. The hypergeometric distribution method was employed to calculate P -values, with significance set at $P < 0.05$, to determine GO terms and KEGG pathways that were significantly enriched in DEGs relative to the entire genomic background.

Quantitative real-time polymerase chain reaction (qRT-PCR) analysis

The total RNA was extracted from rice seedling using an RNA extraction kit (Beijing Tiangen Biotechnology Co., Ltd., China), and subsequently reverse transcribed into cDNA using a reverse transcription kit (Shanghai YEASEN Biotechnology Co., Ltd., China). *Actin* was used as the internal reference, and the CFX96 Real-Time PCR Detection System (Bio-Rad, USA) was employed for qRT-PCR amplification. The reaction steps were conducted

according to the protocols provided by the manufacturers of the real-time fluorescent quantitative PCR kit (Shanghai YEASEN Biotechnology Co., Ltd., China). Relative expression levels were calculated using the $2^{-\Delta\Delta C_t}$ method following three independent experiments. The primers utilized for qRT-PCR analysis are showed in Supplementary Table S1.

Determination of Cu content in roots and shoots

The rice seedling culture was performed as those in phenotype analysis. The shoots and roots were collected at after treatment, oven-dried at 105 °C for 48 h. All prepared samples were acid-digested, and their Cu contents were determined using ICP-MS.

Data analysis

Data were curated using Microsoft Excel, and statistical analyses were conducted with SPSS version 20.0. Significance testing was performed using one-way analysis of variance (ANOVA) followed by Duncan's multiple comparison test (*, **, and *** indicate significant, highly significant, and very significant differences at the $p < 0.05$, $p < 0.01$, and $p < 0.001$ levels, respectively). Graphical representations were created using GraphPad Prism 9 software.

Results

Effects of copper on the growth of rice seedlings

In the present study, rice seedlings were treated with nCuO and $CuSO_4$ and their fresh weight, shoot and root length were subsequently evaluated (Fig. 1). The results indicated a significant inhibition of seedling growth following Cu treatment. The plant height, root length, fresh weight, chlorophyll content, and survival rate of rice seedlings treated with nCuO or $CuSO_4$ were significantly lower than those of the control group. Compared to $CuSO_4$, the leaf curl in rice seedlings treated with nCuO was more pronounced. Additionally, the root length, plant height, fresh weight, chlorophyll content, and survival rate of nCuO-treated rice seedlings were also lower. These results indicated that the growth of rice seedling was significantly inhibited by nCuO and $CuSO_4$.

Additionally, Cu treatment significantly impacted rice root growth, with roots in the control group found to be longer than those in the treatment group. Specifically, under nCuO treatment, rice roots exhibited enhanced particle adsorption on the surface and a reduction in lateral roots, whereas $CuSO_4$ treatment resulted in increased lateral root development (Fig. 2). Staining of rice root tips at 0.5 cm with PI revealed significant differences in cell length and width between the control and treatment groups. Compared to the control group, the length of cells treated with nCuO decreased by 27.7%, while the width increased by 14.1%. In contrast, after

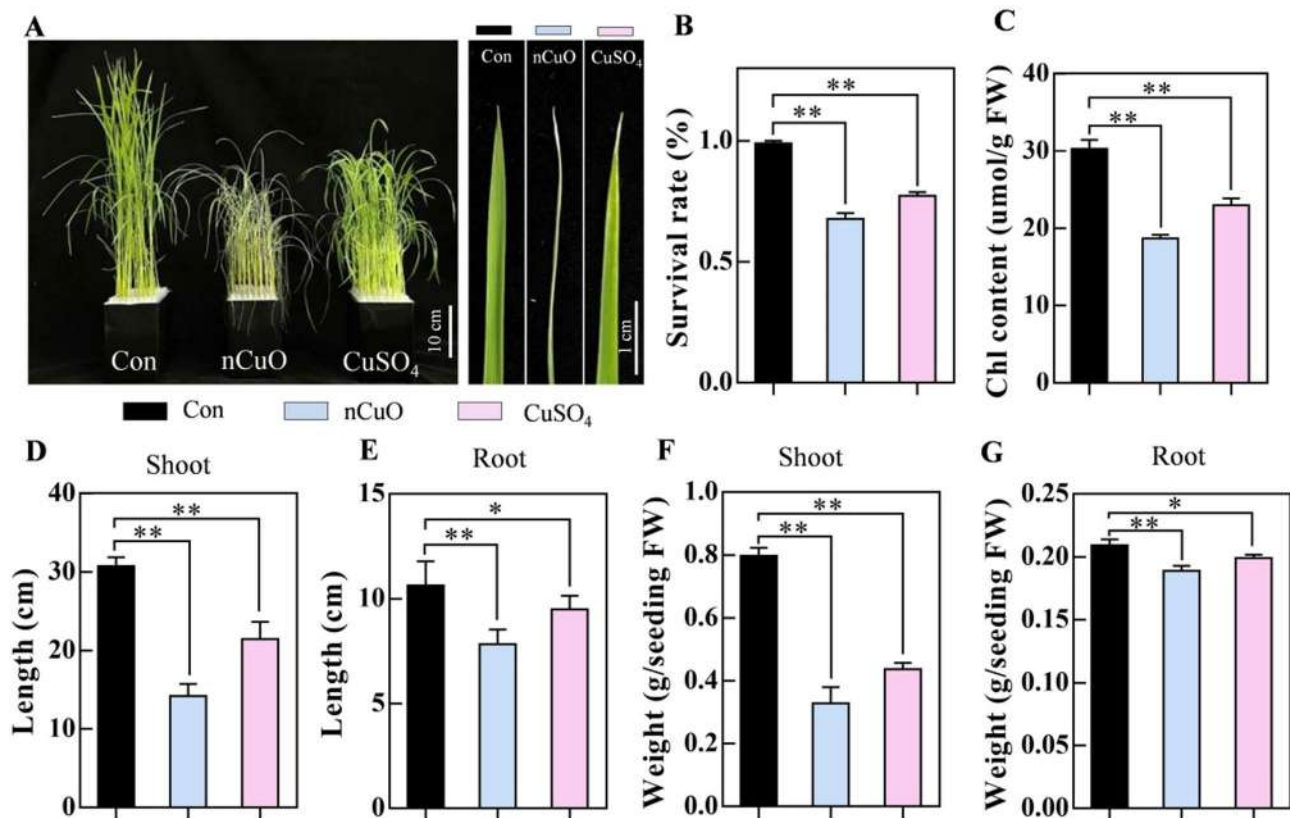


Fig. 1 Morphological and physiological responses of rice to nCuO and CuSO₄ treatments. **A** Observation of rice phenotypes under nCuO and CuSO₄ treatments. **B–G** Effects of nCuO and CuSO₄ treatments on survival rate, plant height, root length, fresh weight (shoot and root), and chlorophyll content of rice. The black, light blue and light pink columns represent Con, nCuO and CuSO₄ respectively. Con, nCuO and CuSO₄ represent nutrient solution, nutrient solution + nCuO treatment, and nutrient solution + CuSO₄ treatment, respectively. Error bars represent the standard error ($n = 10$). * $P < 0.05$, ** $P < 0.01$

CuSO₄ treatment, the length of the cells decreased by 11.4%, and the width increased by 27.8%.

Effects of copper on the physiological and biochemical parameters of rice seedlings

SOD is a vital enzyme involved in defense of plant against oxidative damage. As illustrated in Fig. 3, the treatment groups demonstrate significantly higher SOD activity compared to the control group, indicating the toxicity of both nCuO and CuSO₄ to rice. Notably under nCuO treatment, SOD activity increases by 29.77%. This trend is also reflected in CAT activity, which implies the induction of antioxidant enzyme production in response to copper stress. Furthermore, the substantial increase in MDA content in the nCuO treatment group-1.55 times higher than that of the control group-and 1.36 times higher in the CuSO₄ treatment group.

The action of peroxidase on hydrogen peroxide generates free oxygen, which leads to the oxidation of DAB and the formation of a brown precipitate. Staining results of DAB indicate darker pigmentation and prominent dark brown particles in rice leaves treated with nCuO and

CuSO₄, suggesting an increased accumulation of H₂O₂ in treated plants.

Analysis of differential gene expression

In order to investigate the molecular mechanisms by which Cu inhibits rice growth, we conducted a transcriptome analysis under treatments with nCuO and CuSO₄. DESeq was employed to identify differentially expressed genes (DEGs) between the treatment and control groups. The most significant differences in gene expression were observed between the nCuO treatment group and the control group, with 1194 upregulated genes and 1186 downregulated genes. However, CuSO₄ treatment group found 917 upregulated genes and 1041 downregulated genes compare to control group (Fig. 4B). Additionally, a comparison between the nCuO and CuSO₄ treatment groups revealed 288 differentially expressed genes, consisting of 260 upregulated genes and 28 downregulated genes. These findings suggest potentially distinct molecular mechanisms underlying the stress induced by CuSO₄ and nCuO in rice.

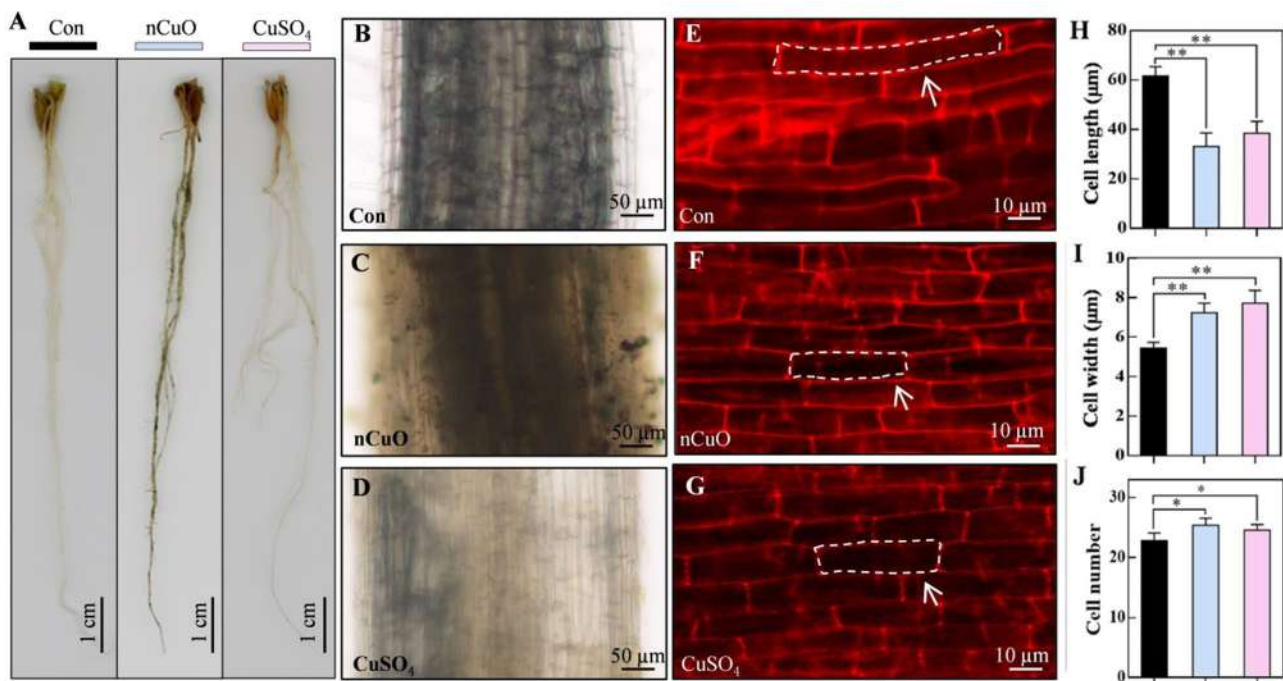


Fig. 2 Effects of nCuO and CuSO₄ treatment on cell number and morphology in rice roots. **A** Observation of phenotypic changes in roots. **B–D** Observation of root surface attachments. **E–G** Cellular changes following Propidium Iodide (PI) staining in roots. **H–J** Statistical analysis of cell length, width and number. The black, light blue and light pink columns represent Con, nCuO and CuSO₄ respectively. Con, nCuO and CuSO₄ represent nutrient solution, nutrient solution + nCuO treatment, and nutrient solution + CuSO₄ treatment, respectively. Vertical bars represent the mean ± SE ($n = 10$). *: $P < 0.05$; **: $P < 0.01$

H₂S may play a role in regulating the resistance of rice to nCuO and CuSO₄

In this study, we conducted a comprehensive analysis of transcriptome across two treatment groups and a control group. Utilizing Venn diagram analysis, we identified 39 intersecting differentially expressed genes (DEGs), with 10 candidates potentially regulating H₂S signaling pathways (Fig. 5). The relative expression of five of these genes was examined through RT-qPCR. Calmodulin-binding proteins *Os01g0134700*, *Os11g0669100*, and *Os12g0556200* may regulate the H₂S signaling pathway by modulating calcium ion levels. Our research revealed that, compared to the control group, the relative expression levels of *Os01g0134700*, *Os11g0669100*, and *Os12g0556200* increased following copper treatment. Among them, the relative expression levels of *Os01g0134700* and *Os11g0669100* increased by 6.9-fold and 6.1-fold, respectively, following nCuO treatment, while the expression level of *Os12g0556200* increased by 12.8-fold after CuSO₄ treatment. *Os04g0165700* and *Os06g0564500* encode cysteine synthase (CS), which plays significant roles in the decomposition and synthesis of H₂S. The expression of *Os04g0165700* was downregulated following copper treatment, while the expression of *Os06g0564500* decreased by 44.6% after nCuO treatment and increased by 148% after CuSO₄ treatment. The data

suggest that H₂S may play a significant role in copper stress tolerance in rice.

Influence of exogenous H₂S on rice seedling growth under stress conditions

To investigate the impact of H₂S on the growth of rice seedlings treatments with nCuO and CuSO₄, we applied NaHS, a compound that releases H₂S, under nCuO and CuSO₄ treatment and monitored the phenotypic changes. The results indicated that NaHS treatment alone had no significant effect on the various growth parameters of rice seedlings. Compared to rice seedlings subjected solely to nCuO or CuSO₄ stress, those treated with NaHS demonstrated improved growth (Fig. 6). The application of NaHS following stress can significantly reduce leaf yellowing and inhibit of aboveground growth. Under nCuO and CuSO₄ stress, the plant height increased by 26.7% and 51.9%, respectively, while the root length increased by 10.1% and 7.5%, respectively, following the application of NaHS. After the application of NaHS, both the survival rate and chlorophyll content were also significantly higher than those of the stress groups. Compared to the copper treatment, the fresh weight of the aboveground and underground parts of the seedlings increased significantly by 92.2% and 19.7%, respectively, after the addition of NaHS.

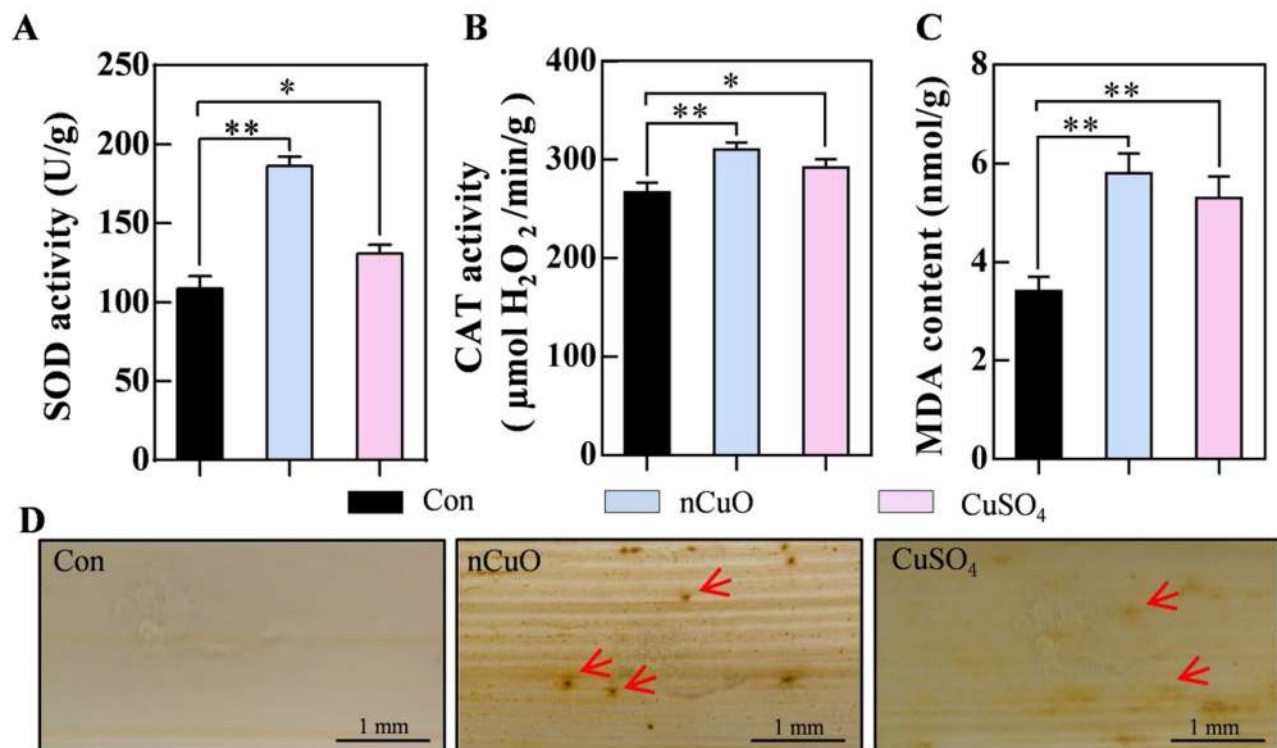


Fig. 3 Biochemical indices affected by nCuO and CuSO₄ treatment in rice. **A–C** Changes of SOD activity, CAT activity, and MDA content in rice leaves under nCuO and CuSO₄ treatment. **D** DAB staining of rice seedlings subjected to nCuO and CuSO₄ treatments. The black, light blue and light pink columns represent Con, nCuO and CuSO₄ respectively. Con, nCuO and CuSO₄ represent nutrient solution, nutrient solution + nCuO treatment, and nutrient solution + CuSO₄ treatment, respectively. Vertical bars represent the mean $\pm$ SE ($n=3$). *: $P < 0.05$; **: $P < 0.01$

Subsequently, the effects of NaHS on the root growth of rice seedlings under stress conditions induced by nCuO and CuSO₄ were investigated. The results demonstrated that exogenous NaHS could enhance the growth of the rice seedling root system under both stress conditions. Under nCuO stress, the application of NaHS reduced the particle adsorption on the surface of rice roots. Microscopic observations of PI staining of root cells showed that the length and width of rice root cells were partially restored after NaHS application. Compared to the nCuO and CuSO₄ treatments, the length of the cells after NaHS treatment increased by 38.5% and 12.9%, respectively, while the width decreased by 14.1% and 28.6%. Similarly, there was a recovery in the number of cells. These findings collectively suggest that an appropriate concentration of exogenous H₂S can alleviate the toxic effects induced by nCuO and CuSO₄ stress on rice seedlings, thereby enhancing their tolerance to these stressors (Fig. 7).

Influence of exogenous H₂S on physiological and biochemical parameters of rice under copper stress

The results indicate that, compared to the control group, NaHS treatment did not significantly affect the SOD activity in rice seedlings, however, it did lead to a slight

increase in catalase CAT activity and MDA content. When NaHS was added to the nCuO group, both SOD activity (which decreased by 19.9%) and CAT activity (decreased by 22.7%) were significantly decreased. Similarly, the addition of NaHS to the CuSO₄ group resulted in significant decreases in both SOD and CAT activities, suggesting that copper stress was alleviated. Further analysis revealed that MDA content significantly decreased by 42.3% and 48.7% under nCuO and CuSO₄ stress, respectively, following NaHS application, effectively inhibiting MDA generation induced by stress. In conclusion, NaHS treatment can effectively alleviate the oxidative stress caused by nCuO and CuSO₄. Additionally, DAB staining results showed no significant difference in leaf color between the control group and the NaHS treatment group (Fig. 8D). After NaHS application under nCuO and CuSO₄ stress, the brown area of the leaves significantly decreased, indicating a reduction in H₂O₂ content, which was consistent with the findings from the antioxidant enzyme analysis.

Influence of exogenous H₂S on the Cu content in rice seedlings under stress conditions

To explore the influence mechanism of Cu stress on rice seedlings, we determined the concentration of Cu ions

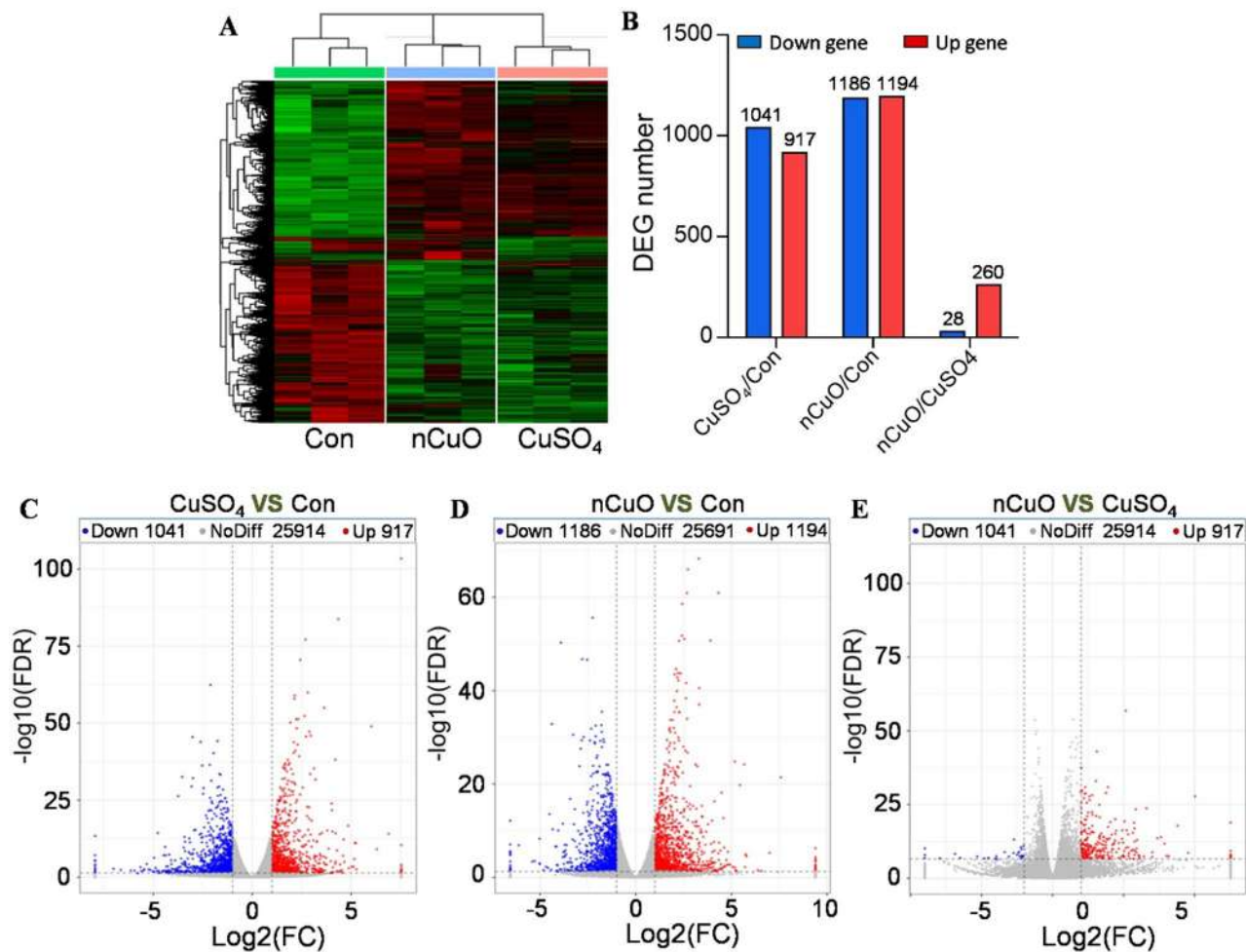


Fig. 4 Differential gene analysis under nCuO and CuSO₄ stress. **A** Heatmap of differentially expressed genes (DEGs) clustering. **B** Differential DEG counts among different treatment groups. **C-E**, Volcano plots illustrating differential gene expression between different treatment groups. The X-axis represents the log₂ fold change (FC), and the Y-axis represents -log₁₀ (FDR). Red dots indicate up-regulated genes, and blue dots indicate down-regulated genes

in rice seedlings. The results showed that in both root and shoot, there was no significant difference in Cu ion between the NaHS treatment group and Con. However, the Cu ions in the nCuO and Cu treatment groups increased significantly, but addition of NaHS could significantly reduce Cu ions accumulation in root and shoot. Further analysis revealed that Cu content significantly decreased by 43.92% and 25.95% in root under nCuO and CuSO₄ stress, respectively, following NaHS application (Fig. 9). The results indicate that H₂S may alleviate the damage caused by Cu ions to plants by regulating the accumulation of Cu ions.

Influence of exogenous H₂S on the expression of stress-responsive genes in rice seedlings under stress conditions

To elucidate the molecular mechanisms underlying the mitigating effects of H₂S on stress induced by nCuO and CuSO₄ in rice, we conducted a comparative analysis of stress-related gene expression under various treatment

conditions (Fig. 10). Fluorescence quantitative analysis of the rice roots revealed that the expressions of *OsHMA5*, *OsNramp1*, *OsNramp5*, *OsNAS2*, *OsPCS1*, and *OsATX1* were induced by nCuO and CuSO₄ stress, with relative expression levels significantly increasing. Among these, *OsHMA5* exhibited the most substantial induced, with upregulation of 3.2 times under nCuO stress and 5.1 times under CuSO₄ stress. Compared to the treatment groups of nCuO and CuSO₄, the relative expression levels of *OsHMA5* (decreased by 7.8% and 51.3%, respectively), *OsNramp1* (decreased by 74.1% and 35.2%, respectively), *OsNramp5* (decreased by 24.8% and 18.7%, respectively), *OsNAS2* (decreased by 26.4% and 61.9%, respectively), and *OsATX1* (decreased by 30.2% and 44.7%, respectively) significantly decreased following the application of NaHS. In contrast, the relative expression level of *OsPCS1* increased significantly. The expression levels of *OsHMA2* and *OsIRT2* did not change significantly under nCuO treatment but exhibited an increase and decrease,

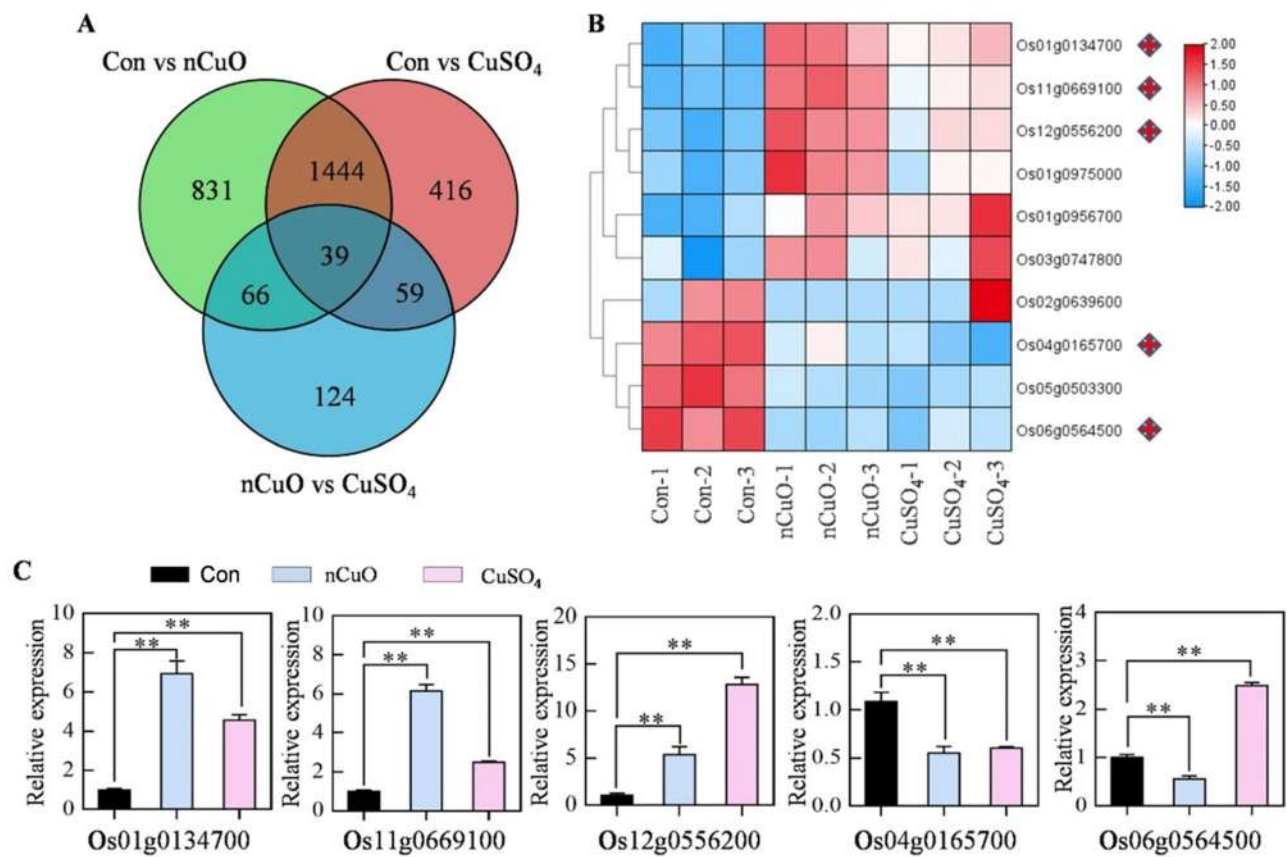


Fig. 5 Key gene analysis under nCuO and CuSO₄ stress. **A** Venn diagram illustrating the overlap of differentially expressed genes. **B** Heatmap clustering of key differentially expressed genes. **C** Comparative expression levels of five key genes under various treatments. The black, light blue and light pink columns represent Con, nCuO and CuSO₄ respectively. Con, nCuO and CuSO₄ represent nutrient solution, nutrient solution + nCuO treatment, and nutrient solution + CuSO₄ treatment, respectively. Vertical bars represent the mean $\pm$ SE ($n = 10$). *: $P < 0.05$; **: $P < 0.01$

respectively, under CuSO₄ stress, and decreased after NaHS inhibition. The expression of *OsMT2a* was inhibited by both nCuO and CuSO₄ stress, resulting in a significant decrease in relative expression levels; however, it was upregulated following NaHS application. In summary, H₂S may alleviate the toxic effects of nCuO and CuSO₄ stress on rice seedlings by regulating the above-mentioned genes.

Discussion

Comparative impact of nCuO and CuSO₄ on rice seedling growth

Our study revealed that nCuO exerts a more pronounced inhibitory effect on rice seedling growth compared to CuSO₄. The results aligns with previous studies, which highlights the enhanced reactivity and bioavailability of nanoparticles, often leading to more severe physiological disruptions than their ionic counterparts [21, 22]. nCuO have been shown to inhibit plant development and metabolism through various mechanisms, including its size, concentration, chemical properties, and the chemical environment at subcellular deposition sites.

Furthermore, the tendency of nCuO to aggregate into larger particles in solution may hinder root absorption, exacerbating its toxic effects [23, 24]. In our study, the nCuO-treated plants exhibited significant reductions in height, severe leaf curling, and chlorosis compared to the CuSO₄ treatment group. These results suggest that nanoscale copper penetrates plant tissues more effectively and interacts more intimately with cellular structures, resulting in greater toxicity. These findings are consistent with previous research indicating that treatment with metallic nanomaterials inhibits plant growth [25, 26].

Root morphology analysis further corroborated these observations, as nCuO exposure resulted in particle accumulation on root surfaces and a significant reduction in lateral root formation. Such disruptions could impair the plant's ability to absorb water and nutrients, which are critical for sustaining growth under stress [27]. On the contrary, CuSO₄ treatment promoted the formation of lateral roots, which may serve as an adaptive response to maintain nutrient acquisition despite the stress imposed by copper ions.

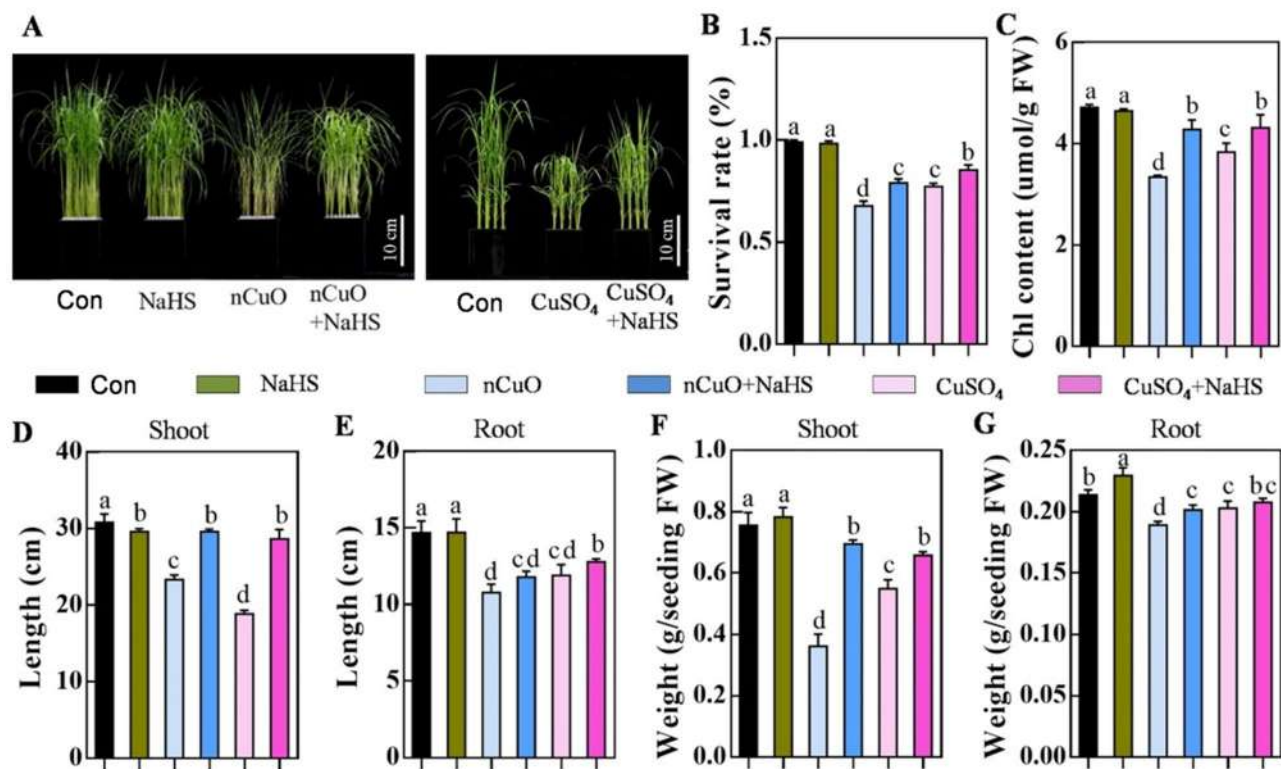


Fig. 6 H₂S mitigates the damage caused by nCuO and CuSO₄ in rice. **A** Phenotypic assessment of rice seedlings under various treatment conditions. **B–G** H₂S reduced the adverse effects of nCuO and CuSO₄ treatments on survival rate, plant height, root length, fresh weight (shoots and roots) and chlorophyll content of rice. The black, light green, light blue, dark blue, light pink, dark pink columns represent Con, NaHS, nCuO, nCuO+NaHS, CuSO₄, and CuSO₄+NaHS respectively. Vertical bars represent the mean $\pm$ SE ($n = 10$). Different letters indicate a statistically significant difference at $p < 0.05$

Physiological and biochemical responses to nCuO and CuSO₄ stress

Oxidative stress is a well-documented response in plants exposed to heavy metals, as demonstrated by our findings of significant increases in antioxidant enzymes, such as SOD and CAT, under both nCuO and CuSO₄ treatments. The more pronounced increase in oxidative stress markers observed with nCuO exposure aligns with studies indicating that nanoparticles generate ROS more efficiently due to their greater surface area and reactivity [28].

The elevated levels of MDA and hydrogen peroxide (H₂O₂) observed in our study further confirm the oxidative damage induced by copper stress. MDA, a key marker of lipid peroxidation, was significantly higher in seedlings treated with nCuO, indicating extensive membrane damage [29]. Similarly, the increased levels of H₂O₂ under nCuO stress suggest that the plant's antioxidant defenses were overwhelmed, leading to more substantial oxidative damage compared to CuSO₄ treatment [30]. The distinct physical and chemical properties of nCuO and CuSO₄ may account for their differing oxidation responses, as the enhanced reactivity of nCuO results in the generation of more aggressive ROS.

Transcriptomic analysis of gene expression under nCuO and CuSO₄ stress

Transcriptomic analysis provided further insights into the distinct molecular responses of rice seedlings to nCuO and CuSO₄. nCuO treatment induced more significant transcriptional changes, consistent with the observed oxidative stress and cellular damage. The differentially expressed genes under nCuO stress are primarily involved in plant hormone signal transduction, amino sugar and nucleotide sugar metabolism, MAPK signaling pathways, and photosynthesis. In contrast, the differentially expressed genes triggered by CuSO₄ are mainly associated with phenylpropanoid biosynthesis, amino sugar and nucleotide sugar metabolism, and diterpenoid biosynthesis, which contribute to enhanced resilience against CuSO₄ stress. A notable aspect of our findings is the upregulation of genes related to the H₂S signaling pathway, which suggests a critical role for H₂S in the plant's response to copper-induced stress. Genes involved in H₂S biosynthesis, were significantly upregulated under nCuO stress, indicating that H₂S may act as a signaling molecule to activate defense mechanisms against nCuO toxicity [31, 32]. This is supported by previous studies demonstrating that H₂S enhances antioxidant

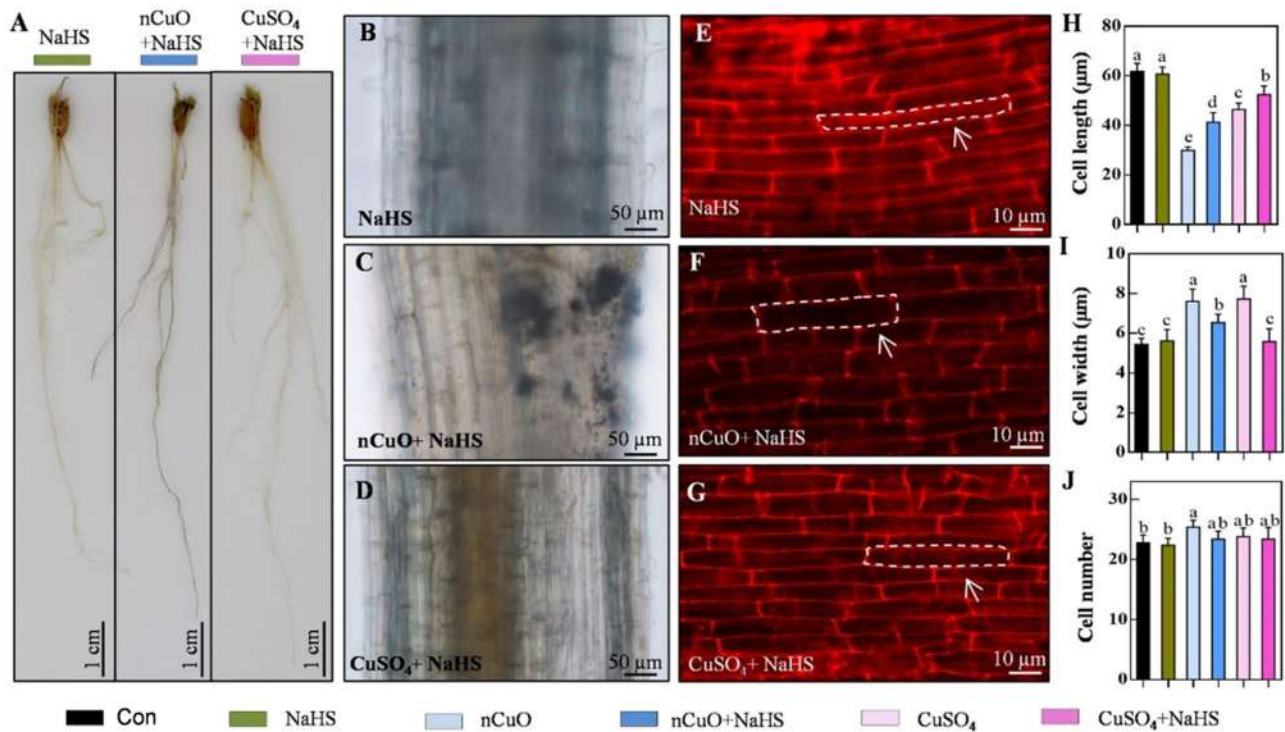


Fig. 7 H₂S can alleviate the damage of nCuO and CuSO₄ on rice growth. **A** Observation of root phenotypic changes with exogenous H₂S. **A–D** Observation of root surface attachments with exogenous H₂S. **E–G**, Cellular changes with exogenous H₂S after PI staining in root. **H–J**, Statistical analysis of cell number, length, and width. The black, light green, light blue, dark blue, light pink, dark pink columns represent Con, NaHS, nCuO, nCuO + NaHS, CuSO₄, and CuSO₄ + NaHS respectively. Vertical bars represent the mean ± SE (*n* = 10). Different letters indicate a statistically significant difference at *p* < 0.05

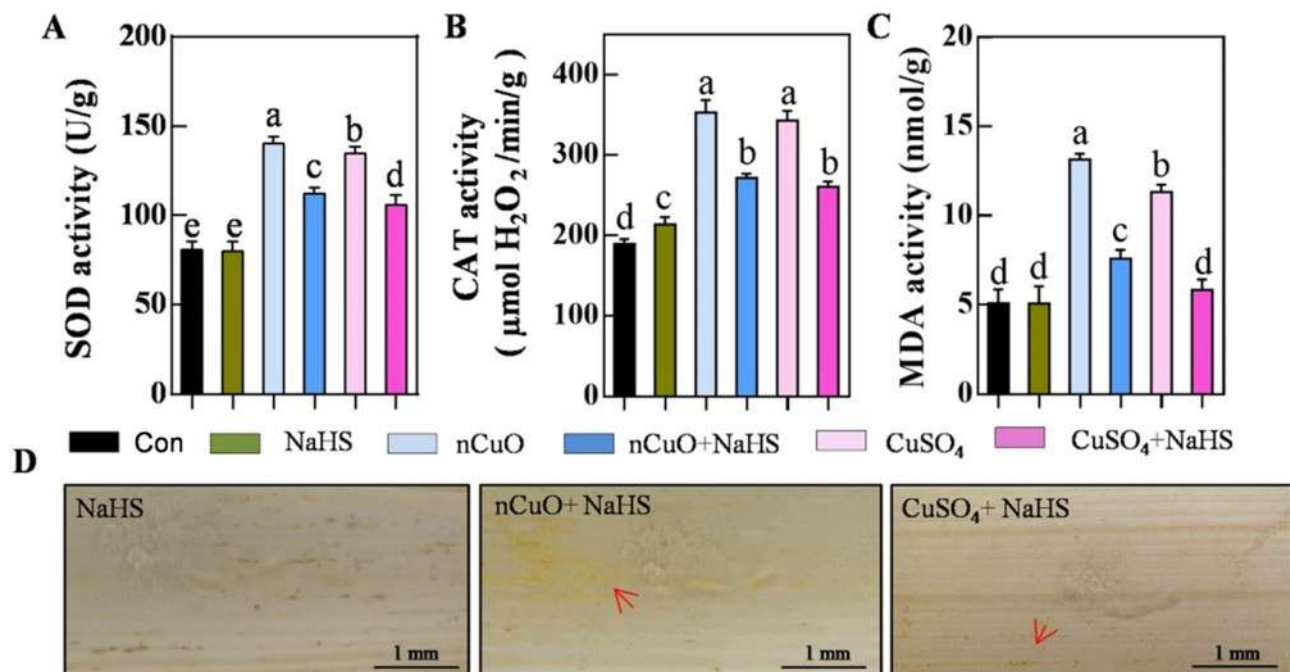


Fig. 8 H₂S alleviates the effects of nCuO and CuSO₄ on the physiology, biochemistry, and cellular structure of rice. **A–C**, Changes of SOD activity, CAT activity and MDA content in rice leaves under nCuO and CuSO₄ treatments with exogenous H₂S. **D** DAB staining of rice seedlings under nCuO and CuSO₄ treatments with exogenous H₂S. The columns of different colors represent different treatments. The black, light green, light blue, dark blue, light pink, dark pink columns represent Con, NaHS, nCuO, nCuO + NaHS, CuSO₄, and CuSO₄ + NaHS respectively. Vertical bars represent the mean ± SE (*n* = 3). Different letters indicate a statistically significant difference at *p* < 0.05

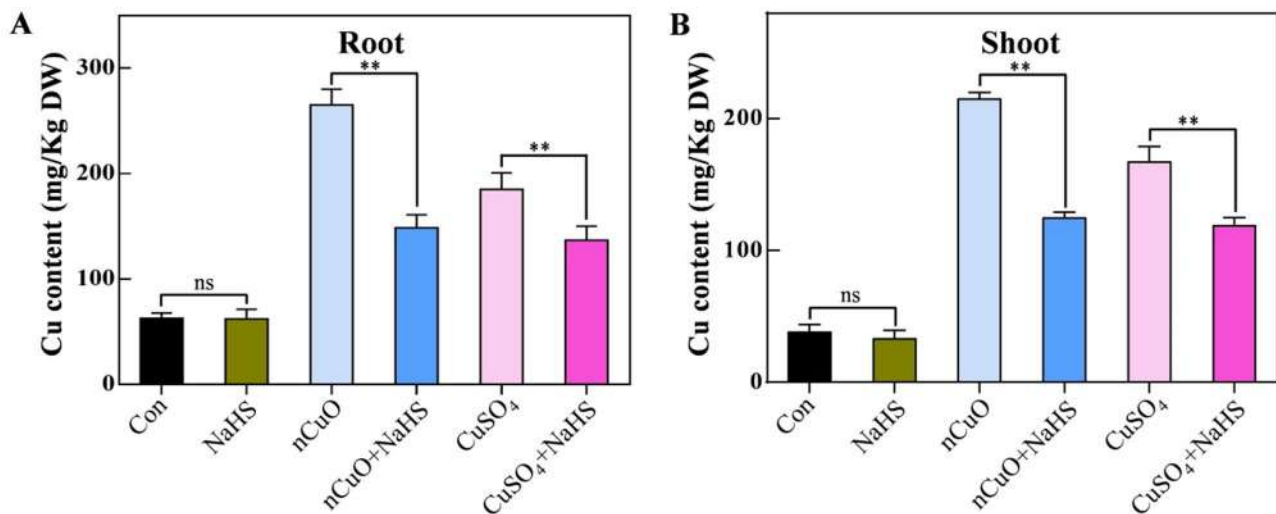


Fig. 9 H₂S reduces the accumulation of copper in root and shoot. (A) The Cu content in the root. (B) The Cu content in the shoot. The black, light green, light blue, dark blue, light pink, dark pink columns represent Con, NaHS, nCuO, nCuO + NaHS, CuSO₄, and CuSO₄ + NaHS respectively. Vertical bars represent the mean $\pm$ SE ($n=5$). **: $P < 0.01$, ns indicates non-substantial differences at that level of significance

defenses and reduces oxidative stress in plants exposed to heavy metals [33].

Moreover, H₂S signaling appears closely linked to the calcium (Ca²⁺)/calmodulin (CaM2)-mediated pathway [34]. For instance, H₂S, in conjunction with the Ca²⁺/CaM complex, has been shown to induce nickel tolerance in zucchini seedlings, thereby preventing oxidative damage [35]. In our study, the expression of calmodulin-binding genes (*Os01g0134700*, *Os11g0669100*, *Os12g0556200*) were significantly induced by nCuO and CuSO₄ treatment. These results indicate that calmodulin-binding genes likely regulate the response to both nCuO and CuSO₄ by modulating H₂S levels [33].

Exogenous H₂S alleviates copper stress in rice seedlings

Exogenous H₂S has emerged as a promising strategy for mitigating various abiotic stresses, including heavy metal toxicity [36, 37]. Our study demonstrated that NaHS, an H₂S donor, effectively alleviated copper-induced oxidative stress in rice seedlings. This was evidenced by the observed reductions in SOD and CAT activities, along with decreased MDA and H₂O₂. These findings underscore highlighting the crucial role of H₂S in enhancing the plant's antioxidant defense system and mitigating oxidative damage [38]. At the cellular level, H₂S treatment not only alleviated oxidative stress but also partially reversed copper-induced morphological changes, particularly in cell length and width. In addition, the application of H₂S significantly reduced the accumulation of Cu ions in plants under Cu stress. These findings suggest that H₂S plays a crucial role in maintaining cellular structure and function under copper stress, further supporting its role as a protective agent [39]. Moreover, the overall growth improvements observed—including increased

plant height, root length, and biomass—underscore the potential of H₂S in enhancing the tolerance of rice seedlings to copper stress.

Importantly, exogenous H₂S treatment via NaHS significantly modulated the expression of several key genes associated with copper stress. Our study highlights the critical roles of *OsHMA2* and *OsHMA5*, which encode P1B-type heavy metal ATPases essential for copper translocation and homeostasis [40, 41]. The upregulation of these genes indicates an increased demand for efficient copper management, particularly under nCuO stress, where copper toxicity is exacerbated. These ATPases facilitate the sequestration of copper into vacuoles, thereby mitigating its cytotoxic effects [41]. Furthermore, the expression of *OsIRT2*, an iron transporter involved in copper transport, was notably modulated, exhibiting differential expression under CuSO₄ stress compared to nCuO, suggesting a copper source-dependent response [42]. The response to copper stress was further evidenced by the upregulation of *OsNramp1* and *OsNramp5*, which encode natural resistance-associated macrophage proteins. The increased expression of these genes, particularly under CuSO₄ treatment, reflects an adaptive mechanism by which rice seedlings regulate metal ion uptake to mitigate toxicity [43, 44]. Additionally, the significant changes in the expression of *OsATX1*, a copper chaperone protein, underscore its vital role in copper homeostasis, ensuring the proper delivery of copper to target proteins while preventing excess accumulation [45]. Finally, the upregulation of *OsPCS1*, which encodes phytochelatin synthase, and *OsMT2a*, which encodes a metallothionein, indicates an enhanced detoxification capacity. These genes play critical roles in chelating and

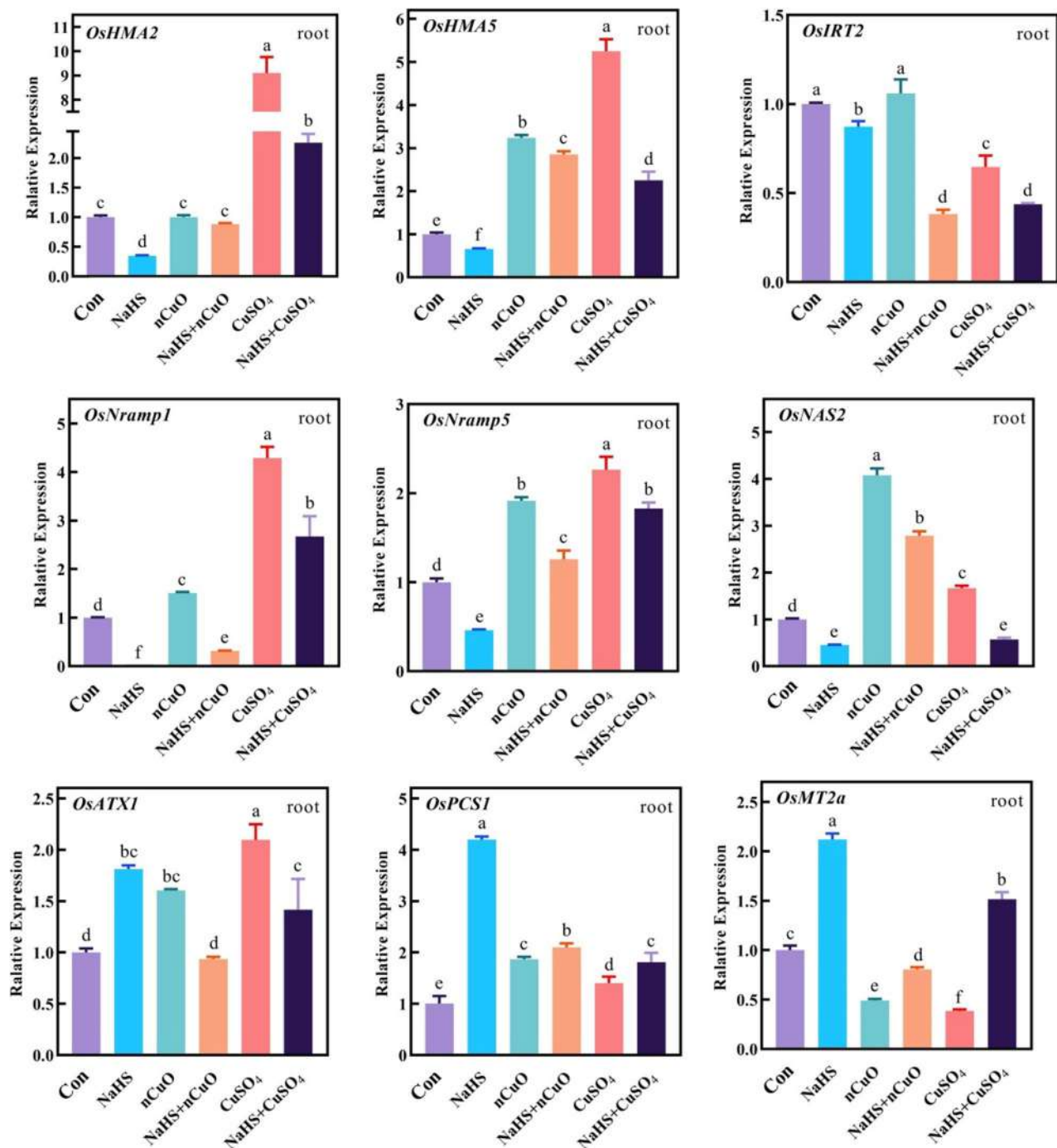


Fig. 10 Effects of various treatments on gene expression in the roots of rice seedlings

sequestering excess copper in vacuoles, thereby bolstering rice resilience to heavy metal stress [46].

Conclusion

In conclusion, our study demonstrates that nCuO exerts greater toxicity on rice seedlings compared to CuSO₄. This is evidenced by more severe oxidative stress, cellular damage, and extensive transcriptional changes.

Transcriptomic analysis revealed distinct gene expression profiles influenced by these two forms of copper, with nCuO inducing broader impacts on pathways related to stress response and metabolism. Crucially, H₂S emerged as a key mitigative agent, significantly enhancing rice tolerance to copper stress by modulating oxidative stress, improving cellular structure, and upregulating critical genes involved in copper homeostasis. These findings

underscore the potential of H₂S as a strategy for alleviating heavy metal stress in plants, offering promising applications for enhancing crop resilience under diverse environmental conditions.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12870-025-07198-y>.

Supplementary Material 1.

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Authors' contributions

D X and Q T designed the experiments. M D, Y P, S L, Y L, T Y, conducted all experimental works, data collection, analysis, and drafting of the manuscript. D X, Q T, X C, Y F, J L and X Z revised and edited the manuscript. All authors have read and agreed to the published version of the manuscript.

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Data availability

All gene sequences used in this article can be obtained on the Rice Genome Annotation Project Database (<https://rice.uga.edu/>) with accession nos. *OsNramp1* (LOC_Os07g15460), *OsNramp5* (LOC_Os07g15370), *OsIRT2* (LOC_Os03g46454), *OsNAS2* (LOC_Os03g19420), *OsHMA2* (LOC_Os06g48720), *OsHMA5* (LOC_Os04g46940), *OsMT2a* (LOC_Os01g05650), *OsPCS1* (LOC_Os05g34290), *OsATX1* (LOC_Os08g10480), *OsActin* (LOC_Os03g50885). The transcriptome data have been deposited at NCBI with the project ID [PRJNA1265380] (<https://www.ncbi.nlm.nih.gov/bioproject/1265380>).

Declarations

Ethics approval and consent to participate

Our study did not involve any human or animal subjects, material, or data. We declare that the plant material in the experiment was collected and studied by relevant institutional, national, and international guidelines and legislation.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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