Foliar Application of Flavonoids (rutin) Regulates Phytoremediation Efficiency of *Amaranthus Hypochondriacus* by Altering the Permeability of Cell Membranes and Immobilizing Excess Cd in the Cell Wall

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Abstract

The gap between the current serious soil heavy metal (HM) contamination situation and the low efficiency of soil remediation has become one of the factors limiting economic development and human health. The aim of this study was to propose a method to improve the efficiency of phytoremediation by exogenous rutin application and to explain the potential mechanism. A series of rutin treatments were designed to evaluate the biomass, cadmium (Cd) accumulation and phytoremediation efficiency responses of *Amaranthus hypochondriacus* to different levels of rutin (0.5, 1.5, and 5 ppm) under different Cd stress levels (10, 25, 50, and 100 ppm). The determination of cell membrane damage indicators, the subcellular distribution of Cd and the establishment of a predictive model for Cd accumulation were also carried out. The results showed a decline in cell membrane damage with rutin application, and more Cd ions were immobilized in the cell wall than in the vacuole, resulting in an increase in Cd tolerance in plants. The addition of rutin caused significant effects on the synthesis of glutathione (GSH), including the advancement of the conversion of GSH to phytochelatins (PCs). Among them, PC₂ and PC₃ in the leaves contributed the most to the high accumulation of Cd in *Amaranthus hypochondriacus* according to the prediction model. Overall, the phytoremediation efficiency and phytoextraction amount of *Amaranthus hypochondriacus* with foliar rutin application were improved significantly by 260% and 319%, respectively. These findings can contribute to the further development of soil remediation in Cd-contaminated fields.

Keywords

Amaranthus hypochondriacus; Phytoremediation; Cadmium; Subcellular distribution; Phytochelatins;

41 Prediction model

42

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44 **Ethics approval and consent to participate**

45 Not applicable

46

47 **Consent for publication**

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49

50 **Availability of data and materials**

51 The datasets used and/or analyzed during the current study are available from the corresponding author

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53

54 **Code availability**

55 Not applicable

56

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58 The authors declare that they have no competing interests.

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64 **Authors' contributions**

65 All authors contributed to the study conception and design. Conceptualization: W C; Writing – review
66 & editing: T A; Supervision: N L; Formal analysis: L Y; Investigation: J L; Methodology: Y W;
67 Writing – original draft: Y K. All authors read and approved the final manuscript.

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1. Introduction

Currently, with the rapid development of cities and industries, soil heavy metal (HM) pollution has become a major problem affecting agricultural production, food safety, human health and environmental safety (Liu et al. 2020, Wang et al. 2021). Compared to traditional chemical and physical remediation methods, phytoremediation is considered to be the most promising, cost-effective and environmentally friendly method of soil decontamination (Liu et al. 2013, Qin et al. 2021). However, the shortcomings of the low remediation efficiency of this method have become the main constraint to its wider application. Improving phytoremediation efficiency can be achieved by hyperaccumulator screening or agronomic manipulation (Wei et al. 2010). Phytomanagement, including water and fertilizer management, is a specific strategy employed in phytoremediation (Li et al. 2021). Phytochelatins (PCs) and their glutathione (GSH) precursors are thiol-rich peptides and critical in HM detoxification in plants and microorganisms (Jacquart et al. 2017). Thus, primary methodologies for the regulation of PCs synthesis were utilized to achieve a better efficiency of phytoremediation, including genetic engineering, photosynthesis regulation and metabolite regulation (Cao et al. 2014, Chaurasia et al. 2008, Yuan et al. 2015, Zayneb et al. 2017). Metabolite regulation by exogenous imposition of metabolites associated with PCs metabolism is of exploratory interest.

A number of studies have identified a link between HM stress and autologous flavonoid metabolism in plants. Flavonoids are considered defensive agents of plants against environmental stress and have a strong ability to scavenge 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals, reduce superoxide anion activity and reduce oxidative stress (Dong et al. 2021). In addition to being free radical scavengers, flavonoids play an important role in enhancing the tolerance of plants by chelating HM and reducing the

mobility of metal ions according to their different molecular structures, thereby inhibiting lipid peroxidation reactions (Stingu et al. 2012). Our research on metabolic responses and correlations with PCs in *Amaranthus hypochondriacus* identified 12 metabolites, such as flavonoids, of rutin that were highly linearly correlated with PCs (Xie et al. 2019). Previously, studies have investigated the effect of exogenous rutin application, providing high protection for the cell membrane and reducing oxidative stress damage in animal and microbial cells (Gong et al. 2010, Singh et al. 2018, Wang et al. 2015). However, little research has focused on the effects of exogenous flavonoids (rutin) on plant stress resistance and determining the related mechanism.

At present, there are no reports on the combined remediation techniques of exogenously applied natural antioxidant flavonoids and plants. *Amaranthus hypochondriacus* is an annual herb with rapid growth, easy cultivation, good adaptability, and Cd hyperaccumulation (Xie et al. 2020). In this study, *A. hypochondriacus* was treated with gradient concentrations of rutin under different concentrations of Cd stress to investigate the dynamic changes in biomass growth, Cd content in various parts, and sulfhydryl compound content. The differences in Cd accumulation were investigated at the subcellular level, and the most critical PCs for altering subcellular Cd accumulation were explored by the establishment of a Cd concentration prediction model. The alleviation of cellular damage after rutin application was explored to confirm the mechanism by which rutin enhances cellular Cd tolerance. Studying the effect of the natural exogenous antioxidant rutin on the resistance of Cd-enriched plants to Cd stress could provide insight for the development of novel phytoremediation techniques.

2. Materials and methods

2.1. Plant materials and growth conditions

The experimental soil (total Cd: $0.21 \text{ mg}\cdot\text{kg}^{-1}$, available Cd: $0.06 \text{ mg}\cdot\text{kg}^{-1}$, pH = 5.86) was collected from the farmland of Mianzhu City, Sichuan Province, China. Cd treatment was carried out by uniformly injecting of $\text{CdCl}_2\cdot 2.5\text{H}_2\text{O}$ solutions into the soil of each pot to the designed soil Cd concentration of 10, 25, 50, and $100 \text{ mg}\cdot\text{kg}^{-1}$. Seedlings of *A. hypochondriacus* were transplanted into experimental pots ($80 \text{ cm} \times 40 \text{ cm} \times 40 \text{ cm}$) with 70 kg of soil after two weeks of soil equilibrium. Ten days after transplanting, seedlings were fertilized with 1.2 g of liquid urea per pot. Exogenous rutin was applied 5 d after Cd application during branching stage (the seedlings grew to 40 cm in height). The treatments of exogenous rutin under different Cd conditions were as follows: R0, $0 \text{ mg}\cdot\text{mL}^{-1}$; R0.1, $0.1 \text{ mg}\cdot\text{mL}^{-1}$; R0.5, $0.5 \text{ mg}\cdot\text{mL}^{-1}$; R1.5, $1.5 \text{ mg}\cdot\text{mL}^{-1}$; and R5, $5 \text{ mg}\cdot\text{mL}^{-1}$. The rutin solution was sprayed on leaf surfaces and leaf backs until dripping. Three plants were randomly collected under each treatment condition after 3 d. The plants were divided into three parts, namely, the roots, stems and leaves, for fresh and dried biomass measurement. For each condition, triplicate analyses were performed.

2.2. Analysis of malondialdehyde, electrolyte leakage and cell vitality

Malondialdehyde (MDA) was determined to be a biomarker of lipid peroxidation and was measured to investigate the impact of rutin on plant cell membranes. A 0.1 g fresh sample was homogenized in 5 mL of 5% trichloroacetic acid (TCA). The cell suspension was centrifuged at 3000 rpm for 10 min. Then, 2 mL of 0.67% thiobarbituric acid (TBA) was added to the 2 mL supernatant and kept in a 100°C water bath for 30 min. After cooling, the mixture was centrifuged again at 3000 rpm for 10 min. The absorbance

of the supernatant was measured at 450 nm, 532 nm, and 600 nm.

The loss of cell membrane integrity in root, stem, and leaf tissues was estimated with electrolyte leakage (EL) assessed by a conductivity meter. A 1.0 g sample was transferred into 20 mL of deionized water and kept for 1 h at room temperature, and the conductivity of the solution was recorded as E_0 . Then, the mixture was kept in a 100 °C water bath for 15 min, and the conductivity was marked as E_1 . EL (%) was calculated by the ratio of E_0 to E_1 .

Cell relative vitality (CRV) (same for cell death) was determined by using the Evans Blue method (Ghasemi et al. 2020). Plant samples were incubated for 20 min with 0.25% (W/V) Evans Blue and then washed extensively with deionized water to remove excess and unbound dye. The dyed sample was boiled in 2 mL of 1.5% (W/V) sodium dodecyl sulfate (SDS) for 10 min. The absorbance of the supernatant at 600 nm was denoted C_0 . The absorbance of the supernatant heated at 100 °C for 10 min was set as 100% cell death ($C_{100\%}$). CRV was calculated as follows:

$$CRV = (1 - \frac{C_0}{C_{100\%}}) \times 100\% \quad (1)$$

2.3. Measurement of Cd content

Dried plant samples were ground into powder by a grinding instrument. Then, 0.1 g samples from root, stem and leaf parts were placed in a polytetrafluoroethylene crucible. Each sample was soaked for one night in 10 mL of mixed acid ($HNO_3:HClO_4 = 9:1$). Then, the samples were digested on an electric hot plate until nearly dry. After brining the volume to 10 mL with 1% HNO_3 and filtration through a 0.45 μm membrane filter, the samples were measured by inductively coupled plasma mass spectrometry.

2.4. Analysis of Cd subcellular distribution

A frozen sample (approximately 0.5 g) was homogenized by a plant grinder in 5 mL of precooled extraction buffer (50 mM Tris-HCl, 250 mM sucrose, 1 mM mercaptoethanol, pH 7.5). The homogenate was separated into four subcellular fractions by differential centrifugation. The mixture was first centrifuged at 1000 rpm for 20 min, and the deposition was sampled as a cell wall fraction (F_{CW}) containing the cell wall and cell wall debris. Then, the supernatant solution was centrifuged at 3500 rpm for 20 min to obtain a deposit that was treated as the organelle fraction (F_O) (excluding the vacuole). To isolate the cell membrane fraction, the supernatant was further centrifuged again at 10000 rpm for 20 min. The resulting supernatant was the soluble fraction (F_S) containing vacuolar solution and cytoplasmic solution, and the precipitate was denoted the cell membrane fraction (F_{CM}). These four fractions used the digestion and detection methods outlined in the previous section.

2.5. Determination of rutin, cysteine, glutathione and phytochelatins

Sneller FE (2000) method was used to determine nonprotein sulfhydryl compounds (cysteine, GSH and PCs). A 0.1 g fresh plant sample with trifluoroacetic acid (0.1%, 2 mL) was ground into a homogenate. Then, the sample was centrifuged (4 °C, 10000 rpm, 10 min) after ultrasonic treatment in an ice bath for 30 min. Standard solutions of five sulfhydryl compounds (0.2 mg·mL⁻¹) were prepared with 0.1% trifluoroacetic acid and diluted to 1.25, 2.5, 5, 10, and 20 ng·μL⁻¹. The procedure of precolumn derivatization of samples and standard series was as follows: 4-hydroxyerhyllpiperazine-1-propanesulfonic acid (0.2 M, 450 μL) and monobromobimane (25 mM, 10 μL) were added to 250 μL of supernatant and reacted in a 45 °C water bath for 30 min. Methanesulfonic acid (1 mM, 300 μL) was

added to terminate the reaction. The mixture was filtered through a 0.45 µm filter membrane and stored at 4 °C. LC-20 high-performance liquid chromatography was used to determine the contents of cysteine, GSH and PCs.

2.6. Data acquisition and statistical analysis

The bioconcentration factor (BCF) is described as the ability of plants to accumulate elements from the substrate. Phytoextraction rate (PER) is broadly defined as the efficiency of the extraction of HMs from soil to plants and are associated with BCFs, plant biomass and soil physics and chemical characteristics.

BCF and PER can be calculated by the following equations:

$$BCF = \frac{\text{Metal conc. (plant part)}}{\text{Metal conc. (soil)}} \quad (2)$$

$$PER = \frac{\text{Metal conc. (plant)} \times \text{plants biomass}}{\text{Metal conc. (soil)} \times \text{soil mass}} \times 100\% \quad (3)$$

Collection and aggregation of raw data was conducted using EXCEL, and figures were generated with GRAPHPAD PRISM8 and HILOT (www.hiplot.com). All data were statistically analyzed using SPSS 24, and significant differences between variables were determined using one-way analysis with the least significant difference (LSD) post hoc test. Differences were statistically significant when $p < 0.05$.

3. Results

3.1. Biomass

Fig. 1 Cumulative Histogram of biomass under different Cd conditions and rutin treatments in *A. hypochondriacus*. The red line of TB-CK represents the total biomass of original control group without Cd stress and rutin addition. The red dotted line represents the S.D. of TB-CK. The total biomass in the histogram represents the sum of the biomass of roots, stems and leaves. Asterisks indicate significant differences: $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***). Each value represents the mean $\pm$ S.D. of three independent experiments.

Growth characteristics are significant indicators used for phytoremediation evaluation. Plant phenotyping was performed to determine the effect of exogenous rutin on *A. hypochondriacus*. Fig. 1 displays an overview of changes in the dried biomass of *A. hypochondriacus* under different Cd stress and rutin treatment levels. What is striking about the biomass data in Fig. 1 is that exogenous rutin can increase the total biomass in all experimental groups in comparison to the nonrutin control groups under all Cd conditions. Notably, in the R1.5 and R5 treatments under 10 and 25 mg·kg⁻¹ Cd stress, *A. hypochondriacus* demonstrated a considerable biomass increase in roots, stems, and leaves, and the total

biomass was significantly increased by 83.79% and 93.48% and 91.98% and 60.27% compared with the R0 treatment. Under 10 and 25 mg·kg⁻¹ Cd conditions, the biomass of roots, stems and leaves increased synergistically, resulting in a distinct increase in total biomass. However, under higher Cd conditions (50 and 100 mg·kg⁻¹), the biomass of roots tended to stabilize, and the accretion of total biomass mainly relied on the stems and leaves. It was noted that almost no conspicuous toxicity symptoms of Cd were found in the biomass accumulation of *A. hypochondriacus* with exogenous rutin application. Furthermore, the biological dry matter in the 10, 25, 50, and 100 mg·kg⁻¹ Cd treatments with rutin spray was 1.41–1.49, 1.07–1.59, 1.10–1.23, and 1.22–1.36 times higher than in the non-Cd-rutin control group, respectively. For maximal biomass results in this study, a higher concentration of rutin spraying was optimum under medium-low Cd stress (10, 25, and 50 mg·kg⁻¹ Cd application), while under higher Cd stress (100 mg·kg⁻¹), relatively low rutin application was sufficient.

3.2. Cd accumulation

Fig. 2 Cd concentration in roots, stems, and leaves and Cd extraction amount of *A. hypochondriacus* under various Cd conditions and rutin treatments. (a) Cd concentration in leaves, (b) Cd concentration in stems, (c) Cd concentration in roots, (d) total Cd extraction amount of whole plant calculated by Cd concentration and biomass of *Amaranthus hypochondriacus*. Each value represents the mean $\pm$ S.D. of three independent

experiments.

The Cd concentrations in roots, stems, and leaves were significantly changed with the application of rutin. According to Fig. 2 (a), the Cd concentration in leaves increased visibly under all Cd conditions under each rutin treatment. Under lower Cd stress (Cd 10), different rutin concentrations (R 0.5, R 1.5, and R 5) did not cause a dose-dependent effect on Cd accumulation in the leaves of *A. hypochondriacus*. However, a significant difference between the rutin groups and the nonrutin groups was evident; the values of the rutin treatments were 1.45, 1.26, and 1.39 times higher than those of the R0 treatment. Under medium Cd stress (25 and 50 mg·kg⁻¹), a dose-dependent effect of rutin appeared. The Cd concentrations in leaves under 25 mg·kg⁻¹ Cd condition were 105.76, 132.96, and 121.02 mg·kg⁻¹ in R0.5, R1.5, and R5 groups, respectively, and 1.76–2.22 times higher than in the R0 group. Under 50 mg·kg⁻¹ Cd application, with increasing rutin addition, the Cd concentration in leaves showed an increasing trend and peaked in the R5 group, which was 2.88 times higher than that in the R0 group, exhibiting a terrific Cd uptake-promoting effect of rutin. Nevertheless, under 100 mg·kg⁻¹ Cd application, relatively low rutin application caused higher Cd accumulation in leaves. For stems of *A. hypochondriacus* (Fig. 2 (b)), the Cd concentration showed no dramatic improvement in all rutin treatments under relatively low Cd conditions (10 and 25 mg·kg⁻¹). An increase in Cd concentration was observed under relatively high Cd conditions (50 and 100 mg·kg⁻¹) in all rutin treatments except in the Cd 100-R0.5 group. The maximal Cd concentrations were 1.56 (Cd 50-R5) and 1.29 (Cd 100-R1.5) times higher than those in the R0 group. As shown in Fig. 2 (c), only the R1.5 and R5 treatments under 25 mg·kg⁻¹ Cd application reported significantly higher root Cd concentrations than the R0 group among all conditions under relatively low

Cd stress (10 and 25 mg·kg⁻¹). Under relatively high Cd stress (50 and 100 mg·kg⁻¹), the rutin treatment groups still had a higher root Cd concentration.

3.3. Cd extraction amount, BCF, and PER

For HM-contaminated soil, Cd extraction of plants calculated by biomass and Cd concentration are critical evaluation indices for phytoremediation effects. Since both biomass and Cd concentration were affected by rutin, the alteration of Cd extraction amount was also obvious under the same Cd stress with different rutin treatments. In Fig. 2 (d), there was a clear trend that in all the rutin treatment groups, the Cd extraction amount changed greatly. The maximal values were 189.82, 526.67, 963.22, and 1699.09 µg·plant⁻¹ under 10, 25, 50, and 100 mg·kg⁻¹ Cd conditions, respectively, which were 2.35, 3.19, 2.72, and 2.86 times higher than that in the R0 treatment.

Table 1 Effect of rutin on BCF, and PER of *A. hypochondriacus* under various Cd conditions

		Cd 10	Cd 25	Cd 50	Cd 100
BCF	R 0	3.02±0.14b	2.29±0.16b	2.16±0.11c	2.26±0.14c
	R 0.5	3.29±0.09ab	2.62±0.10ab	3.62±0.09b	2.88±0.18bc
	R 1.5	3.23±0.13ab	3.82±0.23a	4.27±0.07ab	3.12±0.09b
	R 5	3.68±0.26a	3.36±0.15b	4.79±0.10a	3.86±0.16a
PER (%)	R 0	0.07±0.01b	0.05±0.01b	0.06±0.01b	0.05±0.01b
	R 0.5	0.09±0.01b	0.08±0.01b	0.11±0.01ab	0.11±0.02ab
	R 1.5	0.13±0.02ab	0.18±0.02a	0.15±0.02a	0.11±0.01ab
	R 5	0.16±0.01a	0.13±0.01ab	0.16±0.02a	0.14±0.01a

The results of the BCF and PER are summarized in Table 1. Rutin application at each concentration (R0.5, R1.5, and R5) increased the BCF of *A. hypochondriacus* for the treatments with all Cd stresses. Especially in the R5 group, the BCF varied from 3.678, 3.366, 4.798, and 3.862 and was 1.46–2.22 times

higher than that in the R0 treatment. The presence of rutin also had a significant effect on the PER of *A. hypochondriacus*. The highest PER (0.18%) was found in the Cd 25-R1.5 group, and other treatments followed the trend that an increase in rutin concentration caused a higher PER.

3.4. Malondialdehyde, electrolyte leakage and cell vitality

Fig. 3 Changing MDA, EL, and CRV in *A. hypochondriacus* under various Cd conditions and rutin treatments.

MDA, EL, and CRV represent malonaldehyde, electrolyte leakage, and cell relative vitality. (a) MDA concentration in roots, (b) MDA concentration in stems, (c) MDA concentration in leaves, (d) EL in roots, (e) EL in stems, (f) EL in leaves, (g) CRV in roots, (h) CRV in stems, (i) CRV in leaves. Asterisks indicate significant differences: $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***), $P < 0.0001$ (****). Each value represents the mean $\pm$ S.D. of three independent experiments.

MDA, EL and CRV were measured to evaluate the oxidative damage of membrane lipids in response to rutin application under Cd stress. The MDA content in three tissue parts of *A. hypochondriacus* is shown in Fig. 3 (a)-(c). The MDA content in roots and stems decreased sharply by 53.97% and 48.26% compared to that in the R0 group, respectively, in the R0.5 treatment under low Cd stress ($10 \text{ mg}\cdot\text{kg}^{-1}$), and the MDA content in the R1.5 and R5 groups was still lower than that in the R0 group. Under high Cd stress ($100 \text{ mg}\cdot\text{kg}^{-1}$), with the exception of stems in the R5 group, all experimental groups presented a marked reduction in the MDA content of roots and stems. These decreasing trends occurred in leaves as well. As the rutin concentration increased, the MDA content in leaves showed a gradual decreasing trend under both high and low Cd stress levels, which declined by 19.04%, 21.43%, and 41.86% in the R0.5, R1.5 and R5 treatments under $10 \text{ mg}\cdot\text{kg}^{-1}$ Cd conditions, respectively, and 13.49%, 17.31%, and 14.28% under $100 \text{ mg}\cdot\text{kg}^{-1}$ Cd conditions, respectively. Fig. 3 (d)-(f) shows that there was an alteration in EL with rutin intervention. In relation to that in the R0 group, EL in the roots and stems of *A. hypochondriacus* was decreased by 14.15% and 22.73% in the R1.5 treatment, respectively. In terms of leaves, the EL dropped by 7.12%, 20.05%, and 8.36% in the R0.5, R1.5 and R5 groups compared to the R0 group under $10 \text{ mg}\cdot\text{kg}^{-1}$ Cd conditions, respectively, and 6.12%, 20.73%, and 7.53% under $100 \text{ mg}\cdot\text{kg}^{-1}$ Cd conditions, respectively. The CRV in the rutin treatments was higher than or equal to that in the control group (R0)

280 (Fig 3 (g)-(i)). The peak CRV value occurred in leaves under Cd 10-R5 conditions and was 1.28 times
281 higher than that of the control. Furthermore, MDA, EL, and CRV were significantly affected by rutin,
282 which changed the physiological parameters of the plants.

283 **3.5. Cd subcellular distribution**

284
 285 Fig. 4 Proportion of subcellular distribution of Cd in leaves, stems, and roots of *A. hypochondriacus* under
 286 various Cd conditions and rutin treatments. F_S , F_O , F_{CM} , and F_{CW} represent cell wall fraction, organelles
 287 fraction, cell membrane fraction, and soluble fraction. (a) proportion of subcellular distribution of Cd in

leaves, (b) proportion of subcellular distribution of Cd in stems, (c) proportion of subcellular distribution of Cd in roots.

The proportion of Cd in the subcellular fractions of *A. hypochondriacus* in roots, stems and leaves was in the order of cell wall fraction (F_{CW}) > soluble fraction (F_S) > organelle fraction (F_O) $\approx$ cell membrane fraction (F_{CM}). Interestingly, the Cd proportion of F_{CW} in leaves (Fig. 4 (a)) increased considerably under high Cd stress ($100 \text{ mg} \cdot \text{kg}^{-1}$) with rutin application, which was 62.44%, 59.49%, and 56.34% in the R0.5, R1.5 and R5 groups, respectively, compared to the R0 group value of 30.50%. Nevertheless, under low Cd stress ($10 \text{ mg} \cdot \text{kg}^{-1}$), only the R5 group showed an increase in the proportion of F_{CW} Cd. A surprising correlation was observed between rutin application and the proportion of Cd in the F_{CM} , which showed a slight decreasing trend, and the soluble Cd levels were increased in leaves. The change in the proportion of Cd in the subcellular fractions under $10 \text{ mg} \cdot \text{kg}^{-1}$ Cd conditions was similar in stems and roots (Fig. 4 (b)-(c)), and the proportion of Cd in F_{CW} in the R0.5 and R5 groups was higher than in the R0 group. However, there were no substantial changes in the proportion of Cd in the F_{CW} in stems with or without rutin applied under $100 \text{ mg} \cdot \text{kg}^{-1}$ Cd stress. In contrast, the subcellular distribution of Cd in roots (Fig. 4 (c)) changed with the same rule in accordance with leaves.

3.6. Rutin, cysteine, glutathione and phytochelatin analysis

Fig. 5 Changes in rutin, Cys, GSH, PC₂, PC₃, and PC₄ in leaves of *A. hypochondriacus* under various Cd conditions and rutin treatments. Cys and GSH represent cysteine and glutathione, PC₂, PC₃, and PC₄ are phytochelatins of PC_n which are oligomers of glutathione. Each value represents the mean ± S.D. of three independent experiments.

Rutin was only detected in the leaves of *A. hypochondriacus* and revealed no significant alterations in response to Cd stress (Fig. 5 (a)). Under the same level of Cd stress, exogenous rutin caused a rise in endogenous rutin concentration. However, the rutin concentration in the R5 group was not much higher than that in the R1.5 group, and even occasionally, it was lower than that in the R1.5 group. Moreover, there was a surprising outcome in Fig. 5 (b) that the concentration of Cys in the absence or presence of rutin showed no inductive improvement, but the average level of Cys varied among the different Cd

groups. The maximal level of Cys was found under 50 mg·kg⁻¹ Cd conditions, with no observed improvement with rutin application. The Cys levels in the 10 and 100 mg·kg⁻¹ Cd groups showed very different changes with rutin intervention.

The observed result was also somewhat counterintuitive: *A. hypochondriacus* cultivated solely with Cd displayed a declining trend in GSH concentration with increasing Cd stress (Fig. 5 (c)). However, the concentration of GSH increased drastically with the application of rutin, especially in the R5 group, which was 1.82, 2.31, 2.94, and 2.74 times higher than that in the R0 treatment under 10, 25, 50, and 100 mg·kg⁻¹ Cd conditions, respectively. For PCs (Fig. 5 (d)-(f)), a high level of Cd exposure (50 and 100 mg·kg⁻¹) caused the accumulation of PC₂, PC₃, and PC₄. The concentrations of PC₂, PC₃, and PC₄ reached their peak values in the R1.5 group under 100 mg·kg⁻¹ Cd conditions. Under relatively low Cd stress (10 and 25 mg·kg⁻¹), the PCs showed relatively low response variation levels. Under 10 mg·kg⁻¹ Cd conditions, only PC₂ and PC₄ in all rutin groups showed an increase, and the change response of PC₃ could not be correlated with rutin. Under 25 mg·kg⁻¹ Cd conditions, an upward trend was only found in PC₄ with increasing rutin application.

The concentrations of nonprotein sulphydryl compounds in roots and stems are shown in Fig. S1 and Fig. S2. Following the addition of rutin, a significant increase ($p < 0.05$) in Cys and GSH was recorded in roots and stems. There was no significant difference in Cys and GSH content under different Cd stress levels, but the addition of rutin significantly changed the balance. For PCs, plants cultivated solely with different Cd levels showed alterations, but the effect of rutin was not significant under the same Cd conditions. However, due to the interaction between Cys, PCs and GSH for chelating Cd, their contributions to Cd accumulation are shown in the next section by statistical methods.

3.7. Correlation analysis and multiple regression model

As shown in Fig. S3, a statistically significant correlation was observed in three clustering groups: Cys and GSH contents and rutin content, PCs content and soil Cd concentration, and Cys and rutin contents and PCs contents. This result indicated that the addition of rutin enhanced the chelation response of PCs to Cd. However, as Cys and GSH are the precursors for the synthesis and conversion of PCs, the importance of Cd, rutin and nonprotein sulfhydryl compounds is unclear. Prediction models for Cd content in various organs were established to identify the most significant influencing factors for Cd accumulation.

A stepwise forward multiple regression method was used to predict Cd accumulation in roots, stems, leaves, and whole plants of *A. hypochondriacus*. The models were as follows:

$$Cd_{wp} = 3.652 Cd_{soil} + 2.067 R_{en} - 0.047 (PC_{2R})^2 - 288.584 \quad (4)$$

$$Cd_L = 17.347 (\ln PC_{3L})^2 - 102.74 \quad (5)$$

$$Cd_S = 1.449 Cd_{soil} + 0.272 PC_{2L} \quad (6)$$

$$Cd_R = 1.076 PC_{3L} + 50.449 \quad (7)$$

where Cd_{wp} , Cd_L , Cd_S , and Cd_R are the concentration of Cd in whole plant, leaves, stems, and roots, respectively ($mg \cdot kg^{-1}$ DW); Cd_{soil} is the Cd concentration in soil (designed value, $mg \cdot kg^{-1}$); R_{en} is the endogenous rutin concentration in plants ($mg \cdot kg^{-1}$); PC_{2R} is the PC_2 concentration in roots; and PC_{2L} and PC_{3L} are the PC_2 and PC_3 concentration in leaves, respectively, with $p < 0.001$ in the T-test of each coefficient.

Fig. 6 The comparison between the predicted values and measured values of Cd concentration in (a) whole plant, (b) leaf, (c) stem, and (d) root. The solid blue lines represent regression lines, and the gray area represent 95% prediction intervals. The regression equations and its R^2 are shown in the figure.

The comparison between the predictive value and measured value is displayed in Fig. 6. The model has good fitting, with coefficient of determination (R^2) values of 0.91, 0.84, 0.97, and 0.78 for whole plants, leaves, stems, and roots, respectively. The model of Cd_{wp} suggests that Cd stress in soil, the rutin concentration in plants and the PC_2 concentration in roots are crucial to Cd accumulation in whole plants.

This observation is also in line with a previous observation that rutin can enhance Cd uptake from soil to plants. The PC₂ concentration also indicated that the chelation and fixation of Cd in roots was also the key to increasing Cd accumulation. Additionally, PCs in the leaves play a crucial role in the model of Cd accumulation in leaves, stems, and roots, especially in leaves and roots, and PC₃ is primarily responsible for influencing Cd accumulation. The results showed that Cd content in all plant parts was highly correlated with PCs in leaves, which may be due to the application of rutin leading to the transfer of the Cd content from underground plant parts to aboveground plant parts and the enhancement of Cd fixation ability.

4. Discussion

4.1. Effect of rutin on cell membrane protection and cell tolerance

Lipid peroxidation is one of the most deleterious effects induced by HM exposure in plants and can directly cause biomembrane deterioration (Yadav 2010). It has been reported that rutin, as a natural flavone derivative, plays a protective role in oxidative stress due to a combination of metal chelation via the ortho-dihydroxy phenolic structure and free radical-scavenging activities (de Matos et al. 2020, Moridani et al. 2003, Rice-Evans et al. 1996) showed that rutin provides cytoprotection to lettuce seedlings through the coordination of mercury and cell membrane protection. In this study, *A. hypochondriacus* cultivated with rutin showed a lower MDA level in roots, stems, and leaves (Fig. 3), which indicated a lower degree of membrane lipoperoxidation. Moreover, plants under advisable rutin treatment displayed less EL in all tissue parts under Cd stress. At the same time, a higher CRV also confirmed that rutin could enhance cell tolerance and alleviate oxidative stress. This phenomenon could be attributed to two factors: one factor is that flavonoids could decrease the fluidity of the membrane hydrophobic core, which could impede the diffusion of free radicals, and the other factor is that rutin could prevent the access of several molecules to the hydrophobic region of the lipid bilayers, which induce oxidative damage to membrane components (Arora et al. 2000, Saija et al. 1995). Thus, flavonoids modify membrane-dependent processes such as free radical-induced membrane lipoperoxidation and interact with penetrating lipid bilayers, causing variation in their structure and fluidity (Sanchez-Gallego et al. 2010).

Since rutin can only be detected in the leaves, it is unknown whether the addition of rutin has a direct effect on the roots and stems. However, due to the increased protective effect of rutin on the cell

membrane in leaves, the tolerance of photosynthetic plant organs to Cd is elevated, which promotes this advantage in roots and stems at the same time.

4.2. Effect of rutin on the chelation and retention of cadmium

Most plants have high Cd concentrations in roots due to a restriction effect on the transport of Cd from roots to aboveground parts (Wang et al. 2020). Moreover, hyperaccumulators sequester excess HMs in root vacuoles to mitigate toxic effects in the cytoplasm (Lin & Aarts 2012). Many studies have emphasized the importance of roots in hyperaccumulator tolerance. However, it seems that through this investigation, PCs in leaves play a more critical role in the neutralization and sequestration of excessive Cd ions, which are key processes in maintaining cellular homeostasis (Yazdi et al. 2019). The GSH concentration in plants relates to upstream and downstream metabolites, and the metabolic process mainly includes synthesis, degradation and conversion (Gao et al. 2020); thus, the concentrations of Cys and PCs may not change in conformity with GSH. In this study, rutin addition under Cd stress in *A. hypochondriacus* caused a significant increase in GSH, which is the foundation of Cd immobilization and compartmentalization in vacuoles (Zhan et al. 2017). This study also demonstrated the correlation between rutin and GSH (Fig. S1). As a precursor of PCs synthesis, GSH affects the chelation and fixation of Cd. Rutin has a strong protective effect against GSH oxidation because the structures produced by its oxidized form do not react well with GSH due to the lack of 3-hydroxyl groups in the flavonoid structure (Lopez-Revuelta et al. 2006). Predictive models were used to define the nonprotein sulfhydryl compounds that were most related to the accumulation of Cd. This method can explain the chelation response more obviously than the analysis of the variation trend of PCs alone. The results show that PC₂ and PC₃ in leaves are critical to Cd accumulation in all tissue parts and that rutin does cause accretion in

Cd accumulation in whole plants.

It is well established that the subcellular distribution of HMs is significantly altered by exogenous addition, species differences, or planting patterns (Niu et al. 2021, Waheed et al. 2021, Yu et al. 2019). The outcome of the subcellular distribution of Cd in *A. hypochondriacus* implies that the compartmentalization effect of Cd in the cell wall fraction under Cd stress is the first binding site for Cd accumulation contributing to Cd tolerance. Cd immobilization and compartmentalization in the cell wall is a sustainable approach to maintain cell homeostasis under Cd stress within endurance. However, it can be seen in Fig. 4 that when excessive Cd stress occurs, the fixation of the cell wall without rutin application will reach saturation. At this time, PCs in the vacuole and cytoplasm chelate excessive Cd ions to alleviate Cd toxicity, showing a sharp increase in the soluble Cd fraction proportion. At this point, the cells reach their maximum Cd accumulation capacity. Cd in chloroplasts and mitochondria of cells at higher Cd exposure can cause degradation of the photosynthetic apparatus (Gonçalves et al. 2009, Mwamba et al. 2016). Interestingly, with rutin addition, the Cd proportion in cell walls increased, and the Cd proportion in organelles decreased. This observation confirms that rutin can affect Cd accumulation in *A. hypochondriacus* by altering Cd subcellular distribution to enhance Cd tolerance.

Vacuolar sequestration and cell wall binding are generally considered to be the main storage methods for Cd in plant cells (Xin et al. 2014). There are different hypotheses about whether PCs-driven Cd retention occurs in vacuoles or cell walls. Several studies have suggested that PCs enhance the cytoplasm and vacuole compartmentalization of Cd to decrease Cd-induced oxidative damage (Figueira et al. 2014, He et al. 2011, Huang et al. 2021). However, research has also shown that the action of PCs in Cd tolerance is to temporarily bind Cd in the cytosol, with less contribution to Cd sequestration (Wojas

et al. 2010). Our subcellular studies show Cd accumulation in roots and leaves with an elevated proportion of Cd distribution in the cell wall rather than in the solution fraction. This confirms that PCs-driven Cd retention occurs in the cell wall to cope with high Cd exposure and therefore mitigate Cd toxicity to the cytoplasm and organelles. To date, it has been shown that flavonoids enhance plant tolerance to Cd and influenced Cd uptake in root cell walls (Li et al. 2015), and Das et al. (2021)) reported that S-induced elevated GSH enables PCs to bind with excess Cd, leading to increased Cd in the cell wall but not in the vacuole. Thus, intracellular mechanisms of elevating Cd tolerance in *A. hypochondriacus* are related to subcellular partitioning of Cd and well-coordinated physiological responses.

4.3. Effect of rutin on phytoextraction and phytoremediation efficiency

It is now well established from a variety of studies that exposure to excessive Cd stress could result in a loss of biomass, and plant biomass can be used to estimate the toxic effect of pollutants on plants (Liu et al. 2019). To date, studies have been carried out using exogenous additives to improve plant biomass and phytoextraction to reach a surpassing phytoremediation efficiency. For example, nitrogen fertilizers are most widely used to increase plant biomass and elevate accumulation because of their nutrient supply effect (Eissa &Roshdy 2018, Giansoldati et al. 2012, Ye et al. 2019). Urea was also used in Cd- and pyrene-contaminated soil remediation and increased the accumulation of soil Cd and the plant biomass of different parts of willow (Li et al. 2021). Many studies have shown that not only traditional fertilizer application but also acids, auxin, and chelate have similar or better phytoremediation properties (Guo et al. 2021, Guo et al. 2019, Lu et al. 2020, Wang et al. 2019). In this work, it was proven that exogenous flavonoids (rutin) also increased phytoextraction by increasing the biomass and Cd accumulation concentration in *A. hypochondriacus* (Fig. 1 and Fig. 2). However, the mechanism of function of rutin is

different from most of the preceding examples that are based on nutrition and improve the intensity of the antioxidant system. The main functions of rutin are (1) improving cell tolerance under Cd stress by reducing membrane damage and elevating cell vitality and (2) altering the subcellular distribution of Cd and the synthesis of compounds associated with Cd chelation to drive more Cd binding to the cell wall. It is therefore evident that exogenous rutin actively functions in eliminating morphological retardation and cell damage in *A. hypochondriacus* subjected to Cd stress. The increase in PER and BCF (Table 1) of *A. hypochondriacus* showed that rutin, as a leaf fertilizer additive, can be highly effective in phytoremediation of soil contamination in large fields.

5. Conclusion

The application of rutin showed substantial improvement in biomass, Cd concentration of each tissue part and Cd phytoextraction under Cd stress of *A. hypochondriacus*. It is considered according to the actual Cd concentration of Cd-contaminated soil, foliar application of rutin on the concentration of 1.5 mg·kg⁻¹ at the branching stage of *A. hypochondriacus* is enough to achieve the highest Cd phytoextraction amount. Furthermore, the underlying mechanisms responsible for this change were explored. Rutin maintains and preserves intracellular homeostasis by altering the permeability of cell membranes to alleviate the stress damage caused by Cd. Rutin also significantly affected the subcellular distribution of Cd, causing more Cd ions to be immobilized in the cell wall rather than in vesicles, which allowed the cells to tolerate stronger Cd stress. Robust prediction models of the Cd concentration were established in each tissue part and whole plant by stepwise forward multiple regression. The soil Cd concentration, rutin concentration and root PC₂ matched the amount of Cd in whole plants well. These models determined that PC₂ and PC₃ in leaves are most relevant for PCs-driven Cd retention. In summary, this study is the first to propose the application of endogenous phytoflavonoids to exogenous foliar sprays to achieve higher Cd phytoextraction amount. Furthermore, these findings provide insights into a less costly way to improve the low efficiency of phytoremediation.

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