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Original Research

Effects of Variable Sulfur Supply on the Accumulation, Subcellular Distribution, and Chemical Forms of Cadmium in *Hydrilla verticillata*

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Abstract

Indoor experiments were performed to determine the accumulation, subcellular distribution, and chemical forms of Cd at five S levels in *Hydrilla verticillata*. The Cd content increased from 1.229 mg/g to 3.329 mg/g in leaves, and decreased from 2.794 mg/g to 1.023 mg/g in roots, respectively. Excess S supply stimulated Cd assimilation in leaves as Cd accumulation was inhibited in roots. The Cd content in leaves at subcellular levels revealed that Cd was stored mainly in the soluble fraction (71.9-88.2%), and in small quantities in the cell wall (6.1-22.4%) and cell organelles (4.8-6.9%). As S increased, the Cd content in leaf soluble fractions and cell walls increased remarkably. The content of NaCl-extracted Cd in leaves increased as S supply increased, and this parameter was much higher than that of other Cd forms. In leaves, the Cd concentrations in the cell walls were significantly correlated with the chemical forms extracted by HAc, HCl, and NaCl, with correlation coefficients of 0.985, 0.964, and 0.957, respectively. The high correlation indicated that Cd in soluble fractions or cell walls was mainly in the form of pectates/protein, phosphate, and oxalate. The application of S alleviated Cd-induced oxidative stress by increasing the proline accumulation. Furthermore, sulfhydryl proteins such as glutathione and cysteine may play a crucial role in the reversal of Cd-induced oxidative stress.

Keywords: *Hydrilla verticillata*, sulfur, cadmium, subcellular distribution, chemical forms

Introduction

Heavy metal pollution has been considered a global environmental problem because heavy metals elicit toxic effects, persist in aquatic environments, and accumulate in increasing quantities inside living organisms along the food chain [1]. For example, cadmium (Cd) is a toxic and highly water-soluble element; Cd is readily taken up by plants, although this heavy metal is not essential for plants and is toxic to aquatic plants at cellular, physiological, biochemical, and molecular levels [2].

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Cd toxicity causes several symptoms, including growth retardation, photosynthesis inhibition, enzyme induction and inhibition, water interaction disruption, and ultrastructural changes [3-5].

Less expensive and destructive remediation strategies than current approaches have been extensively investigated [6-9]. For instance, phytoextraction is a valuable technique used to remove pollutants from the environment using plants. This technique has been employed to remediate contaminated water and soils and remove metals from sludge. Compared with other cleanup technologies, this technique provides many advantages, including low cost, in situ development, and minimum environmental impact [10]. Submerged macrophytes, which play important structural and functional roles in aquatic ecosystems, can accumulate metals [11] and be used to remove heavy metals from wastewaters. Metals adsorbed by plants can be trapped by the negative charges of the cell wall or be taken up into the cell cytoplasm and compartmented in some nonfunctional organs, such as vacuoles [12]. Adsorbed metals, such as Cd, can also be extracellularly excreted [13]. Cd-induced stress in plants can be alleviated through numerous strategies. For example, Cd detoxification occurs mainly through chelation via sulfur (S)-containing ligands, such as glutathione (GSH), cysteine (Cys), and phytochelatins (PCs), which are GSH polymers [14].

Submerged plants can absorb nutrients and chemicals from overlying water and sediments because they are exposed to both environments [15]. S is an essential macronutrient that regulates plant growth, development, and responses to biotic and abiotic stresses. Under Cd stress, S improves growth, increases anti-oxidative capacity, and reduces ROS and lipid peroxidation. A high amount of sulfate in a culture medium promotes high Cd tolerance because of an increase in thiol compound biosynthesis [16]. S addition can restrain Cd uptake, increase biomass, and promote nonprotein thiol (NPT) pool synthesis, including PCs and GSH synthesis, in rice to alleviate Cd toxicity [17]. High S availability in soil protects mustard from Cd toxicity by improving AsA and GSH content in leaves [18]. S deficiency increases the level of oxidative stress and restricts the GSH biosynthesis pathway in *Arabidopsis thaliana* under Cd stress [19]. Therefore, high S levels can stimulate high GSH levels to enhance Cd tolerance in plants. S can decrease Cd accumulation and alleviate Cd toxicity in plants because of the formation and precipitation of detectable CdS [20]. However, to our best knowledge, the mechanisms by which S affects the subcellular distribution or chemical forms of Cd in plants have yet to be described.

The subcellular distribution and chemical forms of heavy metals may be associated with metal tolerance and detoxification in plants [21]. Ramos et al. [22] observed that Cd is mainly present in the cell wall fraction of lettuce. Conversely, Wang et al. [23] found that the highest amount of Cd is in the form of pectates/protein-integrated Cd and insoluble Cd-phosphate complexes. Hence, consistent results have yet to be obtained, and the relationship between subcellular distribution and chemical forms of heavy metals was still unclear.

Hydrilla verticillata is a common aquatic angiosperm, which is distributed worldwide and characterized by a rapid growth rate; this species has been described as a potential accumulator of heavy metals, such as Pb, Hg, Cu, Cd, Cr, Ni, and As [24-27]. However, the effects of S on the subcellular distribution and chemical form of Cd in *H. verticillata* have been rarely reported. Antioxidants such as GSH are important molecules contributing to the tolerance of plants to Cd. Therefore, studies should be performed to determine whether the variation of a source of S in the medium affects the subcellular distribution and chemical forms of Cd through an interactive effect.

This study aimed to assess the accumulation, subcellular distribution, and chemical forms of Cd in *H. verticillata* under the effect of S and to investigate the existence of an interaction between Cd and S as a factor that altered Cd toxicity. This study provided useful information for the phytoremediation of Cd and the development of techniques to reduce Cd toxicity in aquatic plants in the presence of S.

Materials and Method

Plant Materials and Cadmium Treatments

H. verticillata was collected from unpolluted bodies of freshwater in Nanjing, China. For experimental studies, plants of approximately the same height (15 cm) and fresh weight (8.0 g) were selected and washed with running tap water and distilled water; then they were kept in a glass aquarium containing 1/10 Hoagland solution for 2 weeks at photosynthetic photon flux density of 114 μmol $(m^2\ s)^{-1}$ for a photoperiod of 14 h at a temperature of 25/20ºC (day/night). There were five sets of experiments, each having a different level of S supply (0, 0.25, 0.5, 1, and 2 mmol/L). After keeping the plants in different sets of S supply for 5 days, they were exposed to several S and Cd treatments carried out as follows: S (Na_2SO_4) 0, 0.25, 0.5, 1, and 2 mmol/L with Cd ($CdCl_2$) 100 μmol/L. At the end of the experiment (after 7 d exposure to Cd and S), plants were separated into roots, stems, and leaves, and then immediately frozen in liquid N_2 and kept frozen until use. All experiments were performed in triplicate.

Cadmium Subcellular Distribution

Fresh plant materials (0.20 g) were homogenized in prechilled extraction buffer (comprising 0.25 mol/L sucrose, Tris-HCl buffer solution (pH 7.5), and 1.0 mmol/ DL-dithioerythritol) with a chilled mortar and pestle. Cells were separated into three fractions,

namely cell wall, soluble fraction, and an organelle-containing fraction, using the differential centrifugation technique as suggested by Weigel and Jager [28] with some modifications. The homogenate was centrifuged at 3,000 rpm for 15 min, and the precipitation was designated as cell wall fraction, consisting mainly of cell walls and cell wall debris. The resulting supernatant solution was further centrifuged at 15,000 rpm for 45 min. The resulting precipitate was referred to as the "cell organelles fraction" and the supernatant solution was referred to as the "soluble fraction." All steps were performed at 4ºC. The three separated fractions were then dried and wet-digested for Cd analysis.

Chemical Formation of Cadmium

The chemical forms of Cd were determined using the method described by Wu et al. [29]. The experiment was carried out with the designated solutions in the following order to determine the chemical forms of Cd in different parts of *H. verticillata*: 1) 80% ethanol, extracting inorganic Cd giving priority to nitrate/nitrite, chloride, and aminophenol Cd; 2) deionized water, extracting water-soluble Cd-organic acid complexes and $Cd(H_2PO_4)_2$; 3) 1 M NaCl, extracting pectates and protein-integrated Cd; 4) 2% acetic acid (HAc), extracting undissolved Cd phosphate, including $CdHPO_4$, $Cd_3(PO_4)_2$, and other Cd-phosphate complexes; and 5) 0.6 M HCl, extracting Cd oxalate. Cd was not detected in the residues because of its low concentration in the samples.

Frozen tissues were homogenized in extraction solution with a mortar and a pestle, diluted at a ratio of 1:100 (w/v), and shaken for 22 h at 25ºC. Afterward the homogenate was centrifuged at 5,000×g for 10 min, obtaining the first supernatant solution in a conical beaker. The sediment was resuspended twice in the extraction solution and shaken for 2 h at 25ºC and centrifuged at 5,000×g for 10 min. The resulting supernatant was pooled from the suspension and each of the five extraction solutions was centrifuged. Each of the pooled supernatant solutions was evaporated on an electric plate at 70ºC until a constant weight was obtained and digested with concentrated nitric acid.

Digestion and Analysis of Cadmium Content

Before metal analysis was performed, all plant parts, cell walls, and cell organelle fractions were wet-digested in 5 mL of concentrated HNO_3 and then diluted with ultrapure water. In the case of the soluble fraction, 5 mL of concentrated HNO_3 was added to 1 mL of solution before metal content analysis. Cd concentration was measured using either a flame or a furnace atomic absorption spectrophotometer (AASM6, Thermo Elemental, USA). A reagent blank and a certified reference material (bush twigs and leaves, GBW07602 from the National Research Center for Standard Materials in China) were included for quality assurance. Repeated analysis of the reference material gave 0.135± 0.08 mg Cd kg^{-1} DW, which is in accordance with the certified value of 0.14±0.06 mg Cd kg^{-1} DW.

Determining the Contents of Proline, Cys, and GSH

Proline content in leaves was determined spectrophotometrically by adopting the ninhydrin method of Bates et al. [30]. Fresh leaf samples (about 0.3 g) were homogenized in 3 mL of 3% sulphosalicylic acid. The homogenate filtrate was reacted with 1 mL each of acid ninhydrin and glacial acetic acid for 1 h in a test tube and placed in a water bath at 100ºC. The mixture was extracted with toluene and absorbance was measured at 520 nm using L-proline as a standard.

Cys content in leaves was determined spectrophotometrically by adopting the method of Gaitonde [31]. Fresh leaf (0.5 g) tissue was homogenized in 5% (w:v) ice-cold perchloric acid. The suspension was centrifuged at 2,800×g for 1 h at 5ºC, and the supernatant was filtered. One mL of filtrate was treated with acid ninhydrin reagent and the absorption was read at 560 nm. The amount of Cys was calculated using calibration curve obtained for standard Cys.

GSH content was determined following the method of Anderson [32]. Fresh leaves (0.5 g) were homogenized in 2.0 mL of 5% sulphosalicylic acid under cold conditions. The homogenate was centrifuged at 10,000×g for 10 min. To 0.5 mL of supernatant, 0.6 mL of phosphate buffer (100 mM, pH 7.0) and 40 µL of 5'5'-dithiobis-2-nitrobenzoic acid (DTNB) were added. After 2 min the absorbance was read at 412 nm.

Data Analysis

All experimental data as means of three replicates were processed through Origin Pro. 8.0. One-way ANOVA was used to calculate statistical significance followed by Dunnett's test as a post-hoc test to independently compare each exposure group. All statistical analyses were run separately using SPSS 16.0 software, and p value of less than 0.05 was considered to be statistically significant. The data were shown as mean ± standard error (SE).

Results

Cadmium Content in Roots, Stems, and Leaves

Cd content in roots and stems generally declined with increasing S concentration following a dose-response relationship, whereas in leaves, Cd levels showed the opposite trend (Fig. 1). As the S levels increased,

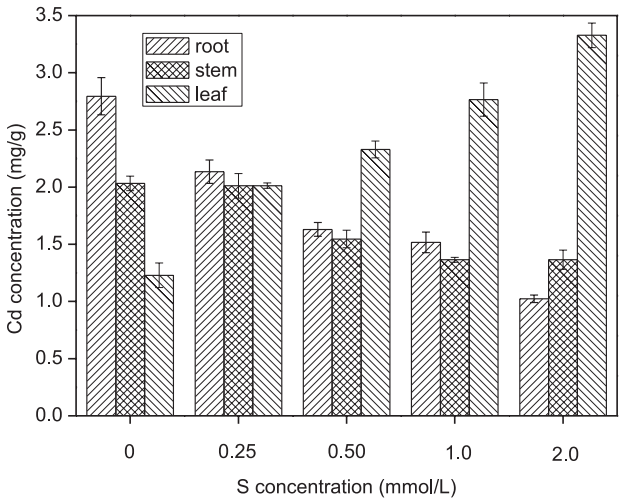

Fig. 1. Cd concentrations in different parts of *H. verticillata* after 7 d exposure to Cd.

the Cd content decreased from 2.794 mg/g to 1.023 mg/g in roots and decreased from 2.023 to 1.365 mg/g in stems, respectively; however, Cd content in leaf increased from 1.229 mg/g to 3.329 mg/g. This indicated that S supply might inhibit Cd assimilation in roots while stimulating Cd accumulation in leaves. Significant correlation was found between Cd concentrations in leaves and S concentration in the medium ($R = 0.979$, $p<0.01$).

Effect of Sulfur on Subcellular Distribution of Cadmium

As shown in Table 1, most Cd accumulated in soluble fractions and in cell walls in *H. verticillata*, with less Cd present in organelle fractions (Table 1). Analysis of Cd content in leaves at the subcellular level showed that Cd was stored mainly in the soluble fraction (71.9-88.2%) and in smaller quantities in cell wall (6.1-22.4%) and in cell organelles (4.8-6.9%). As S concentration increased, Cd contents in root-soluble fractions and cell walls declined gradually; however, Cd content increased remarkably in cell walls and soluble fractions in leaves. Significant correlation was found between S concentration and Cd concentrations in cell walls or the soluble fractions in leaves, which indicated that S played an important role in Cd accumulation and detoxification in *H. verticillata*.

Effect of Sulfur on Chemical Forms of Cadmium

The different concentration of chemical forms of Cd in leaf, root, and stem of *H. verticillata* are shown in Figs 2, 3, and 4, respectively. The results clearly indicate that chemical forms of Cd extracted with 1 M NaCl (F_{NaCl}) and 2% HAc (F_{HAc}) were predominant in all parts of *H. verticillata*. The chemical forms of Cd extracted with 1 M NaCl in leaves increased from 1.053 mg/g

Table 1. Subcellular distribution of Cd in different parts of *H. verticillata* after 7 d exposure; the different letters represent significant differences at $p<0.05$ level under five S levels.

Parts of *H. verticillata*	S Level	Cd in subcellular fractions (mg/g, FW)			
		Cell wall	Cell organelles	Soluble fraction	Total
Leaf	S1	0.102±0.021 c	0.059±0.022 a	1.047±0.051 b	1.207±0.094 b
	S2	0.126±0.032 c	0.117±0.037 a	1.821±0.085 a	2.064±0.154 a
	S3	0.307±0.022 b	0.165±0.069 a	1.905±0.116 a	2.377±0.207 a
	S4	0.535±0.102 a	0.150±0.093 a	2.062±0.293 a	2.745±0.488 a
	S5	0.724±0.135 a	0.182±0.072 a	2.326±0.307 a	3.232±0.514 a
Root	S1	0.433±0.027 a	0.117±0.003 a	2.280±0.421 a	2.829±0.451 a
	S2	0.382±0.051 a	0.134±0.011 a	1.689±0.279 a	2.205±0.241 a
	S3	0.358±0.100 a	0.120±0.020 a	1.172±0.185 b	1.649±0.305 a
	S4	0.260±0.045 a	0.097±0.006 b	1.143±0.164 b	1.499±0.215 a
	S5	0.203±0.008 a	0.056±0.010 c	0.930±0.107 b	1.188±0.125 a
Stem	S1	0.368±0.052 a	0.090±0.010 a	1.761±0.125 a	2.219±0.187 a
	S2	0.313±0.039 a	0.075±0.016 a	1.710±0.197 a	2.099±0.252 a
	S3	0.291±0.091 a	0.065±0.019 a	1.163±0.189 b	1.519±0.299 b
	S4	0.299±0.027 a	0.063±0.025 a	1.030±0.050 b	1.393±0.102 b
	S5	0.240±0.036 a	0.063±0.036 a	1.156±0.147 b	1.459±0.219 b

to 2.334 mg/g, when S concentration increased from 0 mmol/L to 2 mmol/L. This result demonstrated that as S supply increased, inorganic forms of Cd decreased, and the proportion of Cd associated with pectates and proteins increased, indicating that S stimulated the production of sulfhydryl proteins, which then chelated more Cd. The chemical forms of Cd extracted with 0.6 M HCl increased from 0.39 mg/g to 0.64 mg/g, and the proportion of Cd extracted by 0.6 M HCl increased from 2.53% to 4.67%, suggesting that chelation by oxalate could be an essential detoxification mechanism for *H.verticillata* to tolerate Cd in the presence of S. The chemical forms of Cd extracted with 1 M NaCl decreased from 2.256 mg/g to 0.767 mg/g in roots, and decreased from 1.760 mg/g to 0.984 mg/g in stems when S concentration increased from 0 mmol/L to 2 mmol/L, indicating that S supply may help Cd transfer to leaves.

Relationship between Subcellular Distribution and Chemical Forms of Cadmium

The correlations between subcellular fractions and chemical forms of Cd in *H. verticillata* were determined. In leaves, very significant correlation of Cd concentrations was found between cell walls and the chemical forms extracted with HAc, HCl, and NaCl (Fig. 5), with correlation coefficients of 0.985, 0.964, and 0.957 ($p<0.01$), respectively. Very significant correlation of Cd concentration was also found between the soluble fractions and the chemical forms extracted with NaCl and HCl, with correlation coefficient of 0.977 and 0.974 ($p<0.01$). In roots, significant correlation of Cd concentration was found between cell wall and chemical forms extracted with HAc ($R = 0.966$, $p<0.01$) or between soluble fractions and chemical forms extracted with NaCl ($R = 0.967$, $p<0.01$). In stems, the correlations between subcellular fractions and chemical forms of Cd were quite similar to those in roots. These results

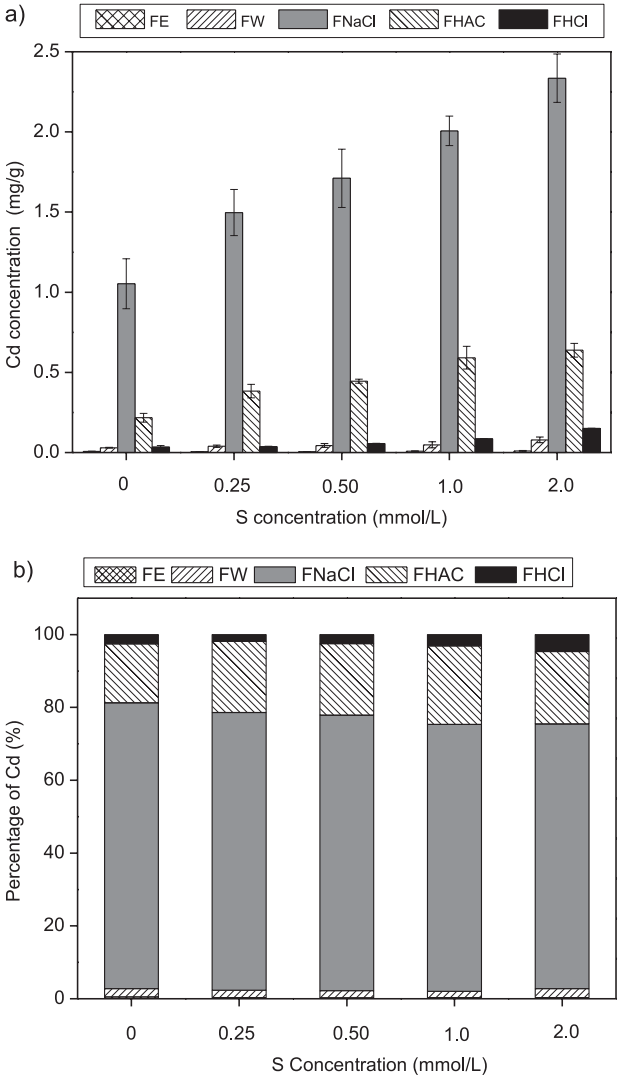

Fig. 2. Cd concentrations a) and percentages b) of different chemical forms of Cd in leaves of *H. verticillata* after 7 d exposure to Cd.

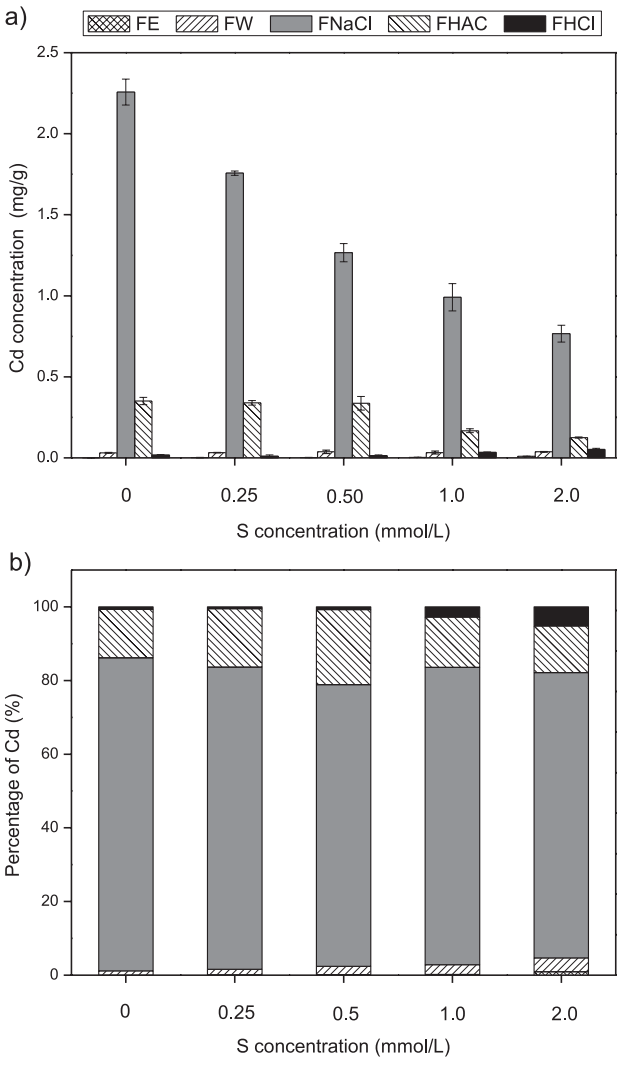

Fig. 3. Cd concentrations a) and percentages b) of different chemical forms of Cd in roots of *H. verticillata* after 7 d exposure to Cd .

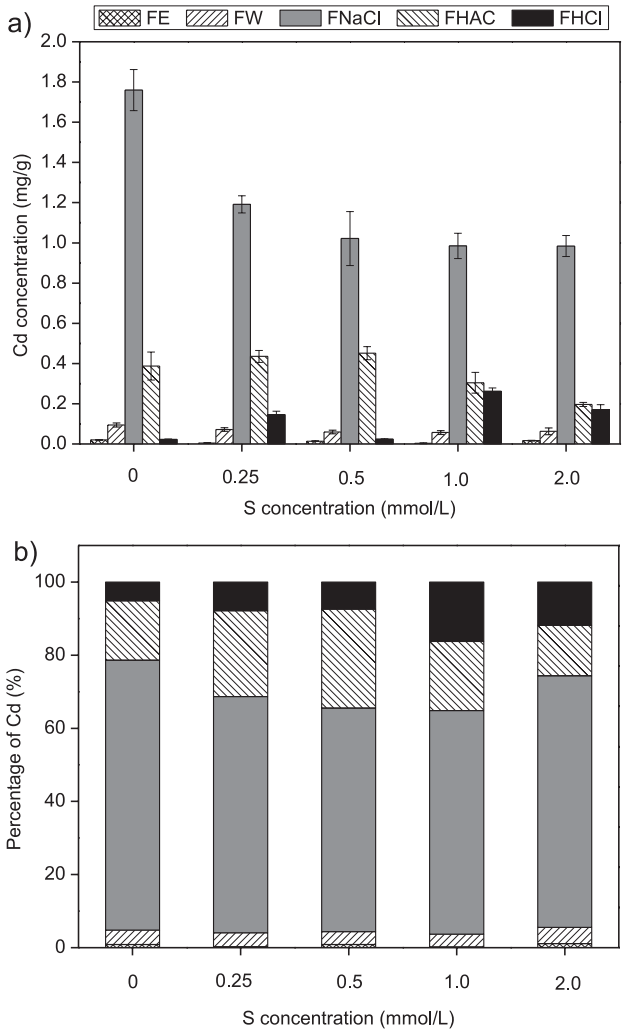

Fig. 4. Cd concentrations a) and percentages b) of different chemical forms of Cd in stems of *H. verticillata* after 7 d exposure to Cd**.**

indicated that Cd in the soluble fraction or cell walls was mainly in the form of pectates/protein, phosphate, and oxalate.

Influence of S on Contents of Proline, Cys, and GSH under Cd Stress

The content of proline in *H. verticillata* under S conditions was significantly higher than that of the control ($P<0.05$), and the proline content was increased with the application of S under Cd stress. With the treatment of 2 mmol L^{-1} S, the content of proline was about 2.1 times compared to control under no S condition (Fig. 6a). As to the treatment for 7 days, the content of Cys increased with the application of S under Cd stress in comparison to control. At 2 mmol L^{-1} S treatment level, S application increased the content of Cys by 85% compared to control under no S condition (Fig. 6b). The GSH content of *H. verticillata* under each treatment was significantly higher than that of the control ($P<0.05$), and the GSH content of *H. verticillata* was firstly increased and then decreased with the increment of S concentration (Fig. 6c). At 1 mmol L^{-1} S treatment level, the GSH content of *H. verticillata* achieved the highest level of about 3.1 times as compared with the control.

The Correlation Matrix for S Concentration and Cd Content with the Physiological Characteristics

The correlations of S concentration and Cd content with the physiological characteristics were determined (Table 2). In this study, the proline and Cys contents in leaves of *H. verticillata* were significantly correlated with S concentrations in the medium and the Cd content in leaves ($P<0.05$). Furthermore, significant correlation was found between Cd contents and GSH contents in leaves ($P<0.01$).

Discussion

Aquatic plants are useful vehicles for treating heavy metal pollution in water. Islam et al. observed a higher Cd content in leaf than in other tissues in *Micranthemum umbrosum*. However, Weng et al. [33] observed higher Cd content in roots than in other tissues in *Kandelia obovata*. These results indicate that heavy metals could be accumulated in different plant parts. Analysis of Cd content in root, stem, and leaf of *H. verticillata* showed that S could affect the distribution of Cd in different parts of *H. verticillata*. S supply significantly restrained Cd accumulation in roots and stems of *H. verticillata*. Other reports have shown that S could inhibit Cd from entering rice roots, suggesting that S supply enhances the formation of iron plaque – a protective defense of rice roots against heavy metal uptake [34]. Hassan et al. [20] observed that S supplementation decreased Cd accumulation and alleviated Cd toxicity in plants. Such results were similar to those observed in the present study. Furthermore, the shoots of *H. verticillata* have the ability to take up metals directly from water since they are completely inundated and have a very thin cuticle. Hence, we deduced that differences in Cd accumulation in *H. verticillata* tissues with increasing S supply might be explained by the following mechanisms: 1) S supply changed the availability of Cd and 2) S induced the production of antioxidants, which could chelate Cd, forming complexes that alleviate Cd toxicity in leaves.

In our study, Cd analysis at the subcellular level of *H. verticillata* roots demonstrated that a large proportion of Cd (80.6–71.1%) was stored in the soluble fraction (Table 1), which has a high migration capacity and will further translocate to the shoot [35]. As the vacuole is a dynamic organelle that comprises as much as 90% of the total cell volume in some cell types, we may deduce that the vacuole was the predominant sink for Cd. These results were in line with those presented for Cd-treated eucalyptus [36], barley [29], and soybean plants [37]. In

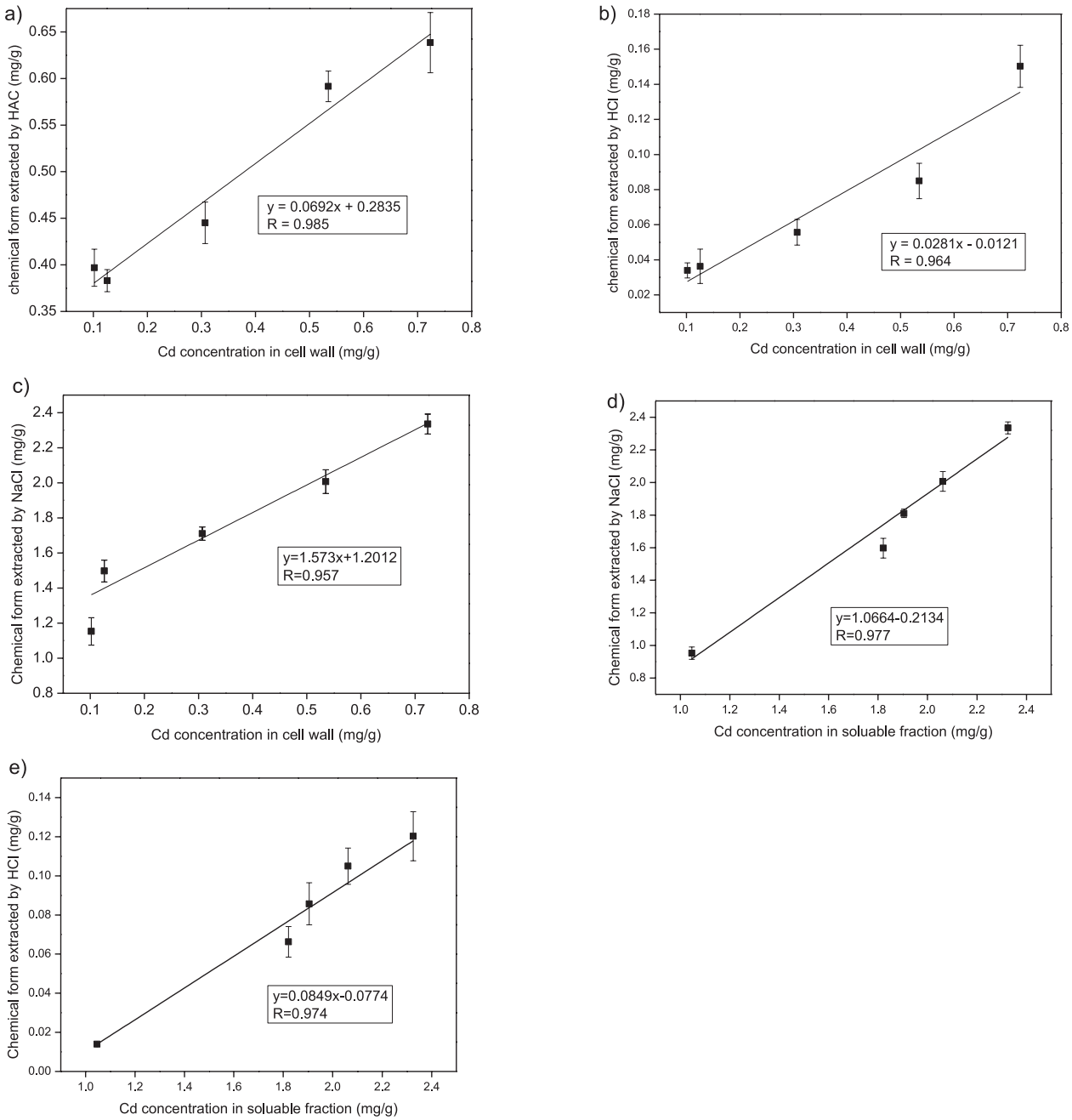

Fig. 5. Correlation of Cd between subcellular distribution and chemical forms in leaf: a) F_{HAc} versus Cd in cell wall; b) F_{HCl} versus Cd in cell wall; c) F_{NaCl} versus Cd in cell wall; d) F_{NaCl} versus Cd in soluble fraction; e) F_{HCl} versus Cd in soluble fraction.

contrast to the present results, several studies found that the main site of Cd accumulation in plant roots is the apoplast, particularly cell walls [23]. These differences may be attributed to the distinct Cd concentrations used by the different authors and also to the variable levels of Cd tolerance of plants.

Plant cell walls, which function as the first barrier protecting the protoplast from Cd toxicity, are mainly composed of polyose (including cellulose, hemicellulose, and pectin) and protein, providing negative charge sites on their surfaces. Therefore, plant cell walls can bind Cd ions and restrict their transportation across cytomembrane. In our study, a proportion (6.1–22.4%) of Cd was bound to the cell wall fraction, suggesting that the cell wall is another large buffer that can accumulate heavy metals and is believed to play a role in metal tolerance. Meanwhile, we found that in plant leaf, the proportion of Cd in the cell wall fraction increased following the increase of S concentration in solution (Table 1), indicating that the cell walls prevented Cd entry into the cells. The cell walls can work as a barrier and effectively sequestrate heavy metal ions. Therefore, the cell walls are another key factor in defense against heavy metal toxicity of aquatic plants.

Fu et al. [38] observed the subcellular distribution of Cd in *Phytolacca americana* L. and deduced that

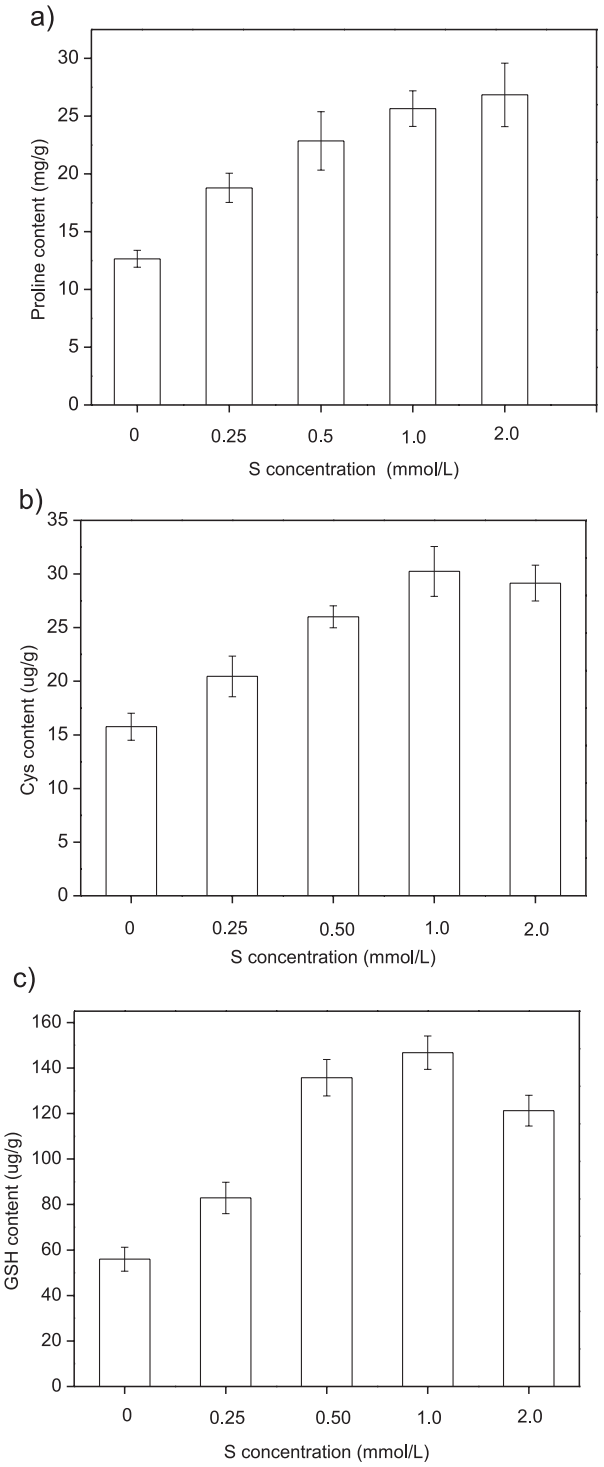

Fig. 6. Effects of S application on proline, Cys, and GSH content in Cd-treated *H. verticillata* (a: proline; b: Cys; c: GSH) .

both vacuoles and cell walls might be involved in Cd-tolerance mechanisms to protect metabolically active cellular compartments. We obtained similar experimental results. It can be inferred that the variability of Cd concentration in the cell may be regulated by three processes: Cd binding to biological molecules, chelation of Cd through oxalate, and cellular compartmentalization in vacuoles.

Table 2. The correlation matrix for S concentration and Cd content with the physiological characteristics of *H. verticillata.*

	S	Cd	Proline	Cys	GSH
S	1				
Cd	0.523	1			
Proline	0.872*	0.893*	1		
Cys	0.849*	0.91*	0.985**	1	
GSH	0.589	0.941**	0.909*	0.949**	1

* Correlation is significant at the 0.05 level (2-tailed).
** Correlation is significant at the 0.01 level (2-tailed).

The chemical forms of Cd are closely related to their biological toxicity. For instance, water-soluble Cd in inorganic form (extracted with 80% ethanol) and organic form (extracted with deionized water) has stronger harmful effects than the undissolved Cd in pectates/protein (extracted with 1M NaCl) and phosphate (extracted with 2% HAc) [23, 38]. For *H. verticillata*, the major chemical form of Cd was Cd in pectates/protein, followed by the undissolved Cd-phosphate complexes. The Cd forms of pectates/protein and phosphate were mainly associated with the vacuoles and cell walls of the *H. verticillata*. The 80% ethanol-extractable Cd consisted of inorganic forms, which contributed the most to Cd stress in plants, whereas NaCl-extractable (organic) Cd might be responsible for plant adaptation to Cd stress [23]. Thus, inorganic Cd was changed to organic (NaCl-extractable) Cd after S supply increased, causing less harm to *H. verticillata*. In addition, *H. verticillata* leaves have an intrinsically high content of oxalate [39]. Chelation of Cd by oxalate could be an essential detoxification mechanism to render excess Cd inactive; therefore, oxalic acid could play an important role in Cd accumulation and detoxification in *H. verticillata*.

The formation of Cd-phosphate complexes is an important detoxification mechanism for plants. P content was increased in the cell of *Rhodotorula* sp. Y11 under Cd toxicity [40]. Polyphosphate accumulation in extraradical hyphae enhances the resistance of *Rhizophagus irregularis* against Cd [41]. *Cunninghamella elegans* may accumulate polyphosphate as a detoxification mechanism to precipitate and tolerate Cd [42]. *Penicillium chrysogenum* deposited Cd inside or outside of the cells by forming $Cd_3(PO_4)_2$ with phosphoryl. The conspicuous phosphorus peak in the EDAX spectra of *Rhizopus oligosporus* indicated that Cd was deposited as Cd-phosphate complexes. We suggest that *H. verticillata* can form undissolved Cd in phosphate on the cell walls to decrease Cd toxicity. Forming Cd-phosphate complexes would allow *H. verticillata* to tolerate Cd stress by accumulating Cd on the cell walls of *H. verticillata*. Although P uptake is affected by many factors, including sulfur supply, the relationship between phosphate or polyphosphate

and Cd-detoxifying mechanisms in *H. verticillata* remains unclear and needs further study.

The complexation of metals with organic ligands results in decreased free ion activity, thus reducing their toxicity. The organic ligands involved in compartmenting Cd in vacuoles are mainly sulfur-rich peptides [43]. At high S levels, the concentration of NaCl-extracted Cd (F_{NaCl}) was higher than that of other Cd forms, which mirrored the accumulation of Cd in soluble fractions and cell walls. Therefore, we can assume that *H. verticillata* adapts to Cd stress through the larger percentage of NaCl-extractable Cd in leaves, which supports the theory that compartmentation in vacuoles and sequestration in cell walls is crucial for the detoxification of Cd. The difference of percentage of chemical forms between root and leaf in *H. verticillata* was because the existence of S led to higher synthesis of antioxidants in plants [33], allowing Cd to transfer from roots to leaves. The relationship between subcellular distribution and chemical forms was similar in roots and stems, indicating that subcellular distribution and chemical forms of Cd were relevant to alleviate Cd toxicity.

It has been shown that plants resist stress by increasing components of their intrinsic defensive system. For example, some antioxidants like proline may play a role in inducing resistance to metals [44]. Proline accumulation is an adaptive strategy of plants to stressful environments which maintains the osmotic balance, scavenges excess free radicals, stabilizes cell membrane structure and function [45], regulates cellular redoxpotential [46], sustains PSII electron transport [47], and increases N remobilization and N-use efficiency in *B. napus* [48]. Up-regulation of proline is often encountered in plants under heavy metal stress, and the possibility of proline involvement in the chelation of metal ions is also indicated [49]. Studies on S have shown that they could improve photosynthetic efficiency and proline accumulation under salt stress in *B. juncea* and under chilling stress in *Cucumis sativus* [50]. In the present study, the level of proline also increased significantly in S application plants. Hence, proline accumulation would have acted as a supporting mechanism for the capacity of proline to quench ROS.

The results presented herein show that intercellular Cd enhanced Cys and GSH contents. On the other hand, alleviation of Cd toxicity in high S-treated *H. verticillata* was due to higher contents of Cys and GSH. Similarly, Mera et al. [16] reported that thiol-rich sulphur containing compounds such as Y-Glu-Cys, Cys, GSH, and phytochelatins developed tolerance mechanisms in microalga under Cd stress. Overall results indicate that S addition up-regulates these antioxidants – particularly S-containing non-enzymatic antioxidants, which prevent Cd-induced oxidative modification in test living organisms against Cd. Up-regulation in antioxidants has been reported by several researchers under various abiotic stresses [34, 51-53]. In this study, the GSH contents of *H. verticillata* increased with the increment of S concentration from 0.25 to 1 mmol L^{-1} S treatment level, which reflected that *H. verticillata* accelerated synthesizing GSH in order to improve their tolerant capacity. The GSH contents of *H. verticillata* were first increased and then decreased with the increment of S concentration, perhaps due to the huge consumption of GSH for detoxification, or the inhibition from excessive ROS induced by Cd stress, or the involvement of GSH in PC synthesis as PCs are synthesized using GSH as a precursor [54]. Therefore, S supply might promote the synthesis of sulfhydryl proteins such as GSH and cysteine, and then chelate Cd for transfer to vacuoles, which might be an important mechanism of heavy metal detoxification.

Conclusions

After combined treatments of Cd and S, Cd mainly accumulated in *H. verticillata* leaves. As S supply was increased, Cd content decreased in roots while it increased in leaves. Cd mainly existed in the soluble fractions and cell walls of *H. verticillata*. Furthermore, the Cd contents in root-soluble fractions and cell walls declined gradually as S concentration increased. By comparison, the Cd contents in leaf cell walls and soluble fractions increased remarkably, which indicated that S supply might promote the transfer of Cd into vacuoles in leaves. At high S levels, the concentration of NaCl-extracted Cd (F_{NaCl}) in leaves was higher than that of other Cd forms. This observation was in good agreement with the accumulation of Cd in soluble fractions and cell walls. The formation of Cd-phosphate complexes or Cd oxalate could be considered another important detoxification mechanism in *H. verticillata*. Exogenous S played an important role in ameliorating the Cd tolerance of *H. verticillata* by promoting proline accumulation, the contents of Cys, and GSH. Our results provide insights into the physiological mechanism of S in regulating the uptake, translocation, and tolerance of Cd in plants. Hence, S can potentially be used as a physiological tool in sustainable development of phytoremediation. However, studies are needed to evaluate the S nutrition effect in phytoremediation involving longer-duration assays.

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Conflict of Interest

The authors declare no conflict of interest.

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