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RESEARCH ARTICLE

Cadmium tolerance and detoxification in *Myriophyllum aquaticum*: physiological responses, chemical forms, and subcellular distribution

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Abstract

Submerged macrophytes have been found to be promising in removing cadmium (Cd) from aquatic ecosystems; however, the mechanism of Cd detoxification in these plants is still poorly understood. In the present study, Cd chemical forms and subcellular distributing behaviors in *Myriophyllum aquaticum* and the physiological mechanism underlying *M. aquaticum* in response to Cd stress were explored. During the study, *M. aquaticum* was grown in a hydroponic system and was treated under different concentrations of Cd (0, 0.01, 0.05, 0.25, and 1.25 mg/L) for 14 days. The differential centrifugation suggested that most Cd was split in the soluble fraction (57.40–66.25%) and bound to the cell wall (24.92–38.57%). Furthermore, Cd in *M. aquaticum* was primarily present in NaCl-extractable Cd (51.76–91.15% in leaves and 58.71–84.76% in stems), followed by acetic acid–extractable Cd (5.17–22.42% in leaves and 9.54–16.56% in stems) and HCl-extractable Cd (0.80–12.23% in leaves and 3.56–18.87% in stems). The malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) concentrations in *M. aquaticum* were noticeably increased under each Cd concentration. The activities of catalase (CAT), guaiacol peroxidase (POD), and superoxide dismutase (SOD) in leaves were initially increased under relatively low concentrations of Cd but were decreased further with the increasing concentrations of Cd. The ascorbate (AsA), glutathione (GSH), and nitric oxide (NO) concentrations in stems increased with increasing Cd concentrations. Taken together, our results indicate that *M. aquaticum* can be used successfully for phytoremediation of Cd-contaminated water, and the detoxification mechanisms in *M. aquaticum* include enzymatic and non-enzymatic antioxidants, subcellular partitioning, and the formation of different chemical forms of Cd.

Keywords Cadmium · *Myriophyllum aquaticum* · Subcellular distribution · Chemical forms · Physiological responses

Introduction

The contamination of aquatic ecosystems due to heavy metals has been extensively highlighted in recent decades (Chen et al. 2019). In order to be specific, cadmium (Cd) refers to the most phytotoxic element with no known biological functions in various types of plants. The main sources of Cd contamination are industrial development, mining, and application of sewage sludge and phosphate fertilizer to the land (Shi et al. 2016). Plants under the influence of Cd contamination display damaged photosynthetic system, promoted senescence, and defects related to mineral deprivation. The high levels of Cd can be concentrated through the food chain in humans and might cause adverse impacts on health.

The conventional techniques used to clean up polluted water are effective but expensive. Phytoremediation, a plant-based green technology, is the most promising, economical, effective, and friendly approach for removing metals from moderately polluted water bodies (Bello et al. 2018). Nowadays, a wide range of wetland plant species have been investigated for their effectiveness in removing heavy metals (Rezania et al. 2016). Since submerged macrophytes grow underwater, the chances of exposure to heavy metals are higher compared with the emergent plants; thus, they may have a relatively higher potential to take up Cd (Xing et al. 2013). Many types of submerged macrophytes, e.g., *Vallisneria natans* (Li et al. 2018), *Elodea nuttallii*

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(Beauvais-Fluck et al. 2019), and *Microsorum pteropus* (Lan et al. 2019), have been tested for their ability to accumulate Cd and promising results have been found. This study also found promising results confirming that large underwater plants may be used to remove Cd from aquatic ecosystems.

Cadmium manifests its toxic effects on plants in numerous ways, such as damage to genomic DNA, inhibition of damaged DNA repair systems, degeneration of mitochondria and chloroplasts, interaction with bioelements, stimulation of oxidative stress, and induction of cell death (Đukić-Ćosić et al. 2020). In order to protect plants from Cd toxicity, plants have evolved numerous strategies to tolerate metals and initiate detoxification mechanisms, including isolation in vacuoles and/or deposition in cell walls or other subcellular parts (Lai 2015). Large amounts of Cd are found in the cell walls of *Phytolacca americana* (Fu et al. 2011) and vacuoles of *Capsicum annuum* (Xin and Huang 2014). Nevertheless, the mechanism involved in Cd sequestration in tolerant plants has not been elucidated (Zhou et al. 2016). Moreover, the biologically active state of Cd in plants is related to its chemical form, which may affect its biological toxicity (Shi et al. 2017). Among the different forms of Cd, inorganic (harvested with ethanol) and water-soluble (harvested with deionized water) Cd exhibited the maximum activity, followed by insoluble Cd phosphates (harvested with acetic acid) and pectate- and protein-bound Cd (harvested with NaCl), while Cd oxalate (harvested with HCl) and residues were the least active (Xu et al. 2018). A Cd-resistant genotype of barley showed more pectate- and protein-integrated Cd compared with a Cd-sensitive genotype (Wu et al. 2005). Therefore, changing the chemical morphology of heavy metal pollutants may be a critical detoxifying mechanism (Zhao et al. 2015).

Though Cd does not belong to a redox-active metal complex, it can induce oxidization stress to plants by forming reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2), and superoxide anion ($O_2^{\cdot-}$). Many studies showed that Cd hyperaccumulators are highly capable to cope with ROS accumulation and oxidative stress caused by high Cd in the environment (Boominathan and Doran 2003). *Solanum nigrum*, a hyperaccumulator of Cd, showed lower ROS accumulation and less cell structure disorders compared with the non-hyperaccumulator, *Solanum melongena*, under Cd stress (Sun et al. 2007). However, most aquatic macrophytes were found to be non-hyperaccumulator of Cd, and a significant increase in ROS accumulation was observed in *Wolffia arrhizal* (Piotrowska et al. 2010), *Lemna minor*, and *Lemna gibba* (Varga et al. 2013). Many enzymatic and non-enzymatic antioxidizing pathways are stimulated to overcome the excess production of ROS in plant cells (Singh et al. 2010). The active states of antioxidant enzymes, e.g., guaiacol peroxidase (POD), superoxide dismutase (SOD), and catalase (CAT), increase in response to Cd stress under specific conditions and subsequently decrease at higher levels of Cd stress (Hediji et al. 2015). Thus, antioxidant enzymes in plants are not efficient to mitigate to the toxic effects under severe Cd stress. Non-enzymatic scavengers, including glutathione (GSH) and ascorbate (AsA), scavenge ROS accumulated overly as well (Dogan et al. 2009; Spengler et al. 2017). It was recently evidenced that nitric oxide (NO), a vital signaling molecule, is capable of regulating numerous physiological activities (Akram et al. 2018). Numerous studies have been conducted to examine the ameliorative effects exerted by NO on Cd toxicity in plants such as tomato (Ahmad et al. 2018) and wheat (Kaya et al. 2019). However, the effect of Cd on the NO content in submerged macrophytes remains unclear.

Myriophyllum aquaticum (*M. aquaticum*) is a commonly occurring aquatic angiosperm with a worldwide distribution and rapid growth. This plant species can offer a large contact area for phytoremediation as its stems can extend over 1 m in length underwater. Due to its high potential to accumulate pollutants, it has been used as an alternative method to accumulate lead, zinc, and copper (Caillat et al. 2014; Harguinteguy et al. 2015). However, its capacity to remove and detoxify Cd remains unknown. It is very important to identify the localization and assimilation of Cd in subcellular fractions as well as its chemical forms to understand this plant's defense system in response to Cd stress.

The present study aimed (1) to characterize the chemical forms of Cd and its subcellular distributing behaviors in *M. aquaticum*, (2) to explore their participation in Cd tolerating process, and (3) to delve into the variations in non-enzymatic antioxidant concentrations and antioxidant enzyme activities in *M. aquaticum* under Cd stress. The results would contribute to a better understanding of Cd phytoremediation potential of *M. aquaticum* and the physiological mechanisms that are associated with Cd accumulation and stress adaption in submerged macrophytes.

Experimental materials and methods

Plant materials and Cd treatments

Myriophyllum aquaticum plants originating from uncontaminated freshwater bodies in Xiamen, China, were collected. For experimental studies, we harvested plants that were of the almost same height and weight, which were cleaned with distilled and flowing tap water. Using a glass aquarium supplemented with 1/10 Hoagland solution, the plants were kept for 2 weeks at a photosynthetic photon flux density of 114 μmol/(m^2 s) for a photoperiod of 14 h and a temperature of 25/20 °C (day/night). After 2 weeks, fresh plant materials (8.0 ± 0.1 g) were transferred to glass beakers containing different Cd concentrations (0, 0.01, 0.05, 0.25, and 1.25 mg/L), supplied as $CdCl_2{\cdot}2.5H_2O$ (analytical reagent) in 1.6 L 1/10 Hoagland

nutrient medium for 14 days. Subsequently, under the mentioned conditions, the beakers were set in a growth chamber. After harvesting, the plants were separated into leaves and stems and frozen in liquid nitrogen immediately. They were frozen at appropriate conditions until further use. All experiments were repeated thrice.

Subcellular distribution of Cd

Based on a method described by Weigel and Jäger (1980), the plant cells from fresh leaf and stem tissues were separated into three parts (soluble fractions, organelle, and cell wall). Using a pestle and a chilled mortar and 20 mL pre-cold extraction buffer (1.0 mM dithioerythritol, 250 mM sucrose, and 50 mM Tris-HCl (pH 7.5)), frozen plant samples (2.0 g) were homogenized. The homogenate was transferred into 50-mL centrifuge tubes and centrifuged for 15 min at 1250×*g*. The precipitate was collected as the cell wall fraction, primarily including cell wall debris and cell walls. The supernatant was then centrifuged for 45 min at 20,800×*g*. The cell organelle and soluble fractions were obtained as the precipitate and the supernatant, respectively. All procedures were performed at 4 °C. The samples with Cd obtained as three fractions were analyzed after drying and wet digestion.

Extraction of different chemical forms

With a method proposed by Lai (2015), we extracted the six chemical forms of Cd. The steps for Cd extraction by specific solutions were as follows: (1) 80% ethanol for harvesting inorganic Cd (F_E), including aminophenol, chloride, and nitrate/nitrite Cd; (2) deionized water (d-water) for harvesting water-soluble Cd-organic acid complexes and $Cd(H_2PO_4)_2$ (F_W); (3) 1 M NaCl for harvesting protein- and pectate-combined Cd (F_{NaCl}); (4) 2% acetic acid (HAc) for harvesting insoluble Cd phosphate (F_{HAc}), covering $Cd_3(PO_4)_2$ and $CdHPO_4$; (5) 0.6 M HCl for harvesting Cd oxalate (F_{HCl}); (6) the residual (F_R). Since the concentration of Cd in the residues was very low, it could not be detected.

The frozen plant tissues were homogenized with an extracted solution (*w*/*v* = 1/10) and then shaken at 25 °C for 22 h. The homogenate was centrifuged at 5000×*g* for 10 min. The first supernatant obtained was transferred into a beaker. The sediment was re-extracted twice with the same extracting solution and then was shaken at 25 °C for 2 h. The three supernatants were pooled and using the next solution in the solvent sequence, the sediment was subjected up to five extraction processes. Each extraction solution underwent the same operational steps for the same duration as the first extraction solution. The pooled supernatant solution was then dried on an electric plate at 70 °C.

Cadmium content analysis

Before the metal analysis was performed, all plant parts, including the cell wall and cell organelle fractions, were wet digested at 145 °C with an $HNO_3/HClO_4$ (2:1, *v*/*v*) oxidation acid mixture and then diluted using ultrapure water. Every sample of Cd concentration was measured thrice using inductively coupled plasma mass spectrometry (ICP-MS, 7500cx, Agilent, Santa Clara, CA, USA). In order to achieve quality assurance, we used a certified reference material (bush twigs and leaves, GBW07602 from the National Research Center for Standard Materials in China) and a reagent blank. The reference material was analyzed repeatedly, and then 0.137 ± 0.05 mg Cd/kg dry weight (DW) was obtained, which was consistent with the certified value 0.14 ± 0.06 mg Cd/kg DW.

Analysis of H_2O_2, malondialdehyde, and antioxidant enzyme activities

The concentration of H_2O_2 was determined colorimetrically, according to Jana and Choudhuri (1981). H_2O_2 was extracted by homogenizing 50 mg of stem tissues or fresh leaves with 3 mL of phosphate buffer (50 mM, pH 6.5). For measuring the H_2O_2 content, 3 mL of extraction solution was mixed with 1 mL of 0.1% titanium sulfate in 20% (*v*/*v*) H_2SO_4, and the mixture was centrifuged at 6000×*g* for 15 min. The absorbance of the yellow supernatant was measured using a spectrophotometer at 410 nm.

The lipid peroxidation level in the leaf and stem tissues was determined in terms of the malondialdehyde (MDA) (a product of lipid peroxidation) concentration using an approach described by Farooq et al. (2016). Of the plant sample, 0.25 g was homogenized in 5 mL of 0.1% trichloroacetic acid (TCA). The homogenate was centrifuged at 10,000×*g* for 5 min; 4 mL of 20% TCA supplemented with 0.5% thiobarbituric acid was added to 1 mL of the supernatant. The mixture was heated at 95 °C for 30 min and then quickly cooled using an ice bath. After the mixture was centrifuged at 10,000×*g* for 10 min, the absorbance of the supernatant was measured at 532 nm, and the value of the nonspecific absorption at 600 nm was subtracted.

In 100 mM chilled potassium phosphate buffer (pH 7.0) supplemented with 0.1 mM EDTA and 1% polyvinylpyrrolidone (*w*/*v*), 0.20 g of the plant sample was homogenized at 4 °C. The homogenate was centrifuged at 15,000×*g* under 4 °C for 20 min, and the supernatant was used for determining the CAT, POD, and SOD activities (Dominguez et al. 2010).

Following a method proposed by Beauchamp and Fridovich (1971), the active state of SOD was ascertained. The reaction mixture (3 mL) contained a suitable aliquot of enzyme extract, 0.1 mM EDTA, 2 μM riboflavin, 75 μM nitroblue tetrazolium, 13 mM methionine, and 40 mM phosphate buffer (pH 7.8). After shaking, the test tubes were

placed at 30 cm below a 15 W fluorescent light source, and the absorbance was recorded at 560 nm.

The POD activity was determined as mentioned by Meng et al. (2007). In total, 0.1 mL of supernatant was used for the analysis. The activity was expressed as an increase in the absorbance at 470 nm under the influence of guaiacol oxidation.

The method proposed by Srivastava et al. (2006) was used to determine CAT activity. A reaction mixture, in 3 mL, contained a suitable aliquot of the enzyme, 20 mM H_2O_2, and 50 mM sodium phosphate buffer (pH 7.0). A decline in absorbance at 240 nm was taken as the CAT active state.

Determination of AsA, GSH, and NO contents

Using an approach proposed by Tanaka et al. (1985), reduced ascorbic acid was determined. The frozen samples were ground in liquid nitrogen and quickly homogenized in 5% TCA in an ice bath. Then, the homogenate was centrifuged at 12,000×*g* and 4 °C for 10 min. We subsequently mixed the supernatant with 0.2 mL 0.3% (*w*/*v*) $FeCl_3$, 0.4 mL 4% a,a′-dipyridyl in 70% ethanol, 0.4 mL 44% *ortho*-phosphoric acid, and 0.4 mL 10% TCA. After vortex mixing, the color was developed in the reaction mixtures. The mixtures were incubated for 60 min at 37 °C, and the absorbance of the supernatant was read at 525 nm.

The concentration of GSH was determined, according to Anderson (1985). A total of 0.3 g of fresh plant sample was homogenized in 2.0 mL of 5% sulfosalicylic acid in a cold environment. The homogenate was centrifuged at 10,000×*g* for 10 min. Then, 40 μL of 5′5′-dithiobis-2-nitrobenzoic acid and 0.6 mL of phosphate buffer (100 mM, pH 7.0) were added to 0.5 mL of the supernatant. After 2 min, the absorbance was read at 412 nm.

The concentration of NO in the plant tissue samples was determined using a modified protocol (Zhou et al. 2005). Using a mortar and pestle, plant samples (0.6 g) were ground in 3 mL of 50 mM cool HAc buffer (pH 3.6, supplemented by 4% zinc diacetate). The homogenates were centrifuged at 10,000×*g* for 15 min, and then the supernatant was harvested. The pellet was washed with 1 mL of extracting buffer and then centrifuged according to the previous procedure. The two supernatants were pooled, and 0.1 g of charcoal was added. After vortexing, the sample was filtered. The mixture of 1 mL of filtrate and 1 mL of Griess reagent was incubated for 30 min at room temperature, and the absorbance was read at 540 nm.

Statistical analysis

Data are denoted as a mean ± standard deviation (SD). All statistical analyses were performed using the SPSS (version 22.0) statistical software package (SPSS, Chicago, IL, USA). Mean differences among the treatments were compared by an analysis of variance (one-way ANOVA) followed by Duncan's multiple range test at a significance level of $P < 0.05$. With the use of Pearson's correlation analysis (two-tailed), a correlation matrix was created for Cd concentrations against the physiology of *M. aquaticum*. All data were plotted using the Origin 8.5 statistical package.

Results

Cadmium accumulation and subcellular distribution

The overall Cd content and its subcellular distribution in plant tissues showed variations when different Cd concentrations were used in the culturing solution. The total Cd content in stems and leaves increased significantly when Cd concentration was increased in the solution (Table 1). Furthermore, Cd accumulation in leaf tissue was slightly higher than that in stems. Most Cd was distributed in the soluble fraction (57.40–66.25%), with low concentration in the cell wall fraction (24.92–38.57%), and the organelle fraction accounted for only 0.97–12.04% of the total. Cadmium ratio in the cell wall fraction in the leaves increased with the increased amount of Cd added to the solution, yet the ratio of the cell wall fraction in stems decreased notably. Furthermore, Cd ratio in the soluble fraction in the stems increased significantly with the increasing Cd content in the solution.

Chemical forms

Cadmium bound to pectates and proteins (harvested with NaCl) was found to be dominant in all treatments (Fig. 1a). Furthermore, the concentrations of the different Cd chemical forms in the plant tissues were increased in a concentration-dependent manner. When Cd concentration in solutions increased to 1.25 mg/L, Cd harvested using 1 M NaCl was 44.09-fold higher in the leaves and 51.09-fold higher in the stems compared with that treated with 0.01 mg/L Cd. The concentrations of Cd harvested using 2% HAc from the leaves and stems of plants grown at 0.05, 0.25, and 1.25 mg/L Cd were 1.36-, 2.70-, and 5.78-fold, and 2.22-, 13.72-, and 28.39-fold higher compared with that exposed to 0.01 mg/L Cd, respectively.

Figure 1b shows the ratios of different chemical forms of Cd observed in *M. aquaticum* exposed to Cd at four levels of concentrations. Cadmium proportion in the leaves harvested with 2% HAc decreased considerably with the increased Cd concentration in the treatment solution, while the ratio of Cd harvested with 1 M NaCl was increased significantly. In the stems, the proportion of Cd extracted using 1 M NaCl increased significantly as Cd concentration in the treatment solution increased from 0.01 to 1.25 mg/L. The proportion of Cd

Table 1 Cadmium concentrations and its subcellular distribution of Cd in leaves and stems of *M. aquaticum*. Data are means of three replicates ± SD (standard deviation, $n = 3$). Different letters in the same row represent statistically significant differences between treatments within the different Cd concentrations ($P < 0.05$). F1, F2, and F3 refer to the cell wall, cell organelle, and soluble fractions, respectively

Tissues	Cd treatment (mg/L)	Cd concentration (mg/kg DW)				Relative Cd allocation (%)		
		F1	F2	F3	Total	F1	F2	F3
Leaves	0.01	1.562 ± 0.239 d	0.336 ± 0.171 a	3.151 ± 0.136 d	5.049 ± 0.545 d	30.944 b	6.654 a	62.402 a
	0.05	3.847 ± 0.650 c	0.522 ± 0.166 a	6.249 ± 0.278 c	10.618 ± 0.817 c	36.233 a	4.914 b	58.853 a
	0.25	23.084 ± 2.119 b	0.780 ± 0.605 a	21.283 ± 0.467 b	45.147 ± 2.915 b	37.841 a	1.729 c	60.430 a
	1.25	49.070 ± 1.655 a	1.184 ± 0.388 a	72.276 ± 1.746 a	122.530 ± 3.622 a	38.415 a	0.966 d	60.618 a
Stems	0.01	1.008 ± 0.200 c	0.105 ± 0.037 d	1.500 ± 0.246 c	2.612 ± 0.393 d	38.570 a	4.029 d	57.401 d
	0.05	1.438 ± 0.226 c	0.638 ± 0.105 c	3.225 ± 0.307 c	5.301 ± 0.525 c	27.127 b	12.044 a	60.828 c
	0.25	8.479 ± 0.260 b	3.519 ± 0.302 b	20.830 ± 1.412 b	32.828 ± 0.878 b	25.828 c	10.720 b	63.452 b
	1.25	27.867 ± 1.823 a	9.874 ± 0.506 a	74.087 ± 2.399 a	111.828 ± 4.646 a	24.919 c	8.829 c	66.251 a

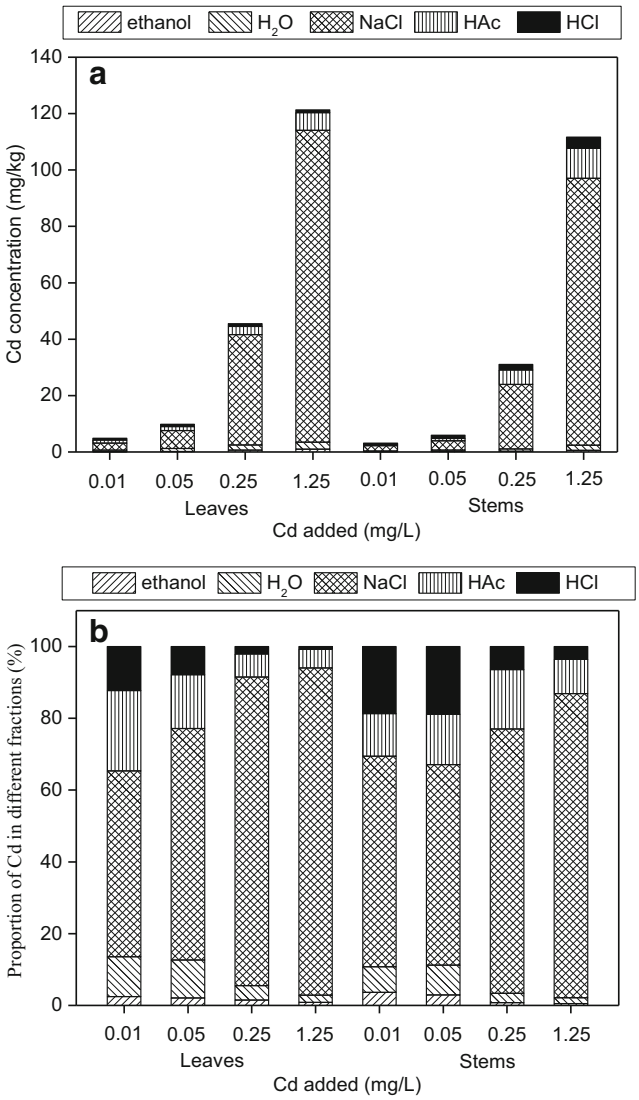

Fig. 1 Different chemical forms of Cd (**a**) and its proportion (**b**) in leaves and stems of *M. aquaticum*. Values are means of three replicates ($n = 3$). Proportion of Cd in fraction (%) = Cd concentration in fraction / (sum of Cd concentrations in all fractions) × 100

extracted using 2% HAc increased considerably when the plants were exposed to 0.01–0.25 mg/L Cd, and then decreased at higher Cd concentrations. As Cd concentration increased in the treatment solutions, the percentage content of Cd-organic acid complexes (harvested with d-H_2O) and Cd oxalate (harvested with HCl) tended to decrease in the leaves and stems. The overall change in the percentage content of inorganic Cd (harvested with ethanol) was small for all Cd concentrations in the treatment solution (Fig. 1b).

Evaluation of oxidative stress and activities of antioxidative enzymes

A dose-response effect was observed in the accumulation of H_2O_2 in leaves and stems of *M. aquaticum*. Following Cd application, there was a considerable increase in ROS in *M. aquaticum*, with enhanced levels of H_2O_2 at all levels of Cd concentrations (Table 2). When exposed to 1.25 mg/L treatment, Cd toxicity caused a considerable increase in H_2O_2 concentration in stems and leaves by 3.37- and 1.75-fold, respectively, compared with the unstressed plants.

Furthermore, by determining the MDA concentration in plant parts, the effect of Cd on lipid peroxidation was assessed. The MDA concentration in stems and leaves increased significantly at all levels of Cd concentration after 14 days of treatment, and the concentration was higher in leaves compared with that in stems (Table 2). Compared with the control group, 1.25 mg/L Cd (after 14 days) treatment resulted in the maximum and gradual accumulation of MDA, with a 5.43- and 41.05-fold increase in the stems and leaves, respectively.

Table 2 shows the changes in the active state of antioxidative enzymes, including POD, CAT, and SOD, in leaves and stems, triggered on exposure to Cd concentration. In comparison with the control group, SOD activity in leaves initially increased but then declined with a further increase in Cd concentration. The SOD activity in leaves was highest at 0.05 mg/

Table 2 Effects of Cd stress on H_2O_2, MDA contents, and SOD, POD, and CAT activities in *M. aquaticum.* Data are means of three replicates ± SD (standard deviation, $n = 3$). Different letters in the same row represent statistically significant differences between treatments within the different Cd concentrations ($P < 0.05$)

Tissues	Cd treatment (mg/L)	H_2O_2 (mmol/g FW)	MDA (nmol/g FW)	SOD (U/g FW)	POD (U/g FW)	CAT (U/g FW)
Leaves	0	2.497 ± 0.123 c	0.151 ± 0.012 d	293.085 ± 17.928 b	8.889 ± 0.399 d	4.065 ± 0.363 e
	0.01	3.535 ± 0.324 b	0.554 ± 0.060 d	312.234 ± 25.928 ab	126.667 ± 8.298 a	7.227 ± 0.655 d
	0.05	3.829 ± 0.373 ab	2.928 ± 0.180 c	324.468 ± 14.293 a	138.889 ± 11.924 a	31.165 ± 2.840 a
	0.25	4.130 ± 0.383 ab	4.194 ± 0.356 b	129.255 ± 8.929 c	94.445 ± 7.823 b	14.905 ± 1.098 b
	1.25	4.374 ± 0.342 a	6.173 ± 0.533 a	150.036 ± 4.398 c	50.012 ± 3.392 c	12.195 ± 1.002 c
Stems	0	0.726 ± 0.056 c	0.554 ± 0.034 d	184.043 ± 18.028 a	18.898 ± 1.293 d	4.968 ± 0.274 c
	0.01	1.114 ± 0.078 b	1.472 ± 0.102 c	156.383 ± 1.375 b	25.556 ± 1.283 c	5.872 ± 0.287 c
	0.05	1.154 ± 0.086 b	1.187 ± 0.098 c	112.766 ± 6.338 c	26.647 ± 1.938 c	7.678 ± 0.567 c
	0.25	1.042 ± 0.043 b	2.662 ± 0.209 b	114.894 ± 10.203 c	34.454 ± 27.745 b	19.873 ± 1.086 b
	1.25	2.443 ± 0.153 a	3.007 ± 0.258 a	86.702 ± 4.294 d	44.435 ± 3.284 a	94.850 ± 4.384 a

L Cd, which was almost 1.11-fold higher than the control. However, the SOD activity in stems declined with an increase in Cd concentration. At the concentration of 1.25 mg/L Cd, the activity of SOD in stems decreased by approximately 52.89%, which was the lowest compared with the control.

The activity of POD in the leaves initially increased but then declined with the increasing Cd concentration. The activity of POD was at the highest level in leaves at the treatment concentration of 0.05 mg/L Cd, which was approximately 14.71-fold higher than the control (Table 2). In the stems, the POD activity increased significantly with an increase in Cd concentration.

The CAT activity in leaves showed a significant increase when Cd concentration was between 0.01 and 0.05 mg/L, but then the activity declined with elevated Cd concentration (Table 2). The highest level of CAT activity in the leaves was approximately 7.67-fold higher than the control. In the stems, the CAT activity increased significantly when Cd concentration increased in the range of 0.05 to 1.25 mg/L.

Effects of Cd stress on AsA, GSH, and NO contents

The concentration of the antioxidants, AsA and GSH, was determined. As shown in Fig. 2a, the AsA concentration in *M. aquaticum* plants showed a considerable increase with exposure to Cd compared with the control. Furthermore, the GSH concentration demonstrated a concentration-dependent increase with Cd treatment (Fig. 2b). The GSH concentration increased by 2.77 to 6.87% in the stems and by 2.04 to 13.99% in the leaves when Cd concentration was increased from 0.01 to 1.25 mg/L in the treatment solutions.

The NO concentration in leaves initially increased but then declined with the increasing Cd concentration in the treatment solutions (Fig. 3). At 0.01 mg/L Cd concentration, the NO concentration in the leaves of *M. aquaticum* attained its

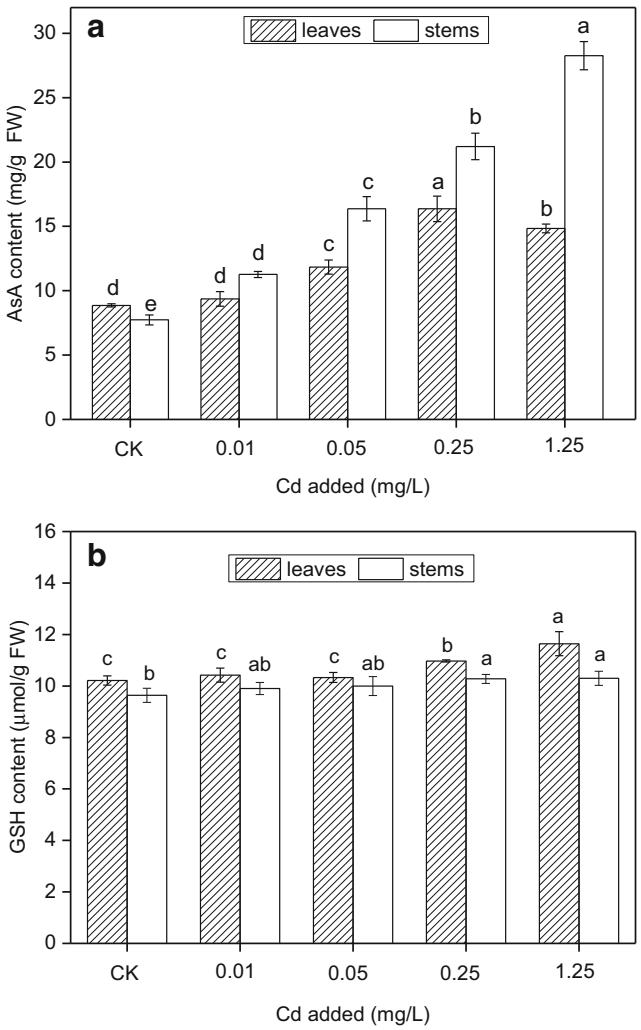

Fig. 2 Effects of Cd stress on AsA and GSH contents in plant tissues of *M. aquaticum* (**a** AsA, **b** GSH). Data points and error bars represent mean and SD (standard deviation, $n = 3$), respectively. Different letters within the same pattern indicate statistically significant differences ($P < 0.05$) according to Duncan's multiple range tests

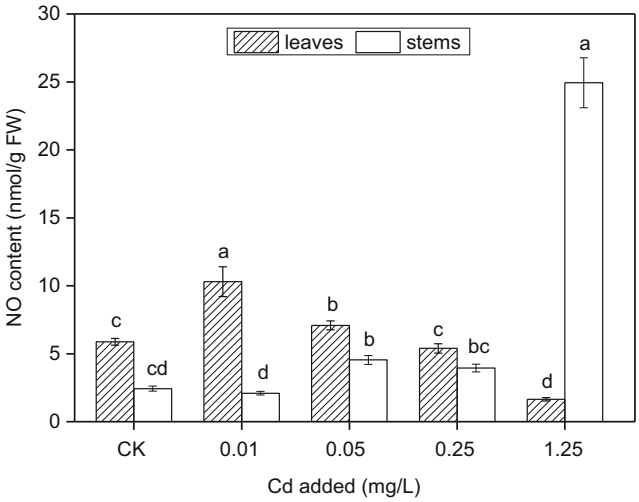

Fig. 3 Effects of Cd stress on NO content in plant tissues of *M. aquaticum*. Data points and error bars represent mean and SD (standard deviation, $n = 3$), respectively. Different letters within the same pattern indicate statistically significant differences ($P < 0.05$) according to Duncan's multiple range tests

highest level, an increase of approximately 75.34% was observed compared with the control. The NO concentration in stems increased with the increase in Cd concentration in the treatment solutions. The 1.25 mg/L Cd treatment caused a remarkable 10.26-fold increase in the NO concentration in the stems.

Correlation coefficient

The correlation coefficient was calculated to determine the relationship between Cd concentration and antioxidant potential of *M. aquaticum* (Table 3). The NO and H_2O_2 concentrations and the CAT activity in the stems displayed a significant positive association with Cd concentration ($P < 0.01$). The MDA concentration in leaves displayed a significant positive association with the GSH and AsA concentrations ($P < 0.05$). The MDA concentration in stems showed a significant positive correlation with the GSH concentration and POD activities ($P < 0.01$).

Discussion

Myriophyllum L. represents a genus of submerged aquatic macrophytic plants. They can accumulate Cd ions, as observed in several studies. In this study, the content of total Cd in the leaf and stem of *M. aquaticum* exposed to 1.25 mg/L Cd could reach 122.530 and 111.828 mg/kg, respectively. The ability to accumulate Cd ions was higher than that of *M. alterniflorum* (Ngayila et al. 2007), *M. heterophyllum* (Sivaci et al. 2008), *M. triphyllum*, and *M. spicatum* (Sivaci et al. 2004) exposed to the same concentrations of Cd ions. This indicated that *M. aquaticum* has a great potential for phytoremediation of Cd in this genus.

The distribution of toxic metals in various metal-tolerant plants suggests that the detoxifying process through the partition of the metals into plant subcellular components is a common mechanism of detoxification (Xin et al. 2018). Here, Cd analysis at the subcellular tissue level suggested that Cd in *M. aquaticum* was primarily stored in the soluble fraction (57.40–66.25%). These results comply with those reported previously in duckweeds (Su et al. 2017) and rice (Li et al. 2016). The vacuole of a cell comprises up to 90% of the total cell volume (Pittman 2005). The organic acids (e.g., amino acids), sulfur donor ligands (e.g., metallothioneins (MTs) and phytochelatins (PCs)), and oxygen donor ligands (e.g., xalate, citrate, malate, and carboxylates) can chelate metals in vacuoles, resulting in a decrease of free metal ion at the active states of enzymes, thus decreasing toxicity (Bhatia et al. 2005). As a result, the vacuoles store the maximum of heavy metals among the intracellular compartments of a submerged macrophyte. As can be seen in Table 1, the content of Cd present in the cell wall fraction was second to that present in the soluble fraction. The cell wall is the first barrier to protect the protoplast from metal toxicity. In this study, 30.94–38.42% and 24.92–38.57% of Cd were stored in the cell wall fraction in the leaves and stems, respectively (Table 1). This indicated that the cell wall noticeably prevented Cd from entering the cells of this submerged macrophyte. These results are similar to the study by Xu et al. (2012) and Zhao et al. (2015), which stated that the maximum fraction of the accumulated Cd was bound to the cell wall in *Potamogeton crispus* and *Porphyra yezoensis*, respectively. The cell wall contains carboxyl, hydroxyl, amino, and other functional groups of polysaccharides and proteins that provide a large number of binding sites to metal ions. Thus, cell walls can fix metal ions, limit their transmembrane transport, and downregulate metal ion concentration in protoplasts (Yang et al. 2018b).

The chemical forms of heavy metals are associated with their biological functions, and the toxicity levels of different forms vary depending on the solvent system by which they are harvested. In terms of plants that contain high Cd concentration exhibiting no or little toxicity, Cd was present in a chemical form that caused low or no phytotoxicity. The contents of insoluble Cd phosphate (extracted by 2% HAc) and pectate- and protein-integrated Cd (extracted by 1 M NaCl) indicate the adaptability of plants to Cd stress (Zhao et al. 2015). In the present study, most Cd was integrated with pectates and proteins (extracted by 1 M NaCl) in stems and leaves (Fig. 1). Similar results were reported previously in the studies on *Brassica napus* (Mwamba et al. 2016) and *Solanum nigrum* L (Wei et al. 2014). Accordingly, Cd is assumed to undergo a chelating process by several particular polar materials (e.g., hydroxyl, carboxyl, PCs, and MTs) to form a nontoxic complex (Lu et al. 2017). The second most abundant chemical

Table 3 The correlation matrix for Cd concentration with the physiological characteristics of *M. aquaticum* treated for 14 days

Tissues		Cd treatment	MDA	AsA	GSH	NO	SOD	POD	CAT	H_2O_2
Leaves	Cd treatment	1								
	MDA	0.845	1							
	AsA	0.597	0.898*	1						
	GSH	0.690	0.906*	0.785	1					
	NO	− 0.847	− 0.789	− 0.634	− 0.790	1				
	SOD	− 0.686	− 0.790	− 0.893*	− 0.858	0.725	1			
	POD	− 0.314	0.019	0.089	− 0.193	0.579	0.276	1		
	CAT	− 0.049	0.367	0.325	− 0.043	− 0.012	0.125	0.644	1	
	H_2O_2	0.640	0.877	0.832	0.772	− 0.402	− 0.629	0.456	0.471	1
Stems	Cd treatment	1								
	MDA	0.786	1							
	AsA	0.870	0.929*	1						
	GSH	0.946*	0.959**	0.943*	1					
	NO	0.987**	0.695	0.825	0.612	1				
	SOD	− 0.724	− 0.819	− 0.947*	− 0.918	− 0.706	1			
	POD	0.907*	0.959**	0.982**	0.928*	0.855	− 0.892	1		
	CAT	0.999**	0.768	0.855	0.668	0.991**	− 0.709	− 0.895*	1	
	H_2O_2	0.961**	0.741	0.848	0.679	0.974**	− 0.768	0.889*	0.965**	1

*Correlation is significant at the 0.05 level (two-tailed)

**Correlation is significant at the 0.01 level (two-tailed)

form of Cd in *M. aquaticum* shoots was HAc-extractable Cd (5.17–22.42% in leaves and 9.54–16.56% in stems), followed by HCl-extractable Cd (0.80–12.23% in leaves and 3.56–18.87% in stems). These results demonstrated that Cd linked with undissolved phosphate and/or oxalate could be accounted for the tolerance in the submerged macrophytes. It can be assumed that the large percentages of NaCl-, HAc-, and HCl-extractable Cd in shoots were responsible for the adaptation of submerged macrophytes to Cd accumulation and stress. This suggested that the compartmentation of Cd into vacuoles and sequestration in the cell wall are likely to be critical to detoxify Cd as well as tolerate metal stress.

Reactive oxygen species include commonly occurring free radicals that probably cause oxidative stress. They can attack nucleic acids, pigments, proteins, and lipids, thereby causing enzyme inactivation, membrane damage, and lipid peroxidation; accordingly, cell viability is affected (Dixit et al. 2001). Cadmium cannot catalyze Fenton-type reactions that yield ROS; however, it has been extensively evidenced that Cd exposure results in the generation of ROS in numerous plants (Bari et al. 2019; Berni et al. 2019). Here, it was demonstrated that Cd uptake induced a strong antioxidative response in both leaves and stems of *M. aquaticum*, which was evident with the presence of H_2O_2 stimulated by all stress treatments (Table 2). These results have also been reported for many other plants (Kaya et al. 2019; Naderi et al. 2018). Malondialdehyde is produced by the per-oxidized membrane lipids; it accumulates when plants are under stress. Here, MDA concentration in *M. aquaticum* increased due to Cd treatments (Table 2), revealing that Cd stress varied the structure and function of the cell membranes and promoted reactive oxygen radicals generating process in the submerged macrophyte. This agrees with the results of Li et al. (2013) and Yang et al. (2018a), who reported that Cd stress promotes the accumulation of MDA in *Pistia stratiotes* and *Salix matsudana*, respectively.

In order to resist the damage from ROS, the organisms activate their cellular immune system to remove cellular ROS through the secretion of antioxidant enzymes. The first line of defense against the ROS is SOD, which converts superoxide radicals to H_2O_2 and molecular oxygen (Li et al. 2019). Both CAT and POD also contribute to the decrease in the H_2O_2 concentration in cells, by breaking H_2O_2 down to H_2O and oxygen (Irfan et al. 2014). Besides, with chlorogenic acid as a substrate, H_2O_2 may be removed by SOD (Takahama and Oniki 1997). In this study, the highest level of POD, CAT, and SOD activities in leaves increased by 13.72-, 6.67-, and 0.11-fold, respectively, under Cd stress in comparison with the control (Table 2). Existing research suggested that the activity of antioxidative enzymes can be elevated in *Ceratophyllum demersum* (Kováčik et al. 2017) and *Oryza sativa* (Yu et al. 2013) by Cd stress. These antioxidant enzymes contribute to the detoxification of Cd. The POD and CAT activities in leaves initially increased and then decreased

to a certain extent with the increasing Cd concentration in the treatment solutions, whereas they increased continuously in stems. This indicated some differences in the detoxification mechanism between the different plant tissues. The active state of antioxidant enzymes in metal-stressed plants is largely unstable, which depends on plant species, metal ions, concentration, and duration of stress, whereas these processes show variations in the redox state of the stressed cells, as suggested by Sharma and Dietz (2009).

Glutathione and ascorbic acid are antioxidants of low molecular weight and play critical roles in the GSH-AsA cycle, helping to sustain the cellular redox status. According to previous studies, AsA and GSH are effective oxidative stress defense systems against Cd (Semida et al. 2018; Singh et al. 2018). Here, at a Cd concentration between 0.01 and 1.25 mg/L in the growth medium, the contents of GSH and AsA in *M. aquaticum* were increased (Fig. 2). Our results agreed with those of previous studies on *Ceratophyllum demersum* (Kováčik et al. 2017) and *Vallisneria spiralis* (Wang et al. 2009). According to these findings, *M. aquaticum* is likely to promote GSH and AsA synthesis to overcome the stress caused by oxidation, thus enhancing its tolerating ability to Cd. In addition, the increase in the GSH concentration in all Cd-treated plants also reflected the biosynthesis of PCs, with GSH being a PC precursor (Hall 2002). The PCs may participate in Cd detoxification and tolerance due to their ability to chelate heavy metals. The ascorbate levels in leaves decreased in response to the exposure of 1.25 mg/L Cd; this indicated that ROS are involved in the oxidation of ascorbic acid to dehydroascorbic acid, leading to the decrease of the ascorbic acid content.

Nitric oxide, a multifunctional gaseous molecule, alleviates the toxicity of heavy metals. Here, the NO concentrations in stems were increased significantly with the increase in Cd concentration, which indicated that the NO concentration may be critical for detoxifying Cd in submerged macrophytes. In *Typha angustifolia*, alleviation of NO against Cd stress and improvement of plant growth and biomass yield have been explored (Zhao et al. 2016). The alleviation of exogenous NO reduced arsenic toxicity in *Oryza sativa* L. through the downregulation of ROS and As^{3+}-reduced MDA content (Singh et al. 2016). These reports demonstrate that NO is involved in a variety of adaptive mechanisms (e.g., overall plant growth to withstand heavy metal stress, promotion of cell wall expansion, protection of phospholipid bilayer, and cell wall relaxation) (Nabi et al. 2019). Other mechanisms of NO regulation include osmotic pressure maintenance, which in turn maintains cytoplasmic viscosity and protects chlorophyll pigments, chloroplast membranes, and related components against the negative effects of Cd on photosynthesis (Ahmad et al. 2018). Likewise, a possible mechanism by which NO alleviates Cd stress may be by inducing Cd-related domains containing metal chaperone genes (Imran et al. 2016).

The results of Pearson's correlation analysis (Table 3) showed a significant positive correlation between MDA, GSH, and AsA contents ($P < 0.05$) in both leaves and stems, indicating that GSH and AsA played an irreplaceable role in protecting *M. aquaticum* from oxidative stress. This result was consistent with that of Xu et al. (2016), who found a significant correlation between GSH, AsA, and MDA. Furthermore, the POD activity in stems was significantly correlated with MDA ($R = 0.959$, $P < 0.01$) indicating that POD has a major role in the antioxidant defense systems in stems. The strong positive correlations between H_2O_2 and antioxidant components such as NO, POD, and CAT in stems may be due to their synergetic role in scavenging H_2O_2 (Murtaza et al. 2019). However, the lack of correlations between antioxidant components and H_2O_2 levels in the leaf may be due to the dual role of H_2O_2 in mediating the balance between antioxidant response and oxidative stress (Qu et al. 2014). The correlation between physiological response indexes of stem and leaf under Cd stress was different. The higher correlation between antioxidant enzymes and/or non-enzymatic detoxifying metabolites in stems indicated that the detoxification mechanism in the stem has better cooperative action (Hu et al. 2019).

Conclusion

In summary, the distribution of Cd at the subcellular level suggested that most Cd existed in the soluble fraction and in the cell wall. Besides this, NaCl-, HAc-, and HCl-extractable forms of Cd were also the dominant ones in stems and leaves. These results indicated that the incorporation into pectates and proteins, phosphates, and oxalates, and the physical sequestration in the cell wall are two major strategies employed by *M. aquaticum* to tolerate and detoxify Cd ions. Cadmium uptake induced strong oxidative stress in both leaves and stems of *M. aquaticum*, as reflected by the overproduction of H_2O_2 and MDA. Oxidation resistance enzyme and non-enzymatic antioxidants, such as GSH, AsA, and NO, were critical in detoxification and accumulation of Cd. Our results indicated that *M. aquaticum* has good adaptability to Cd stress, showing its promising potential for Cd phytoremediation in aquatic ecosystems, and further provide novel ideas of the cellular mechanisms in resisting and detoxifying Cd in submerged macrophytes.

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