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Research Article

The Response of Duckweed (*Lemna minor* L.) Roots to Cd and Its Chemical Forms

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The response of duckweed (*Lemna minor* L.) roots to Cd and its chemical forms was investigated. The relative root growth rate and concentrations of Cd and its different chemical forms in the root, that is, ethanol-extractable (F_E-Cd), HCl-extractable (F_{HCl}-Cd), and residual fractions (F_r-Cd), were quantified. Weibull model was used to unravel the regression between the relative root elongation (RRL) with chemical forms of Cd. Parameters assessed catalase (CAT), peroxidases (POD), and superoxide dismutase (SOD), as well as malondialdehyde (MDA) and total antioxidant capacity (A-TOC). Our results show that both the relative root growth rate and relative frond number were affected by Cd concentrations. The chemical forms of Cd were influenced by Cd content in the medium. Relative root elongation (RRL) showed a significant correlation with chemical forms of Cd. Additionally, POD and SOD increased at lower Cd concentrations followed by a decrease at higher Cd concentrations (at more than 5 μM Cd). Moreover, MDA and A-TOC increased and CAT decreased with increasing Cd exposure. Furthermore, CAT showed a significant correlation with F_{HCl}-Cd. Taken together, it can be concluded that the chemical forms of Cd are statistically significant predictors of Cd toxicity to duckweed and to the other similar aquatic plants.

1. Introduction

Cadmium (Cd) is a major contributor to heavy metal pollution. It is found in both natural and waste waters and is produced by agriculture through pesticides and fertilizers use and wastewater irrigation and by industry through smelting, metalworking, and pigmentation [1, 2]. The metal has a relatively high solubility and mobility in water and then is easily absorbed for aquatic plants [3]. It destroys photosynthetic apparatus and carbohydrate metabolisms of plants [3–5] and can be transferred in the food chain to threat human health [6]. Some evidences also indicated that the presence of Cd affected cell wall construction and vesicular trafficking [7]. Plants have developed several detoxification mechanisms to alleviate Cd toxicity, including the existence of different forms of metals and sequestration into the cell wall [8, 9].

The chemical form of Cd determines the characteristics of Cd migration, potential phytotoxicity, and accumulation in plants and then influences the detoxification and tolerance to Cd of plants [10–13]. Yin et al. [12] found that the different capacity to binding Cd different forms (such as F_{HCl}-Cd and F_r-Cd) played an important role in the genotype difference in Cd accumulation of spinach. Mwamba et al. [14] demonstrated that metals which are both in the form of insoluble phosphate and firmly adsorbed in the cell walls and vacuoles are not free to migrate and have low toxicity, whereas Xu et al. [15] reported that high Cd mobility is often in the forms of the water soluble Cd, inorganic Cd, and Cd complexes with organic acids. Wang et al. [9], in a comparison of watercress genotypes with low- and high Cd-accumulation, found that low-Cd genotypes may convert Cd into pectate/protein-bound forms and insoluble phosphate precipitates to a greater degree than high Cd types, and that this could be the primary method by which Cd toxicity and mobility are reduced in watercress.

Cd has previously been shown to induce reactive oxygen species (ROS) and cause oxidative stress (OS), which damages cell structure and function [16, 17]. Plants have evolved effective scavenging mechanisms to survive the Cd stress [6]. The key components of the ROS-scavenging system are mainly antioxidative enzymes, such as peroxidases (POD), superoxide dismutase (SOD), and catalase (CAT) and also other metabolites, including reduced glutathione, malondialdehyde (MDA), and ascorbic acid [18]. Several studies have found that enzyme activity can be modified by Cd stress before symptoms of toxicity become visible [19]. Considering this, it is inferred that enzymatic activity may signal this biological process and be an indicator of metal toxicity.

Duckweed (*Lemna minor* L.) is a small floating aquatic plant consisting of submerged greenish roots and floating plant bodies (referred to as fronds). In terms of its reproduction, this primarily takes place in a vegetative way through the creation of colonies [20]. To be specific, this involves the formation of daughter fronds at the proximal ends of mother fronds in two meristematic regions (ibid). It is well known that duckweed accumulates heavy metals [21], including Cd [2, 22]. Its physiological response to heavy metal stress [23] has been extensively studied in recent years, and researchers have started to examine its defence mechanisms against heavy metal toxicity [24]. However, relatively few studies have examined the correlation between the heavy metal toxicity (which results from the chemical forms) and antioxidant enzymatic activity in duckweed. This knowledge gap greatly restricts our capability to understand the potential mechanisms of defence against heavy metal toxicity of aquatic plants.

In the present study, an experimental design was developed to achieve the following objectives: firstly, to investigate root growth and the antioxidative enzymatic activity (specifically with respect to CAT, SOD, POD, and MDA) of duckweed roots exposed to different Cd concentration; secondly, to detect the change of different chemical forms of Cd; and thirdly, to quantify the relationship between the antioxidant system and different chemical forms of Cd, which directly reflect different toxicity of different chemical forms. The achievement of these objectives was planned to illuminate the role of chemical forms in detoxification of aquatic plants to heavy metal.

2. Materials and Methods

2.1. Plant Materials and Culture. Sample duckweed seedlings were collected from Yileen Garden in Nanjing, China. The samples were placed in a Hoagland culture solution containing 600 μM $Ca(NO_3)_2$, 300 μM $MgSO_4$, 300 μM K_2HPO_4, 13.8 μM H_3BO_3, 217 μM $MnSO_4$, 0.3 μM Na_2MoO_4, 0.5 μM $CuSO_4$, 16.5 μM Fe(III)-EDTA, and 0.3 μM $ZnSO_4$ under natural light conditions in a greenhouse. The pH of the medium was taken to 5.5 with 0.1 M NaOH and HCl. The medium was replaced every other day. Inoculation of a single 2-3 frond colony was used to initiate experimental cultures for growth measurement and of 10–12 colonies for other analyses.

2.2. Toxicity Experiments. Duckweed plants of similar fronds numbers were selected to place in medium containing Cd (0.1, 0.5, 1, 5, 10, or 20 μM $CdCl_2$). A free Cd treatment (only containing Hoagland medium) was used for control. Each treatment was performed in triplicate. The treatment medium was replaced every other day, and the fresh plant samples were collected after 4 days of treatment. Growth over 4 days was monitored by counting the number of fronds, which were recorded as relative frond numbers after Ensley et al. [25].

To calculate relative root elongation (RRL), the formula $RRL\,(\%) = 100(RL_T - RL_S)/(RL_C - RL_S)$ was applied, where RL_T is the mean root length (RL), where toxicants (i.e., Cd^{2+}) are present, RL_C is RL in a toxicant-free control, and RL_S is RL when the seedling was transferred into the test media. The growth thus quantified can be plotted against toxicant intensity (T) measures such as the overall Cd concentration and its different chemical forms. The results often plot as negatively sigmoidal curves, which have been characterised with a Weibull equation [26, 27]. If T limits growth, $RRL = 100/\exp[(aT)^b]$, where b is a shape coefficient and the strength coefficient a increases with the degree of metal toxicity.

2.3. Extraction of Chemical Forms of Cd. The chemical forms of Cd were determined using a modified version of the following method described by Wu et al. [28]. Cd in its different chemical forms was sequentially extracted as shown below:

(i) 80% (v/v) ethanol, extracting soluble Cd, including chloride, nitrate, and aminophenol cadmium (F_E-Cd),

(ii) 0.6 M HCl, extracting insoluble $CdHPO_4$ and $Cd_3(PO_4)_2$, other Cd-phosphate complexes and Cd integrated with pectates and protein, and so on (F_{HCl}-Cd),

(iii) Cd in residues (F_r-Cd).

Fresh duckweed root samples were rinsed in tap water and deionized water and then dried with paper tissue. Roots were cut into 1-2 mm^2 pieces, which were used separately in the sequential extraction for various forms of Cd. A plant sample of 4 g was placed in a 50 ml centrifugal tube with 25 ml extractant (80% ethanol). The tube was kept in a 30°C water bath for 18 hrs. After the tube was centrifuged for 10 min at 10000 rpm, the supernatant was collected. Another 20 ml of the same extractant was added to the extract for 2 h, and the supernatant was collected and mixed with the previous supernatant collection. The entire extraction solution was collected, evaporated to a constant weight, and then digested using HNO_3–$HClO_4$ (3 : 1, v/v). After one extraction solution had been collected, the next was added to the plant materials still in the beaker using identical procedures. To determine the Cd content in residues, HNO_3–$HClO_4$ (3 : 1, v/v) was used to digest the plant material after the sequential extraction. Atomic absorption spectrophotometry (TAS-986, Beijing, China) was used to determine the concentrations of the different chemical forms of Cd.

2.4. Determination of Antioxidative Enzyme Activities. 5.0 ml of extraction buffer solution (0.05 M NaH_2PO_4 + Na_2HPO_4, pH 7.4) was used to homogenize 0.5 g samples of fresh root material. The resulting homogenate was spun in a centrifuge at 10000g for 10 min. The procedures were all performed at 4°C. The supernatant was utilized for the enzyme activity assays. The increase in 470 nm absorbency as a result of guaiacol oxidation was used to measure POD [29, 30]. To measure the guaiacol-dependent activity of peroxidase, a reaction mixture containing 50 mmol/l phosphate buffer (pH 7.0), 10 mmol/l H_2O_2, 50 mM guaiacol, and enzyme was used. Assay of SOD was based on its inhibition of photochemical reduction in nitro blue tetrazolium [31]. The reaction mixture was made up of 50 mM phosphate buffer (pH 7.4), 75 mM nitro blue tetrazolium, 13 mM methionine, 100 nM EDTA, 0–200 μL of enzyme extract, and 2 mM riboflavin, with the riboflavin being the last to be added. The absorbance of this mixture was read at 560 nm to measure SOD. One unit of SOD activity (U) was defined as the amount of enzyme that caused 50% inhibition of the initial reaction rate in the absence of enzyme. The total activity of SOD was given as U/mg protein. The method of Aebi [32] was used to measure CAT. This method uses decreasing absorbency at 240 nm as an indicator of H_2O_2 hydrolysis. One unit of enzyme activity (U) was equated for the reduction of 0.1 units at A_{240} in 1 min, and the activity of CAT was expressed in terms of U/mg protein.

2.5. Lipid Peroxidation. The formation of MDA was used as an indirect method to estimate lipid peroxidation in vitro, as this is a by-product of the peroxidation of lipids and reacts with thiobarbituric acid [33]. This was performed through homogenization of 100 mg of fresh plant material in 1 ml of 15% (w/v) trichloroacetic acid, 0.25 M HCl, 0.37% (w/v) 2-thiobarbituric acid, and 0.01% (w/v) butylated hydroxytoluene in a ceramic mortar, followed by incubation of the samples for 30 min at 90°C, chilling with ice, and, finally, centrifuging for 10 min at 10000 rpm at 4°C. The 535 nm and 600 nm absorbances of the resulting chromophore were then measured. As a nonspecific turbidity correction, the latter absorbance was subtracted from the former. An extinction coefficient of 156 mM^{-1} cm^{-1} was then used to calculate the concentration of MDA.

2.6. Determination of Total Antioxidant Potential. Both enzymatic and nonenzymatic reactions (e.g., SOD, GSH, GSH-PX, CAT, VC, and VE) are included in the T-AOC of the protective system. Commercial assay kits from the Nanjing JianCheng Institute, China, were used to measure T-AOC [34] in accordance with the manufacturer's instructions. To summarise, the analysis was a colorimetric assay based on the reduction of Fe^{3+} to Fe^{2+} by the antioxidant and the formation of complexes through the reaction of the Fe^{2+} with the phenanthroline. An absorbance measurement was made at 520 nm, and one unit of antioxidant capacity (U) was characterised as an increase of 0.01 units at A_{520} in 1 min/mg protein. T-AOC was expressed in terms of U/mg protein.

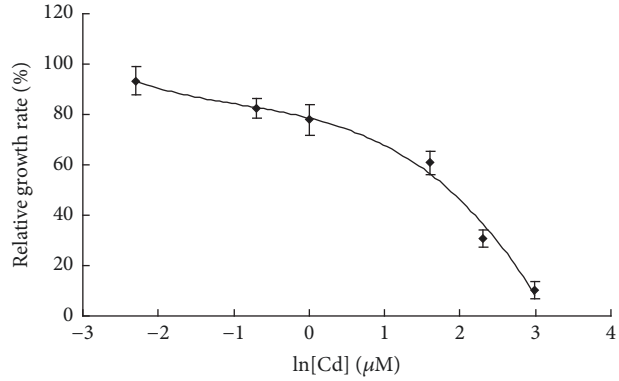

Figure 1: Relative root growth rate (%) as a function of the solution's Cd concentration (μM).

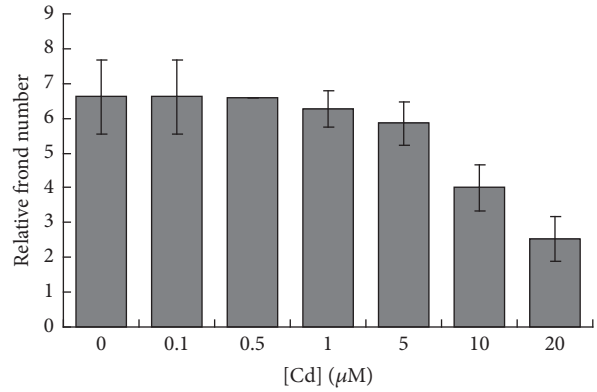

Figure 2: Growth of duckweed during cultivation in medium containing various concentrations of Cd (μM).

2.7. Statistical Analysis. EXCEL 2007 was used for determining the correlation between chemical forms of Cd and toxic response in the data. A one-way ANOVA with a least significant difference (LSD) test was used to identify significant differences, using a confidence level of 95% ($P < 0.05$). Spss was used for the ANOVA analysis.

3. Results

3.1. Effect of Cd on Duckweed Root Elongation and Frond Numbers. Figure 1 shows that the relative root growth rate was significantly related to Cd concentration. The relative root growth rate showed a continuous decrease with an increase of exposed Cd concentrations from 0.1 μM to 20 μM. Critical values corresponding to a 50% reduction of the relative root growth rate were about 10 μM.

It is important to recognize the fact that the relative frond number was also affected by the Cd concentration. Figure 2 indicates that the relative frond number, expressing the growth of Cd-exposed duckweed plants, significantly decreased ($P < 0.05$). As the solution's Cd concentration rose, the frond numbers dropped progressively. The relative frond number was 60.7% lower than that of the control in the solution with a 10-μM Cd concentration. The relative frond

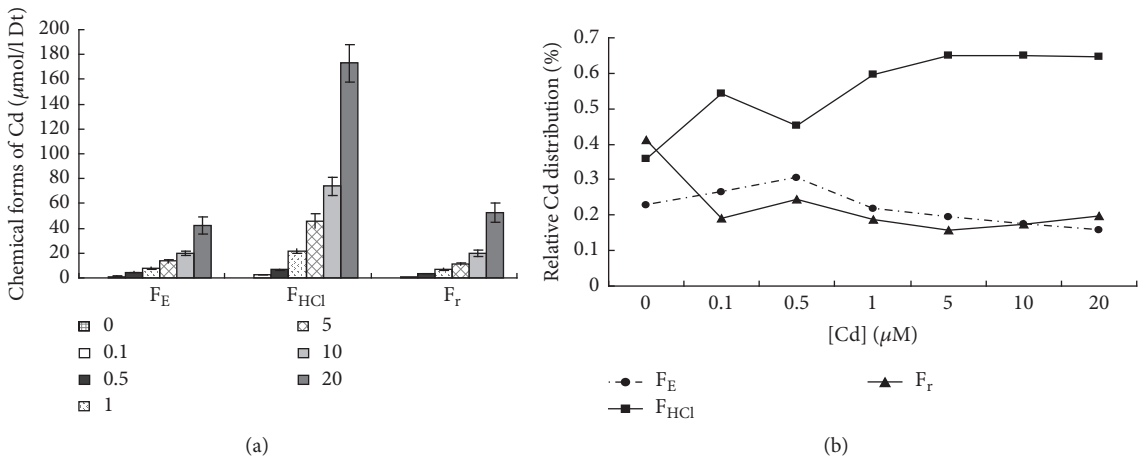

Figure 3: Chemical forms of Cd in duckweed roots (a) and percentage (b) after a 4-day period of exposure to Cd stress.

number dropped to 38.2% when plants were cultivated using a 20 μM Cd concentration medium.

3.2. Chemical Forms of Cadmium in Duckweed Root. The results of chemical forms of Cd and their relative distribution in duckweed were shown in Figure 3. It was found that Cd concentrations bound to different chemical forms in duckweed root increased with an increase of Cd exposure concentrations. For example, when the solution's Cd concentration was 0.1 μM, the F_E-Cd and F_{HCl}-Cd contents were 1.22 and 2.51 mg/kg root, respectively, and these levels increased significantly for duckweed cultivated in 20 μM Cd solution, reaching 42.13 and 172.9 mg/kg root, respectively. When the solution's Cd concentrations were in the range of 0.1–10 μM, F_{HCl}-Cd was the major chemical form, followed by F_E-Cd, and finally F_r-Cd. After exposure to the 20 μM Cd concentration solution, the least prevalent form was F_E-Cd. The relative distribution varied in a concentration-dependent manner with Cd treatment. When the Cd concentration of the solution was 0.5 μM, the relative distribution of both F_E and F_r showed a peak, whereas F_{HCl}-Cd was relatively low. However, when Cd concentration in the solution increased from 0.1 μM to 20 μM, the relative distribution of F_E-Cd and F_r-Cd decreased, while the relative distribution of F_{HCl}-Cd increased. For instance, the percentage decrease for F_E-Cd was from 26.5% to 16.7% (except 30.3% at 0.5 μM), whereas the percentage increase for F_{HCl}-Cd was from 54.4% to 64.5% (except 45.3% at 0.5 μM) (Figure 3(b)).

Figure 4 illustrated the regression results between RRL and F_E-Cd, F_{HCl}-Cd, and F_r-Cd. The data indicated that the relationships conformed to the Weibull model, in which the values of R^2 were above 0.96.

3.3. Effect of Cd on Antioxidant Enzyme Activity. The responses of the antioxidant enzymes to Cd stress were shown in Figure 5. The relative specific activities of SOD and POD increased at lower Cd concentrations until they reached a peak, before decreasing at higher concentrations. Both SOD and POD peaked at 5 μM. The most pronounced 2.67-fold increase of relative specific enzyme activity was measured for SOD, and this occurred when the plants were exposed to the solution with 5 μM Cd concentration. The peak value of POD was 162.0 ± 30.7% (compared to control, $P < 0.01$) at the concentration of 5 μM. At higher Cd concentrations, enzymatic activity decreased significantly and was below the control level reaching 85.2 ± 8.1% of the control value, where the concentration of 20 μM Cd was used. However, the activity of SOD was always above the control level across both low and high Cd concentrations, and it reached 150.8 ± 18.6% of the control value ($P < 0.01$) at 20 μM. At all applied Cd concentrations, T-AOC relative specific activity was greater than in the control samples, showing a gradual, significant increase to 171.6 ± 22.2% of the control value at 0.5 μM Cd ($P < 0.01$). After a 4-day period of exposure to various Cd concentrations, this increased significantly to 410.8 ± 66.7% of the control value at 20 μM Cd. In contrast, the relative specific activity of CAT was lower than that of the control samples at all Cd concentrations, with a gradual decrease at lower Cd concentrations (≤0.5 μM) and with a sharply significant decrease at higher Cd concentrations (>0.5 μM). When the plant was exposed to 20 μM Cd, CAT activity only amounted to 18.9 ± 3.7% of the control value ($P < 0.01$). In addition, a correlation between the chemical form of Cd and Cd was observed (Figure 6). It indicated that the relationships were consistent with the logarithmic curve, in which the values of R^2 were above 0.86. F_{HCl}-Cd exhibited the strongest correlation of the three chemical forms.

3.4. Lipid Peroxidation. The MDA content of the root showed an increase when the medium Cd concentration was increased from 0.1 μM to 10 μM and then dropped slightly at the Cd concentration of 20 μM (Figure 7). That is, the MDA content had a plateau (18.87 U/mg) at 10 μM Cd. The levels of MDA at and above 1 μM Cd significantly exceeded those in the control. For example, the MDA concentration in duckweed roots exposed to 5 μM Cd was 15.8 ± 1.13 U/mg, whereas it was 6.17 ± 0.93 U/mg in the control.

4. Discussion

4.1. Toxicity of Cd on Duckweed. Superfluous heavy metals may inhibit plant root growth and shoot growth [4, 35]. The

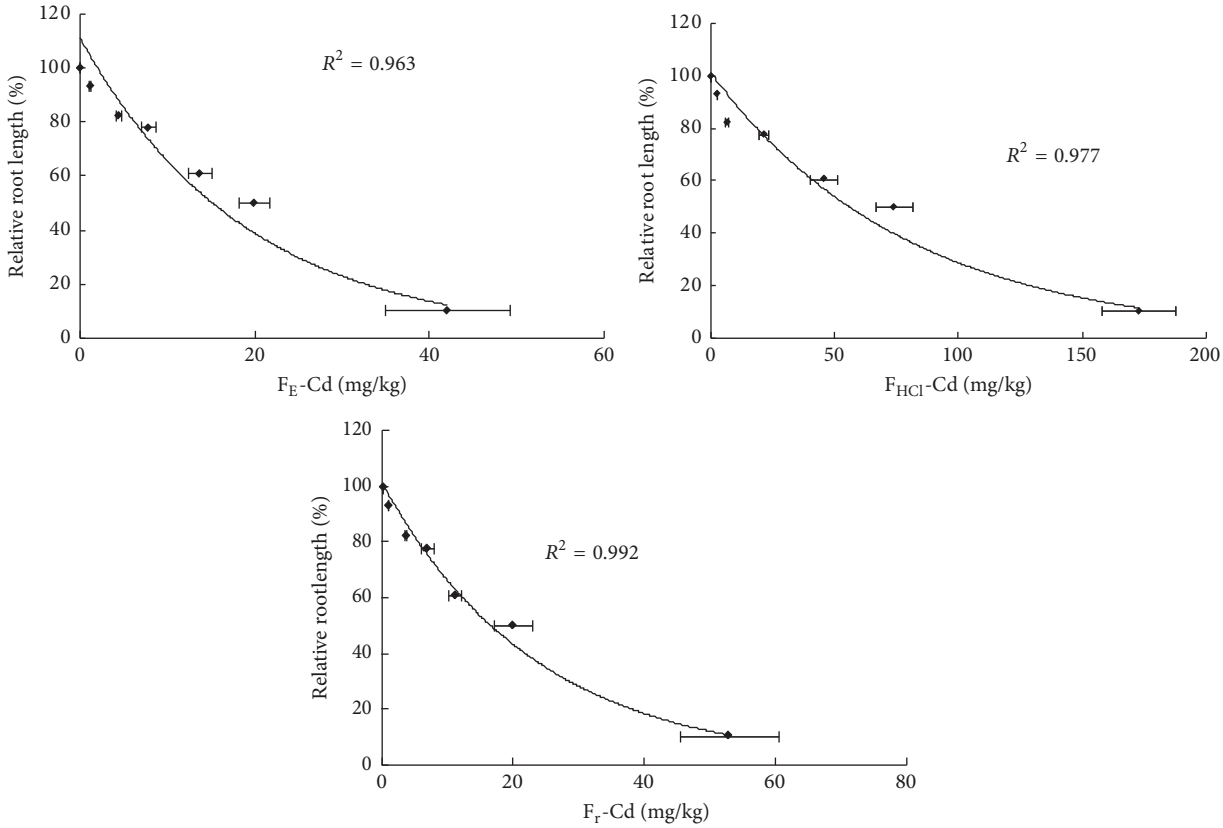

Figure 4: Regression between RRL (%) and the chemical forms of Cd.

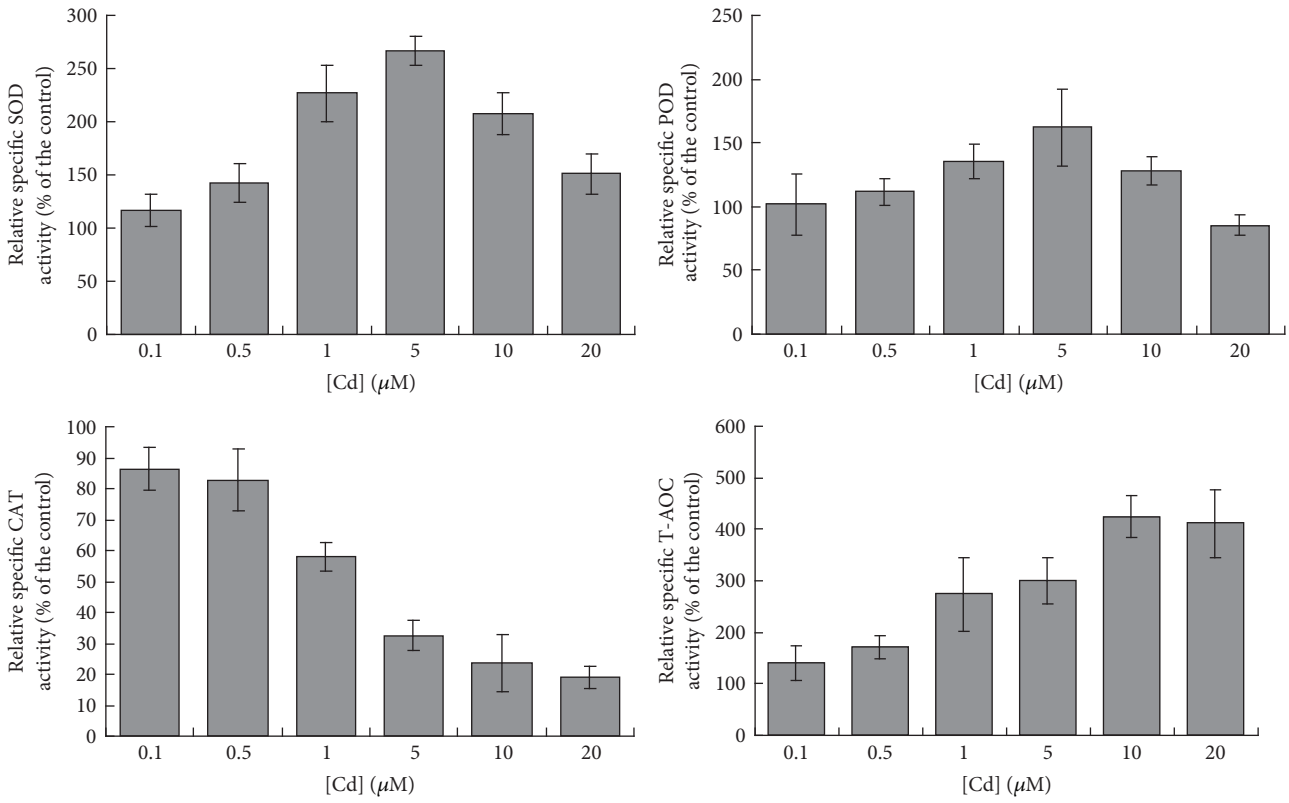

Figure 5: Dependence of relative specific enzyme activities of SOD, POD, CAT, and T-AOC on various Cd concentrations for 4 days.

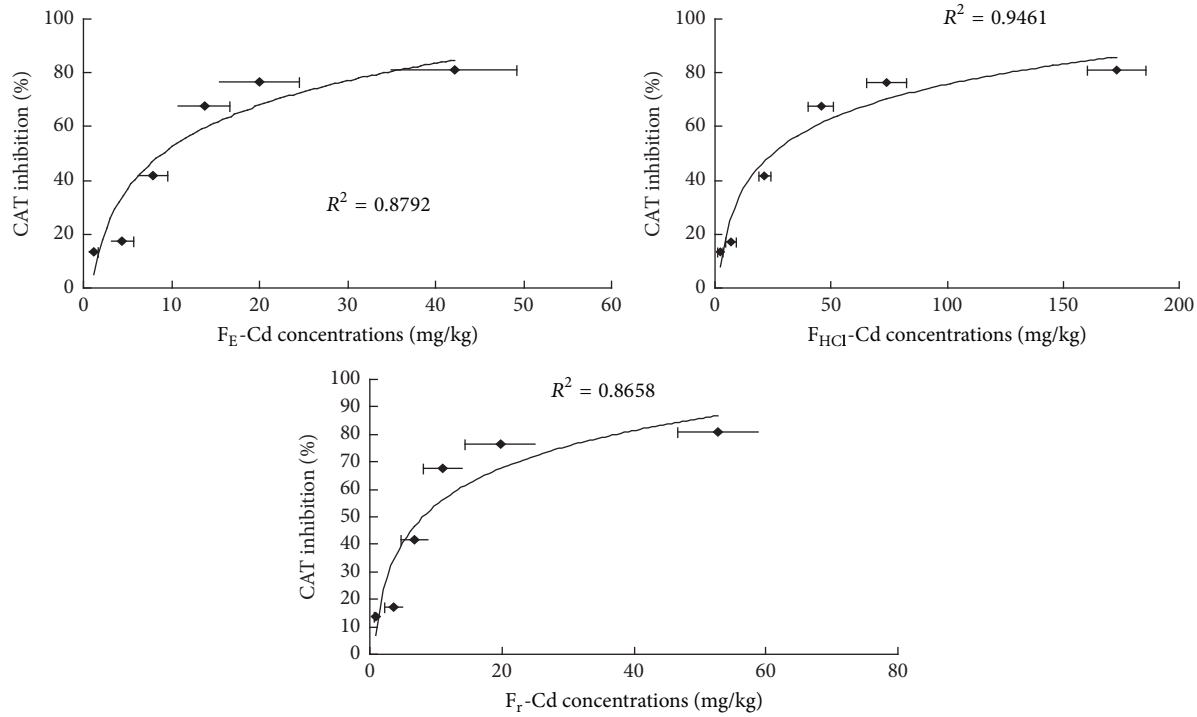

Figure 6: Inhibition of CAT of duckweed root as a function of Cd associated with different chemical forms.

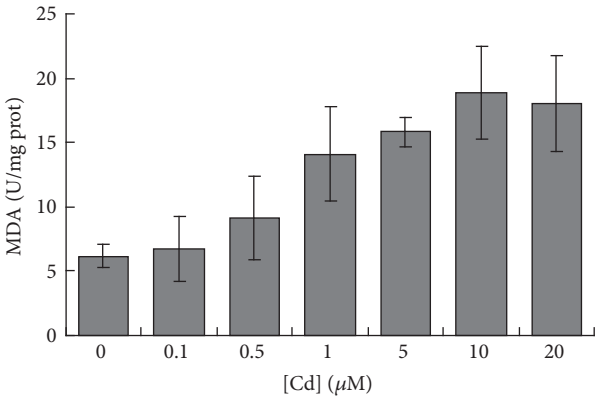

Figure 7: Relation of lipid peroxidation (expressed as amount of MDA) to solutions of various Cd concentrations.

present study found that both root elongation and frond numbers decreased with an increase in the growth medium's Cd concentration. Li et al. [36] found root elongation to be among the most sensitive of the growth parameters to Cd concentration, with an elevated Cd concentration injuring the root [37]. These observations are consistent with the present results (Figure 1). The current results suggested that the elongation of roots was significantly correlated with root Cd concentration, following the Weibull equation. Smith and Kwan [38] demonstrated high Cd toxicity in duckweed and found a Cd level of 1.7 μM as EC_{50} (concentration for 50% of maximal effect) for the reduction in frond number after a 10-day treatment. In the present results, the frond numbers were significantly decreased at the 10 μM Cd concentration after 4 days of treatment. Additionally, the results of the current study indicated the appearance of a hormesis phenomenon at lower Cd concentrations, which accords with results reported in the literature [39, 40].

4.2. Chemical Forms. Different chemical forms correspond to variable transport efficiencies, mobilities, and thus modes of accumulation and distribution [41, 42]. For example, high metal mobility is often associated with ethanol forms (F_E), whereas metals in the form of insoluble phosphates (or those which are firmly adsorbed on the cell wall) cannot migrate freely and effectively [14]. Wu et al. [13] found higher concentrations of protein and pectates when bound by Cd in barley's Cd-resistant genotype compared with that in the Cd-sensitive genotype, implicating the chemical form of Cd as a factor in plant Cd tolerance. The current study recorded higher concentrations of F_{HCl}-Cd than F_E-Cd when Cd concentration in the solution ranged from 0.1 to 20 μM (Figure 3). The implication of this is that F_{HCl}-Cd made contributions to Cd detoxification and, moreover, this was Cd's major chemical form in duckweed. In addition, with increasing Cd, the relative contributions of F_E-Cd and F_r-Cd to total Cd content decreased, except for a peak at 0.5 μM. As the Cd concentration in the solution increased further (up to 10 μM), the F_E-Cd form was less prevalent than the form of F_r-Cd. Moreover, it became the least prevalent chemical form of Cd in duckweed.

These results suggest that duckweed's tolerance to high Cd levels can be attributed to an increase in the relative contribution of forms that are Cd-inactive, along with a decrease in the relative contribution of Cd-active forms. Moreover, F_{HCl}-Cd was depressed relative to the control, suggesting the

action of a protection mechanism. This may also account for the higher root elongation and frond numbers at lower Cd concentrations in the solution. In view of this, we may conclude that it is reasonable to consider the chemical forms in which heavy metals occur to be a useful parameter for the study of metal toxicity in plants.

Ikka et al. [43] found that chemical forms can be used to assess heavy metal toxicity. The results of the present study indicated (Figure 4) a significant correlation between relative root elongation and the F_E-Cd and F_{HCl}-Cd chemical forms of Cd, which not only suggested an association between the chemical forms and Cd toxicity but also suggested that the Cd-sensitive fraction could be a metric for predicting Cd toxicity. A previous study found that the chemical form of F_{HCl}-Cd is more effective for explaining potential toxicity than is the acute toxicity [44]. Assessing the relationship between the chemical forms of heavy metals and their toxicity is therefore crucial. This study provides important evidence that Cd toxicity in duckweed root can be predicted by F_{HCl}-Cd.

4.3. Toxicity of Cd on Antioxidative Enzymatic Activity. Metal phytotoxicity is considered to induce OS [45, 46]. Scandalios [47] found that metals disturbed the normal balance between ROS and antioxidants in all aerobic cells, and a recently conducted study reported that increased antioxidant enzymatic activity has the capability of reducing OS for plants [45]. Under heavy metals conditions, SOD, POD, CAT, and T-AOC play an important protective role against ROS-induced damage [48, 49]. In the present study, the four analysed enzymes responded differently to Cd toxicity (Figure 5). Both SOD and POD were significantly induced at 5 μM Cd, whereas CAT was inhibited at all tested Cd concentrations. In addition, T-AOC was induced with a gradual, significant increase after 4-day exposure to various Cd concentrations. These results are indicative of the plasticity and specificity of duckweed's antioxidative system, which is helping in reducing OS. Similar results were obtained after exposing *Phragmites australis* [50] and *Arabidopsis thaliana* [51] to Cd, with the exception that, for *P. australis*, CAT activity increased. In addition, the inhibition of CAT was significantly and negatively correlated to the chemical form of F_{HCl}-Cd (Figure 6). This indicates that the lower CAT activities may, to some degree, result from the increased fraction of F_{HCl}-Cd.

4.4. Toxicity of Cd on Lipid Peroxidation. The relationship between the toxicity of Cd and OS was also revealed by severe lipid peroxidation, as expressed with reference to MDA [52]. MDA is an indicator of OS and consequent tissue damage [53]. In the present study, MDA increased during Cd exposure in contrast with the reduction in growth of duckweed (Figure 7), which provided support for the potentially disruptive action of peroxidation lipid with respect to Cd. Zhao et al. [23] investigated that MDA content significantly increased when duckweed sp. was exposed to high concentrations of mixed metal contaminants. Singh et al. [54] found similar results when examining an aquatic plant, *Bacopa monnieri*. In the present study, MDA decreased slightly when exposed to high Cd (μM). In the case of *Spirodela polyrhiza*, higher MDA levels were observed with medium exposure concentrations but reduced MDA levels were found at the highest mixed metal exposure concentrations [23], which totally agreed with our results. The researchers stated that these could act either directly or indirectly via toxic derivatives. Zhang et al. [55] found that MDA in leaves of *Kandelia candel* was a concentration-dependent free radical generation. In the leaves of *K. candel*, the collaboration of antioxidative enzyme activities (POD, SOD, and CAT) resulted in the MDA varieties, which are in agreement with the present results.

5. Conclusions

The response of duckweed roots to Cd stress is accompanied by changes in intracellular biological processes, including antioxidant enzymatic activity and the production of different chemical forms of Cd. Taken together, our results attest to the viability of using chemical forms as a means of investigating Cd toxicity in duckweed. This study has confirmed that F_E-Cd is the soluble form of Cd and has high toxicity and that both F_{HCl}-Cd and F_r-Cd are insoluble and have low toxicity. Among the three chemical forms, the potentially toxic Cd fraction (F_{HCl}-Cd) showed the highest level of accumulation. The relative Cd distribution showed more of the Cd-sensitive fraction (F_E-Cd) than the inert fraction (F_r-Cd), but less F_E-Cd than F_r-Cd with increasing Cd concentration. Additionally, relative root elongation was most strongly correlated with chemical forms of Cd. The decrease in CAT activity with increasing Cd concentration and in the activities of POD and SOD at high Cd concentrations indicates that Cd concentration influences the antioxidant enzyme system. As a result of a high sensitivity to Cd, CAT could be a biological indicator of Cd toxicity in duckweed roots. In conclusion, the current study has provided important evidence that illuminates the mechanism of Cd toxicity in duckweed.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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