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Phytoremediation capacity of aquatic plants is associated with the degree of phytochelatin polymerization

Anamaria Török[a,b], Zsolt Gulyás[b], Gabriella Szalai[b], Gábor Kocsy[b,*], Cornelia Majdik[a]

[a] Department of Technological Chemistry, Faculty of Chemistry and Chemical Engineering, Babeş-Bolyai University, Cluj-Napoca, Romania
[b] Agricultural Institute, Centre for Agricultural Research, Hungarian Academy of Sciences, Martonvásár, Hungary

HIGHLIGHTS

- *L. minor* extracted heavy metals more efficiently than *S. natans* or *E. canadensis*.
- A considerable metal-induced phytochelatin synthesis was observed in *L. minor* only.
- The length of produced phytochelatins was associated with metal removal capacity.

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ABSTRACT

Phytochelatins (PCs) play important role in phytoremediation as heavy metal binding peptides. In the present study, the association between heavy metal removal capacity and phytochelatin synthesis was compared through the examination of three aquatic plants: *Elodea canadensis*, *Salvinia natans* and *Lemna minor*. In case of a Cd treatment, or a Cd treatment combined with Cu and Zn, the highest removal capacity was observed in *L. minor*. At the same time, *E. canadensis* showed the lowest removal capacity except for Zn. The heavy metal-induced (Cu + Zn + Cd) oxidative stress generated the highest ascorbate level in *L. minor*. Cd in itself or combined with the other two metals induced a 10–15-fold increase in the amount of ɣ-glutamylcysteine in *L. minor* while no or smaller changes were observed in the other two species. Correspondingly, the total PC content was 6–8-fold greater in *L. minor*. In addition, PCs with higher degree of polymerization were only observed in *L. minor* (PC_4, PC_6 and PC_7) while PC_2 and PC_3 occurred in *E. canadensis* and *S. natans* only. The correlation analysis indicated that the higher phytoremediation capacity of *L. minor* was associated with the synthesis of PCs and their higher degree of polymerization.
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1. Introduction

Heavy metals in toxic concentrations in the environment come from various industrial activities such as mining or battery manufacturing. Phytoremediation is an eco-friendly remediation strategy that uses plants to reduce or to extract heavy metal contaminations from the environment [1]. The primary application of the phytoremediation methods is phytoextraction by which plants are used to bio-concentrate toxic heavy metals in polluted wastewater or soil. The metal uptake of plants depends on the physical and chemical properties of the soil and those of heavy metal ions [1].

Heavy metal toxicity is caused by various factors like the production of reactive oxygen species (ROS; transition metals like Fe and Cu), the blockage of essential functional groups (non-redox-active metals like Cd and Hg), and the removal of metal ions from biomolecules (different kinds of heavy metals). In high concentrations, ROS damage various macromolecules (lipids, proteins and nucleic acids), but they are important regulators of growth and development in controlled, lower amounts [2]. Stress-induced changes in ROS concentrations activate various defence mechanisms. ROS levels are controlled by non-enzymatic and enzymatic antioxidants. Ascorbate (AsA) and glutathione (GSH), as non-enzymatic antioxidants, play important role in the ascorbate–glutathione cycle by controlling the level of hydrogen peroxide. GSH also participates in the detoxification of heavy met-

Abbreviations: bis-γ-Glu-Cys, bis-γ-glutamyl-cystine; Cys, cysteine; Cys-bis-Gly, cystinyl-bis-glycine; Cys-Gly, cysteinyl-glycine; *E. canadensis*, *Elodea canadensis* Michx; $E_{Cystine/2Cys}$, reduction potential of cysteine; $E_{GSSG/2GSH}$, reduction potential of glutathione; γ-Glu-Cys, γ-glutamyl-cysteine; GSH, glutathione; GSSG, glutathione disulphide; *L. minor*, *Lemna minor* L; PC, phytochelatin; PCS, phytochelatin synthase; *S. natans*, *Salvinia natans* L.

* Corresponding author at: Agricultural Institute, Centre for Agricultural Research, Hungarian Academy of Sciences, 2 Brunszvik Str., H-2462 Martonvásár, Hungary. Fax: +36 22 569 576.
E-mail address: kocsy.gabor@agrar.mta.hu (G. Kocsy).

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als and in the maintenance of metal homeostasis [3]. It can remove toxic molecules (peroxides and xenobiotics) through conjugation, and it controls cellular redox state through the degradation of H_2O_2.

GSH removes metals directly through chelation which process is catalyzed by the glutathione S-transferase [4]. As a precursor of the metal-binding phytochelatins (PCs, [γ-Glu-Cys]$_n$Gly), it participates indirectly in the detoxification of metals too [5]. The complexes formed with the GSH or phytochelatins (PCs) are transported to the vacuoles of the cells. It has been established that PC synthesis can be stimulated in cells by their exposure to various metals [6]. For example, PCS activity in tobacco BY-2 cells treated with high concentrations (100 μM) of Cd has been seven times higher than in the control cells [7]. As a response to Pb exposure in *Salvinia minima*, it has also been noticed that the PCS activity has rapidly increased in the roots and slowly in the leaves [8].

Cd is a highly toxic heavy metal to all living beings. Cu and Zn are involved in numerous physiological processes; however, they are also toxic to the cells in higher concentrations [9]. PCs ensure the homeostasis of Cu and Zn by transferring them to the apoenzymes in the necessary amount. The remaining amount of these metals are transferred to the vacuoles [10].

The removal of Cd, Cu, Zn and other heavy metals has become a serious issue worldwide and has drawn the attention of the scientists to the development of efficient bioremediation techniques. Various crops and plant species have been analyzed for metal uptake, translocation and tolerance. Aquatic macrophytes, such as *E. canadensis* Michx., *L. minor* L. and *S. natans* L., are known as fast-growing, heavy metal-tolerant species. They are able to accumulate various heavy metals like Cr [11], Ni [12,13], As [14], Cd [15,16] and, consequently, they can act as biofilters [17].

Previously, it has been observed that Cu is an indicator of ROS formation in *Lemna* species [18]. As a result of the induced oxidative stress, their exposure to Zn and Cd metals has led to a decline in the protein concentration [19,20]. In *Lemna* species ascorbic acid, phenolics, catalase and guaiacol peroxidase have been found to play important roles in heavy metal detoxification processes [21]. Cd accumulation has led to an increase in H_2O_2 and O_2 concentration and in the lipid peroxidation in the leaves of *S. natans* too. In the latter case, it has been accompanied by an elevation of the peroxidase (POX), ascorbate peroxidase (APX) and glutathione peroxidase (GPX) activities [22]. Exposure of *E. canadensis* leaves to Cd has caused apoplastic acidosis due to the enhanced binding of Cd to the cell wall. As a consequence, Cd influx into the cytosol has been decreased [23].

These studies indicate that heavy metals activate protective mechanisms and they can be accumulated in aquatic plants in high concentrations. The objective of the present experiments was the comparison of the heavy metal removal capacities of three aquatic plant species when Cd or Cd combined with Cu and Zn have been added to their nutrient solution. In order to better understand the phytoremediation mechanism, the heavy metal-induced changes in the antioxidants (ascorbate and glutathione), in the phytochelatin levels and phytochelatin synthesis has also been studied.

2. Materials and methods

2.1. Plant material and treatment

E. canadensis, *S. natans* and *L. minor* were grown under laboratory conditions, at room temperature for 60 days, in modified Hoagland nutrient solution [24]. The aquatic plants (4 g of each species, in plastic containers, V = 500 ml) were kept in a growth chamber for a 3-day acclimatization period (Agricultural Institute, Martonvásár) under the following conditions: 12/12 h light/dark period (fluorescent light tubes), 210 μmol m^{-2} s^{-1} light intensity, continuous 22 °C and 75% relative humidity. Subsequently, they were treated with Cd alone (monometallic treatment) or in a combination with Cu and Zn (multimetallic treatment – a model system which was more similar to the natural conditions) adding each metal in a 65 μM/L concentration (concentrations in mg/L: 4.10 mg/L Cu, 4.30 mg/L Zn and 7.30 mg/L Cd). The Hoagland solution contained 0.08 μM/L Cu and 0.19 μM/L Zn which concentrations are negligible compared to those ones added later. Preliminary studies and literature reports had revealed that a 6-day long heavy metal exposure was adequate for the study of phytoextraction capacity [25].

2.2. Determination of heavy metals and their removal

In order to determine the Cu, Zn and Cd contents in the plants, the samples were oven-dried at 70 °C for 24 h. Thereafter, the tissues were ground and digested with nitric acid and hydrogen peroxide (8:3, v:v) solution using a Berghof (Germany) microwave digestion system (MWS-2 type) with 1000 W, the maximum total output of the microwave generator. The following microwave program was chosen: first, heating at 14 °C for 5 min at 75% power, subsequently at 180 °C for 10 min at 90% power, then at 100 °C for 10 min at 40% power.

The concentration of the heavy metals was determined by atomic absorption spectrophotometer (GBC SensAA Dual Australia) with flame atomization systems using Photron P410HCL-D2 lamp at the following wavelengths: 228.8 nm (Cu), 213.9 nm (Zn) and 324.7 nm (Cd). The calibration solutions were made in the range of 1–5 μg/ml for Cu, 0.2–2 μg/ml for Zn, and 0.2–1–8 μg/ml for Cd The phytoextraction process was characterized by the calculation of the heavy metal removal capacity of the plants by the formulas below (1).

Removal capacity:

$$q_{\max} = \frac{(C_f - C_i) \times V}{m} \quad (1)$$

$q_{\max}$ – plant removal capacity (mg/g), C_i – initial concentrations of heavy metals calculated from their amount in plants and the volume of solution (mg/L), C_f – final concentrations of heavy metals calculated from their amount in plants and the volume of solution (mg/L), V – Volume of solution (L), m – plant quantity, fresh weight (g).

2.3. Analysis of proteins

The total protein was estimated according to the method described of Bradford [26] using bovine serum albumin as standard.

2.4. The measurement of ascorbic acid

The plant samples were ground to a fine powder using liquid nitrogen. The ascorbic acid was extracted from 200 mg sample with 1.5 ml 1.5% metaphosphoric acid. The solutions were centrifuged for 10 min at 10,000 g at 4 °C, and then the supernatants were filtered. The total ascorbic acid [reduced (AsA) and oxidized form (dehydroascorbate, DHA) altogether] content was determined by adding 0.26 mM dithiotreitol to the supernatant. The samples were kept at room temperature for 15 min for the complete reduction. For the measurement of reduced ascorbic acid, 20 μl water was added instead of dithiotreitol. The samples were analysed by HPLC (Waters, Milford, MA, USA) using a diode array (W996) detector as described previously [27].

2.5. Analysis of thiols

For the determination of thiols, 200 mg plant sample was homogenized in a mortar with 1 ml 0.1 M HCl solution, and cen-

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