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Research Paper

Investigation of subcellular distribution, physiological, and biochemical changes in *Spirodela polyrhiza* as a function of cadmium exposure

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ABSTRACT

Cadmium (Cd) is considered to be the most phytotoxic heavy metal pollutant. Duckweeds are often used in ecotoxicological investigations as experimental model systems due to their ability to accumulate toxic metals. In this study, accumulation, subcellular distribution, and alterations of metabolic fingerprinting and physiology were evaluated in *Spirodela polyrhiza* exposed to 2.5, 5, and 10 μM Cd for 4 d. The accumulation of Cd increased in a concentration dependent manner. Subcellular fractionation of Cd-containing tissues indicated that 52%–61% of the metal was localized in cell walls and 37%–46% in the soluble fraction, and lowest concentrations were found in cellular organelles. Fourier transform infrared spectrometry analysis indicated that carbonyl, hydroxyl, thiol, and amide groups might be involved in Cd uptake. Cd induced alterations in nutrient elements; for example, it significantly increased iron and calcium and reduced phosphorus and magnesium concentrations in *S. polyrhiza*. Cd-caused oxidative damage was evidenced by increased lipid peroxidation and decreased chlorophyll, protein, and unsaturated fatty acid contents − this was associated with reductions in superoxide dismutase, glutathione reductase, and catalase activities. However, *S. polyrhiza* could combat Cd-induced injury involving a mechanism of non-enzymatic antioxidants and proline and soluble sugar accumulation.

1. Introduction

Aquatic ecosystems are sensitive to pollutants due to the presence of relatively small biomass in a variety of trophic levels, which may lead to accumulation of heavy metals (Singh et al., 2006). Cadmium (Cd), which has no known metabolic function, has been recognized as an extremely significant environmental pollutant due to its high solubility, high toxicity, and widespread distribution in aquatic environments (McLaughlin et al., 1999). Cd concentrations have been reported as 100–900 μg L^{-1} in lake waters (ICdA, 2012) and reached 4500 μg L^{-1} in some heavy-metal polluted waters in China.

Cd is toxic to plants and can easily enter into plant cells through the cell membrane via Cd-permeable channels and/or calcium (Ca), zinc (Zn), and iron (Fe) channels/transporters of low specificity (Perfus-Barbeoch et al., 2002; Clemens, 2006; Dudev and Lim, 2014). It then causes inhibition of plant growth and alterations in photosynthesis, respiration, transpiration, protein metabolism, and nutrient uptake (Clemens, 2006; Dong et al., 2006; Xu et al., 2012b; Andresen et al., 2016). At the cellular level, Cd phytotoxicity is strongly related to the disruption of cellular redox homeostasis and overproduction of reactive oxygen species (ROS) indirectly by disturbing the antioxidant system (Ortega-Villasante et al., 2007; Pérez-Chaca et al., 2014) or disruption of the electron transport chain (Heyno et al., 2008; Andresen et al., 2016). Plants have evolved a complex network of antioxidants, such as non-protein thiol (NPT), glutathione (GSH), and ascorbic acid (AsA), as well as superoxide dismutase (SOD), ascorbate peroxidase (APX), guiacol peroxidase (GPX), catalase (CAT), and glutathione reductase (GR), which function in concert to protect plant cells from oxidative damage by scavenging different types of ROS (Singh et al., 2006; Gallego et al., 2012; Xu et al., 2012a). An imbalance between ROS production and scavenging leads to cellular damage, as observed in several hydrophytes exposed to Cd (Singh et al., 2006; Xu et al., 2012b; Andresen et al., 2016).

Cd is reported to compete with other ions for binding sites (Dudev and Lim, 2014), making deficiency or imbalance of essential nutrients a part of Cd toxicity (Dong et al., 2006; Andresen et al., 2016). In the

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sunflower, *Helianthus annuus* L., inductively coupled plasma-mass spectrometry (ICP-MS) was applied to detect imbalances of copper (Cu), Fe, phosphorus (P), magnesium (Mg), and Zn homeostasis due to the presence of Cd (Lopes Júnior et al., 2014), and to determine contents of Cd, Zn, Fe, Mn, and Cu in leaves of *Solanum nigrum* and *Solanum torvum* (Xu et al., 2012a). In a previous study, we used inductively coupled plasma-atom emission spectrometry (ICP-AES) for elemental profiling studies concerning how the ionome responds to Cd in *Potamogeton crispus*, a submerged macrophyte (Xu et al., 2012b).

Cd can interfere with all parts of plant metabolism. Fourier transform infrared spectroscopy (FT-IR) is an ideal candidate for high-throughput metabolic fingerprinting, which enables a snap-shot of the metabolic composition of samples (Griffiths and de Haseth, 1986). The FT-IR results of *Lemna* biomass showed that biomass has different functional groups and these functional groups are able to react with Cd (II), Cu (II), and nickel (Ni) (II) (Saygideger et al., 2005). Villaescusa et al. (2004) reported that the lignin C—O bond might be involved in metal uptake. Recently, FT-IR analysis was successfully used to identify the active functional groups in *Oryza sativa* related to Cd stress responses (Xie et al., 2015) and in Cd and lead biosorption onto *Ficus carcia* leaves (Farhan et al., 2013). FT-IR spectroscopy suggests that carbonyl groups are involved in the remediation of silver nanoparticles by *Lemna minor* (Üçuncü et al., 2014). However, there is a shortage of toxicological data on in situ usage of this technique for monitoring of toxicity in Cd-exposed giant duckweed (*Spirodela polyrhiza*).

S. polyrhiza, a free-floating macrophyte, has been widely applied as a model organism in ecotoxicological assays to investigate effectiveness for phytofiltration of heavy metals from waste water and accompanying changes therein (Seth et al., 2007; Mishra and Tripathi, 2008; Appenroth et al., 2010; Jiang et al., 2012; Oláh et al., 2015). Many results indicated that *S. polyrhiza* was useful as a phytoremediator species in aquatic environments moderately polluted with different heavy metals (Cd, Cu, Zn, chromium, and Fe) (Seth et al., 2007; Mishra and Tripathi, 2008) and is very useful for biomonitoring of Ni^{2+} contaminated waste water (Appenroth et al., 2010).

Subcellular distribution of metals can provide useful information about metal accumulation, toxicity and tolerance in plants (Gallego et al., 2012; Xu et al., 2012b). Previous evidence supports that subcellular distribution of Cd in cell wall can provide useful information about Cd tolerance in plants (Dalla Vecchia et al., 2005; Chen et al., 2013; Fernández et al., 2014), it was hypothesized that Cd toxicity to plants was related to its distribution in cell organelles and the accompanied alterations in the composition of the membrane lipids and membrane permeability, and, consequently, essential elements across the membrane. In the present study, the toxicities of environmentally realistic Cd concentrations (Ortega-Villasante et al., 2007; Keunen et al., 2016) to *S. polyrhiza* were modeled in a dose-dependent manner. Cd subcellular distribution was evaluated using a tissue fractionation method. The metal-induced oxidative stress and antioxidant (enzymatic and non-enzymatic) response of *S. polyrhiza* was studied in detail. A comparative ionomic approach (ICP-AES) was used to evaluate the interaction between Cd and key elements: Ca, Fe, Mg, Mn, P, and Zn. Whole-frond extracts from control and Cd-grown *S. polyrhiza* were analyzed using FT-IR coupled with chemometrics to determine changes produced by Cd at the level of the metabolic profile. The main aim of this work is to provide insights into the underlying mechanisms involved in Cd uptake, distribution pattern and phytotoxicity by examining the characteristics of Cd subcellular distribution, the active functional groups, lipid peroxidation, the fatty acids composition and metal ion uptake in giant duckweed during a 4-day treatment.

2. Materials and methods

2.1. Plant material and culture conditions

Spirodela polyrhiza (L.) Schleiden was collected from a noncontaminated fresh water pond at Nanjing, China. The plants were further acclimatized in 10% Hoagland's solution for 10 d under laboratory conditions (115 μmol m^{-2} s^{-1} light irradiance, 14-h photoperiod, and 25/20 °C day/night temperature). After at least 1 wk of acclimation to the experimental light conditions, healthy and similar sized fronds (about 6 g) were treated with four different concentrations of $CdCl_2{\cdot}2.5H_2O$ (0, 2.5, 5, and 10 μM) in 2 L of Hoagland's nutrient solution in triplicate. Cd doses used in this work were environmentally realistic and chosen appropriately to expose plants to a low level of Cd. The solutions were changed every 2 d. Plants were harvested 4 d after treatment.

2.2. Fractionation of fronds and Cd analysis

After 4 d of treatment, fronds were washed thoroughly with 10 mM EDTA solution at 4 °C for 30 min under stirring followed by double distilled water to remove metals adsorbed to the surface. Tissues were separated into cell walls, organelle and soluble factions based on their different settling after centrifugation according to the procedure of Xiong et al. (2009). Briefly, the fronds were homogenized in chilled extraction buffer containing 50 mM Hepes (pH 7.5), 500 mM sucrose, 1 mM DTT, 5 mM ascorbate and 1% polyvinylpolypyrrolidone. The homogenate was centrifuged at 500*g* for 5 min to isolate the cell wall fraction. The supernatant from this centrifugation step was then centrifuged at 20 000*g* for 45 min to sediment cell organelles, and the resultant supernatant solution was referred to as the soluble fraction. Cd in cell wall, cell organelles, and the soluble fraction was quantified by ICP-AES (Prodigy, Leeman Labs, Hudson, NH, USA).

2.3. FT-IR spectroscopy

Frond samples (1 g) from each group were frozen in liquid nitrogen, homogenized by crushing, mixed with 100 mg KBr and pressed prior to measurement. The FT-IR experiments were carried out with a Nicolet model NEXUS 670 FT-IR spectrometer (Nicolet Corporation, USA). The spectrometer was purged with CO_2-free dry air for 24 h before recording the spectra, which were recorded over the wavenumber range 4000–400 cm^{-1} at room temperature using a resolution of 0.09 cm^{-1}. Each spectrum was automatically normalized to obtain the relative absorbance.

2.4. Analysis of mineral nutrient concentrations

Fronds were washed thoroughly with 10 mM EDTA solution at 4 °C for 30 min under stirring followed by double distilled water to remove metals adsorbed to the surface, then they were digested with HNO_3:$HClO_4$ (10:1, v/v) at 160 °C. The contents of Ca, P, Mg, potassium (K), Fe, and Zn were determined using ICP-AES (Prodigy).

2.5. Determination of photosynthetic pigment

Chlorophyll (Chl) and carotenoid (Car) contents, from ~0.4 g fresh weight (FW) of samples, were extracted with 80% acetone, and absorbances (A) at 470, 647, and 663 nm recorded on a spectrophotometer (GENESYS 10, Thermo Electron, Waltham, MA, USA). The contents of Chl *a*, Chl *b*, and Car were determined according to Lichtenthaler (1987).

2.6. Lipid extraction and analysis of fatty acids

The fronds were dried at 105 °C for 2 h and then were dried for another 48 h. Exactly 2 g of dry *S. polyrhiza* fronds were extracted with ether for 18 h (Soxhlet extraction) at 50 °C. Then, 3 mL of 0.5 M NaOH in methanol was added and circulation reflux applied for 20 min at 60 °C after the ether had run dry. After this, 3 mL of 13% methanolic BF_3 was added and the solution heated again for 20 min, and extracted