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Subcellular distribution and toxicity of cadmium in *Potamogeton crispus* L.

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HIGHLIGHTS

- Large proportion (48–69%) of the accumulated Cu was bound to the cell wall.
- Sub cellular distribution of Ca precipitates changed markedly after Cd treatment.
- Cd exposure caused significant oxidative stress in Cd-treated *Potamogeton crispus*.
- Cd inhibited the efficiency of electron transfer in PS II in Cd-exposed *P. crispus*.
- Cd strongly affected the uptake of certain major nutrients and micronutrients.

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ABSTRACT

The submerged macrophyte *Potamogeton crispus* L. was subjected to varying doses of cadmium (0, 20, 40, 60 and 80 μM) for 7 d, and the plants were analyzed for subcellular distribution of Cd, accumulation of mineral nutrients, photosynthesis, oxidative stress, protein content, and ultrastructural distribution of calcium (Ca). Leaf fractionation by differential centrifugation indicated that 48–69% of Cd was accumulated in the cell wall. At all doses of Cd, the levels of Ca and B rose and the level of Mn fell; the levels of Fe, Mg, Zn, Cu, Mo, and P rose initially only to decline later. Exposure to Cd caused oxidative stress as evident by increased content of malondialdehyde and decreased contents of chlorophyll and protein. Photosynthetic efficiency, as indicated by the quenching of chlorophyll *a* fluorescence (Fv/Fm, Fo and Fm), decreased significantly, the extent of decrease being directly proportional to the concentration of Cd. Increased amounts of precipitates of calcium were noticed in the treated plants, located either outside the cell membrane or in chloroplasts, mitochondria, the nucleus, and the cytoplasm whereas control plants showed small deposits of the precipitates around surface of the vacuole membrane and in the intercellular space but rarely in the cytoplasm. Photosynthetic efficiency and oxidative stress could be used as indicators of physiological end-points in determining the extent of Cd phytotoxicity.

1. Introduction

Heavy metal pollution has become a global environmental problem because heavy metals are toxic, persist for several decades in aquatic environments, and accumulate inside the living organisms in increasing quantities along the food chain. Cadmium (Cd) is among the most toxic and highly water-soluble elements, and although not essential to plants, extensive studies have revealed that Cd is readily taken up by plants and is toxic to aquatic plants at all levels—cellular, physiological, biochemical, and molecular. The symptoms of Cd toxicity include growth retardation, inhibition of photosynthesis, induction and inhibition of enzymes, disruptions in water relations, and ultrastructural changes (Prasad, 1995; Tripathi et al., 1996; Gu et al., 2002; Xu et al., 2003; Singh et al., 2006; Ding et al., 2007; Rau et al., 2007; Sivaci et al., 2008; Piotrowska et al., 2010; Yang et al., 2010, 2011).

Calcium is important to the growth and development of plants and also plays a crucial role in the response and adaptation of plants to the environment (Bush, 1995). Levels of intracellular calcium change frequently in response to different sources of environmental stress, such as heat (Yan et al., 2002) and cold (Jian et al., 2000). Vanhoudt et al. (2010) found that uptake of Ca was enhanced in *Arabidopsis thaliana* exposed to Cd. At Present, a cytochemical technique, namely antimonite precipitation, is widely used in studying the localization of Ca and changes in its levels in plant cells in response to controlled changes in the environment

Abbreviations: BSA, bovine serum albumin; DTT, dithiothreitol; EDTA, ethylene diamine tetraacetic acid; EGTA, ethylene glycol-bis *N*, *N*, *N*′*N*′-tetraacetic acid; Fo, initial/minimal fluorescence; Fm, the maximal fluorescence; Fv/Fm, the maximum quantum yield of PS II; MDA, malondialdehyde; PBS, phosphate buffered saline; PUFA, polysaturated fatty acids; PVPP, polyvinylpolypyrrolidone; TBA, thiobarbituric acid; TCA, trichloroacetic acid.

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(Jian et al., 2000; Yan et al., 2002; Li et al., 2006). However, the possible relationship between Cd toxicity and intracellular distribution of Ca in aquatic plants has not been elucidated so far.

Potamogeton crispus L. (Potamogetonaceae), a submerged macrophyte of world distribution, produces large quantities of biomass and can remove such toxic metals as Cd, Hg and Pb from wastewater (Ali et al., 1999, 2000; Sivaci et al., 2008; Yang et al., 2010, 2011). In our earlier studies, we could locate Cd in leaves of *P. crispus* using histochemical techniques and traced it to the cell wall, the cell membrane, chloroplasts and tonoplasts in leaf cell of *P. crispus* (Xu et al., 2002). We also found at the same time that exposure to Cd is deleterious to the activity of protective enzymes; retards photosynthesis, polyamines metabolism, and uptake of nutrients; and affects the ultrastructure of the cell and its organelles (Gu et al., 2002; Xu et al., 2003; Yang et al., 2010, 2011). The present paper explores Cd^{2+} toxicity in terms of its interaction with Ca^{2+} by studying the subcellular distribution of Cd and Ca in *P. crispus* and also seeks to explain – through such physiological indicators as nutritional status, lipid peroxidation (malondialdehyde, or MDA) and functioning of photosystem II – the mechanism of plant response to stress in the form non-redox heavy-metal pollutants in a water body. Results from this study could provide useful information in selecting suitable indicators of Cd toxicity in plants.

2. Materials and methods

2.1. Plant materials and Cd-treatments

P. crispus L. was collected from unpolluted bodies of freshwater in Nanjing, China. For experimental studies, plants of approximately the same height and weight were selected and washed with running tap water and distilled water, then they were kept in glass aquarium containing 1/10 Hoagland solution at photosynthetic photon flux density of 114 μmol $(m^2\ s)^{-1}$, a photoperiod of 14 h, and temperature of 25/20 °C (day/night) (Xu et al., 2010).

After 2 weeks, plant materials were transferred to glass beakers and various concentrations (0, 20 μM, 40 μM, 60 μM, and 80 μM) of Cd were supplied as $CdCl_2{\cdot}2.5H_2O$ (analytical reagent) in 2 L 1/10 Hoagland nutrient medium for 7 d, beakers were placed in growth chamber under the conditions mentioned above. All solutions were refreshed every 2 d. After harvesting, leaves were used for the study of physiological and biochemical indexes. All experiments were performed in triplicate.

2.2. Fractionation of leaves and Cd analysis

The subcellular distribution of Cd within the leaves was determined according to the procedure of Xiong et al. (2009). Cd in cell wall, cell organelles and soluble fraction was quantified by GBC 932 Plus Atomic Absorption Spectrophotometer (AVANTA, Australia) using air-acetylene flame. The detection limit of Cd was 0.011 ppm.

2.3. Nutritional status

Leaves were washed thoroughly with 10 mM EDTA at 4 °C for 30 min under stirring followed by double distilled water to remove metals adsorbed to the surface. They were digested with HNO_3: $HClO_4$ (10:1, v/v) at 160 °C. The solution samples were analyzed for nutrient elements by Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES, Leeman labs, Prodigy, USA). The detection limit of Ca, Fe, Cu, Zn, K, Mg, Mn, B, Mo and P was 0.005 ppm, 0.002 ppm, 0.007 ppm, 0.009 ppm, 0.03 ppm, 0.001 ppm, 0.001 ppm, 0.005 ppm, 0.01 ppm, 0.5 ppm, respectively.

2.4. Lipid peroxidation

Lipid peroxidation was determined by measuring the amount of malondialdehyde (MDA) in the plant samples (0.5 g) using thiobarbituric acid (TBA) (Chaoui et al., 1997). The concentration of MDA was calculated by using an extinction coefficient of 155 $mM^{-1}\ cm^{-1}$ and expressed as nmol g^{-1} fresh weight.

2.5. Photosynthetic pigment

Chlorophylls and carotenoids content (~0.4 g fresh samples) was extracted with 80% acetone and absorbances (A) at 470, 647 and 663 nm recorded on a spectrophotometer (Thermo GENESYS 10). The contents of Chl *a*, Chl *b* and Car were determined according to Lichtenthaler (1987).

2.6. PSII efficiency

Chlorophyll fluorescence was performed with a portable fluorescence spectrometer Handy PEA (Hansatech Instruments, Norfolk, UK), in order to determine the efficiency of electron transfer in PS II (Rau et al., 2007). Chlorophyll fluorescence was monitored in expanded leaves of control ($n = 6$) and exposed plants ($n = 6$). Fluorescence measurements were made between 9 and 11 a.m., and after dark adaptation for at least 20 min at room temperature to open all reaction centers. The following parameters were measured: (1) Fo, initial/minimal fluorescence. (2) Fm, the maximal fluorescence. (3) Fv/Fm, the maximum quantum yield of PS II.

2.7. Protein content

The protein content in the leaves (1 g) was estimated according to Bradford (1976) using bovine serum albumin (BSA, Sigma) as a standard protein.

2.8. Ultrastructural distribution of Ca

Subcellular Ca localization from leaves of control and Cd-treated plants of *P. crispus* was analyzed according to the methods of Tian et al. (1998). The sections were stained with uranyl acetate and observed with a Hitachi H-600 transmission electron microscope (TEM).

In order to confirm that the deposits contain Ca^{2+}, chelation of calcium ions with EGTA (ethylene glycol-bis *N*, *N*, *N*′*N*′-tetraacetic acid) was performed. The selected grids with specimens containing calcium precipitates were incubated in 0.1 M EGTA (pH 8.0) at 60 °C for 1 h to remove precipitates.

2.9. Statistical analysis

Data presented in this article are the mean values ± S.D. from at least three individual experiments. To confirm the variability of data and validity of results, all the data were subjected to the analysis of variance (ANOVA).

3. Results

3.1. Subcellular distribution of Cd

Cd content of leaves of *P. crispus* increased in direct proportion to the initial concentration of Cd (Table 1): in plants grown in a culture medium containing 80 μM of Cd, the level of Cd was as high as 1361.8 μg g^{-1} (on fresh weight basis).