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Response of Spirodela polyrhiza to cerium: subcellular distribution, growth and biochemical changes

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ABSTRACT

Rare earth elements are new and emerging contaminants in freshwater systems. Greater duckweed (*Spirodela polyrhiza* L.) is a common aquatic plant widely used in phytotoxicity tests for xenobiotic substances. In this study, the cerium (Ce) accumulation potential, the distribution of Ce in bio-molecules, and ensuing biochemical responses were investigated in greater duckweed fronds when they were exposed to Ce (0, 10, 20, 40, and 60 μM). There was a concentration dependent increase in Ce accumulation, which reached a maximum of 67 mg g^{-1} of dry weight (DW) at 60 μM Ce after 14 d. The Ce concentrations in bio-macromolecules followed the order: cellulose and pectin > proteins > polysaccharides > lipids. In response to Ce exposure, significant chlorosis; declines in growth, photosynthetic pigment and protein contents; and cell death were noted at the highest Ce concentration. Photosystem II inhibition, degradation of the reaction center protein D1, and damage to chloroplast ultrastructure were observed in Ce treated *S. polyrhiza* fronds, as revealed by chlorophyll *a* fluorescence transients, immunoblotting, and transmission electron microscopy (TEM). $O_2^{\cdot -}$ accumulation and malondialdehyde (MDA) content in the treated fronds increased in a concentration dependent manner, which indicated that oxidative stress and unsaturated fatty acids (C18:3) were specifically affected by Ce exposure. These results suggest Ce exerts its toxic effects on photosynthesis, with a primary effect on PS II, through oxidative stress.

1. Introduction

In recent years, the increased use of rare earth elements (REEs) in most modern electronic technology, and in industrial and medical products has led to elevated levels in freshwater systems. This has been caused by waste water emissions, industrial effluents, e-waste, and recycling emissions (Protano and Riccobono, 2002; Olías et al., 2005; Kulaksiz and Bau, 2011). To date, there has been little available information about the actual REE concentrations in aquatic environments, but micromolar concentrations have been found in stream water following anthropogenic inputs (Protano and Riccobono, 2002). Consequently, REEs are important, new and emerging contaminants with many different ways of entering the aquatic environment (Herrmann et al., 2016), although they have limited toxicity and are not considered extremely hazardous to the environment (Thomas et al., 2014). To date, few studies have attempted to predict their bioaccumulation in biota (Chua, 1998; Yang et al., 1999; Tai et al., 2010) or have evaluated the potential health risk of REEs in aquatic ecosystems (Herrmann et al., 2016) following anthropogenic inputs.

Previous toxicological investigations have suggested that the physiological effects of REEs (La, Ce and Tb) toxicity in plants include inhibition of germination and growth (Babula et al., 2015), nutrient deficiencies (Guo et al., 2007), and decreases in the net photosynthesis rate (Wang et al., 2009). At the cellular level, REE toxicity is known to cause disorganization or delayed organization of microtubules and aggregation of microfilaments (Liu and Hasenstein, 2005), a decrease in the fluidity of membrane lipids, and damage to membrane function (Wang et al., 2010). Possible mechanisms by which these disorders are generated are the induction of oxidative stress and the replacement of elements following exposure to high concentrations of REEs (Wang et al., 2009, 2010; Babula et al., 2015). However, the biochemical behavior of REEs in the water- plant system is still not fully known.

Photosynthetic reactions are the most important sites of inhibition by REEs and the toxic effect of REEs on photosynthesis is very complex and different mechanisms are involved. REEs can decrease light utilization, photosynthetic rate, electron transport efficiency, carbon assimilation, and chlorophyll content (Wang et al., 2009, 2014; Wen et al., 2011). The disorder, looseness, and swelling of thylakoids have

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been associated with the administration of REEs (Wang et al., 2009; Wen et al., 2011). Furthermore, studies on REE involvement in thylakoid binding have indicated that REEs mainly influence PS II photosynthesis in *Pronephrium simplex* (Lai et al., 2006). However, the effects of REEs on the photochemical activity of PS II and the main target of REE inhibition have still not been identified.

Duckweeds are found worldwide in different types of aquatic ecosystems. Many species (such as *Lemna minor*, *Lemna gibba* and *Spirodela polyrhiza*) have been extensively used as biological test systems to assess the potential impact of both organic and inorganic pollutants in ecotoxicology (Rahman et al., 2007; Kanoun-Boulé et al., 2009; Appenroth et al., 2010; Zhao et al., 2015). In this study, greater duckweed (*Spirodela polyrhiza*) was used as an ideal model plant because of its fast growth, wide distribution, sensitivity to various pollutants (Appenroth et al., 2003, 2010; Rahman et al., 2007), and it is easy to handle in the laboratory. Cerium (Ce) was used as a representative of various REEs that are known water contaminants because its average abundance in the earth's crust (66 μg g^{-1}) is almost the same as other elements (such as Cu and Zn) which have been studied more, and it is used in new materials and agricultural rare-earth micronutrient fertilizers (Chua, 1998). This study investigated the Ce toxicity to *S. polyrhiza* using a laboratory system. The subcellular distribution of Ce in biological molecules, growth, the composition of the fatty acids in the cell membrane, PSII photochemistry, chloroplast ultrastructure, and cell death were used to evaluate toxicity. We also examined changes to the composition of the thylakoid proteins complexes in PS II (D1 and D2) by immunoblot analysis. This study will improve our knowledge of the main molecular targets of Ce in plant cells and the relationship between the biological toxicity of Ce and its abundance in freshwater.

2. Materials and methods

2.1. Plant material and treatment conditions

S. polyrhiza (L.) Schleiden, clone DR (geographic isolate) plants were collected from a local pond at Nanjing city in 2013. Axenic 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 25/18 °C day/night temperature). Then, these plants were treated with five different concentrations of $Ce(NO_3)_3{\cdot}6H_2O$ (0, 10, 20, 40, and 60 μM) in a 2 L Hoagland's nutrient solution, except that PO_4^{3-} ions were not added to avoid REE precipitation (Wang et al., 2007). Cerium nitrate used was analytical grade from Shanghai Chemical Co. (China) (> 99.0%). A total of 0.1 mM KH_2PO_4 was applied to the foliage at the end of each photoperiod (Hu et al., 2002). The Ce concentrations were set according to previous toxicological investigations of Tai et al. (2010). The solutions were changed every 2 days, and the plants were harvested 14 days after treatment. The experiments were carried out in triplicate.

2.2. Analysis of leaf damage

The leaf discs were scanned with an Epson Perfection V700 Photo (J221A, Japan) and analyzed using WinFolia PRO 2011 software (Regent Instruments Inc., Canada). The proportion of leaf area damaged (*PLAD*) was defined as a fraction of the number of pixels in the damaged area to the total number of pixels for the entire leaf image.

2.3. Plant growth parameters

Relative growth rate was expressed as (*W*t – *W*o) / *W*o, where *W*o is the fresh weight just before Ce treatment and *W*t is the fresh weight after 14 d Ce treatment. The photosynthetic pigments in the treated and untreated fronds (~0.4*g* fresh sample) were extracted in 80% chilled acetone. After centrifugation at 10,000*g* for 10 min at 4 °C, the absorbance (A) of the supernatant was recorded at 470, 647, and 663 nm using a spectrophotometer (Thermo GENESYS 10, USA). The chlorophyll and carotenoid contents were obtained by calculation following the method used by Lichtenthaler (1987). Protein content was estimated according to Bradford (1976) using bovine serum albumin (BSA, Sigma) as the standard protein.

2.4. Cell fractionation

The bio-macromolecules were separated through chemical sequence extraction followed by ICP-AES analysis according to Lai et al. (2006). The first step involved grinding 2.0 g of dry fronds to powder and extracting them with ether for 8 h (Soxhlet extraction) to provide a crude lipid fraction. The second step involved extracting the residue generated after the sample had been boiled in 30 mL water for 3.5 h, and then centrifuging the mixture at 800 g for 10 min. The process was repeated twice and the supernatant solutions were combined after they were concentrated with a rotary evaporator under reduced pressure to generate the crude polysaccharide fraction. The third step involved extracting the residue obtained from the second step with 20 mL of 0.1 M NaOH at 80 °C for 2 h and then centrifuging the mixture at 10,000 g for 10 min. The process was repeated twice and all of the supernatant solutions were combined. The supernatant solution generated is a crude protein extract, and the residue comprises of cellulose and pectin. The Ce concentrations in the biomacromolecules were determined using ICP-AES.

2.5. $O_2^{\cdot -}$ generation rate

$O_2^{\cdot -}$ generation rate was measured using the hydroxylamine chloride method (Wang and Luo, 1990). The supernatant (0.5 mL) was collected and incubated at 25 °C for 60 min in the presence of 1 mM hydroxylamine hydrochloride in 50 mM sodium phosphate buffer (pH 7.8). The reaction mixture was then incubated with 1 mL of 17 mM P-aminobenzene sulfonic acid anhydrous and 1 mL of 7 mM α-naphthylamine at 25 °C for 30 min. The absorbance was measured at 530 nm. A calibration curve was established using sodium nitrite.

2.6. Lipid peroxidation

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

2.7. Lipid extraction and analysis of fatty acids

The fatty acids were extracted and quantified according to Wang et al. (2010) with minor modification. A total of 1 g of fresh *S. polyrhiza* fronds were dried at 105 °C for 5 min. Then 10 mL mixed solution of chloroform and methanol (2/1, v/v) was added and the sample was homogenized. After the addition of 2.0 mL water, the chloroform layer was separated and the solvent was evaporated. Then, 1 mL toluene and 1.5 mL 5% H_2SO_4 in methanol were added. The mixture was left for 1 h at 85 °C, after which, 5% sodium chloride (5 mL) was added and the fatty acid methyl esters formed were extracted with heptane (2 mL) and quantified by GC/MS (VARIAN 3800/2200, USA) equipped with a flame ionization detector and a capillary column DB-5 (30 m × 0.25 mm). The standard reagents for the fatty acids were purchased from Sigma. Peak identification was carried out by comparison with the standards. The results are expressed as the C18:3/(C18:0 + C18:1 + C18:2) ratio.

2.8. Assay of anti-oxidative enzymes and antioxidants

Fresh tissue (0.5*g*) was ground in a pre-cooled mortar with 5 mL