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Effect of cerium on growth and antioxidant metabolism of *Lemna minor* L.

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ABSTRACT

An increasing input rate of rare earth elements in the environment is expected because of the intense extraction of such elements form their ores to face human technological needs. In this study *Lemna minor* L. plants were grown under laboratory conditions and treated with increasing concentrations of cerium (Ce) ions to investigate the effects on plant growth and antioxidant systems. The growth increased in plants treated with lower Ce concentrations and reduced in plants treated with higher concentrations, compared to control plants. In plants treated with higher Ce concentrations lower levels of chlorophyll and carotenoid and the appearance of chlorotic symptoms were also detected. Increased levels of hydrogen peroxide, antioxidant metabolites and antioxidant activity confirmed that higher Ce concentrations are toxic to *L. minor*. Ce concentration in plant tissues was also determined and detectable levels were found only in plants grown on Ce-supplemented media. The use of duckweed plants as a tool for biomonitoring of Ce in freshwater is discussed.

1. Introduction

Rare earth elements (REEs) include elements from lanthanum to lutetium, known as "lanthanides" plus scandium and yttrium (Evans, 1983; Ni, 1995). They share similar chemical properties and accumulate in the same ore deposits. Cerium (Ce) is one of the most abundant element among REEs, displaying a wide range of human applications. The utilization of REEs in a broad array of industrial processes took place in the recent decades, making REEs indispensable ingredients in many technological applications. In addition, REEs utilization in agriculture, in animal husbandry and also in medicine is well known (Tommasi and d'Aquino, 2017; d'Aquino and Tommasi, 2017). Chloride and nitrate forms of Ce and lanthanum (La) are the main constituent of REE micro-fertilisers that are used in China since the 1970s to improve crop yield (Hu et al., 2004). Following the dramatic increase of Ce-containing materials reaching the environment, such as wastes, by-products of REEs extraction, fertilizers, manure etc., biogeochemical cycles of REEs have been heavily altered and abnormal accumulation of such elements in the environment is expected to take place. An increasing amount of data about the biological effects of REEs, often controversial, are available from 1980's about effects on terrestrial plants. For example, Lopez-Moreno et al. (2010a) reported an increase in root growth in cucumber and corn, and a stimulation of reproductive growth and floral initiation was reported in *Arabidopsis thaliana* by He and Loh (2002, 2000). Chen et al. (2004) found that both Ce and La exert stimulatory effects on *Crocus sativus*, enhancing crocin production (Ce) and growth (La). Other authors reported that Ce treatments can alleviate the effects of mineral deficiencies (Chao et al., 2008; Hao et al., 2008; Zhou et al., 2011; Ze et al., 2009) and UV-related stress (Liang et al., 2011). Fashui (2002) reported that Ce treatments on aged *Oryza sativa* seeds contrasted seed aging, promoting seed germination and antioxidant enzyme activities. In addition, different authors reported that Ce enhanced photosynthesis (Fashui et al., 2002; Xiaoqing et al., 2009; Yuguan et al., 2009), mitochondrial activity (Dai et al., 2011) and nitrogen metabolism (Weiping et al., 2003). Ce treatments seem to promote transgene integration in some species (Boyko et al., 2011) and to preserve fruit degradation due to pesticide accumulation (Wu et al., 2010). On the other hand, adverse effects of Ce on plants

Abbreviations: ABTS, 2,2'-Azino-bis-3-ethylbenzthiazoline-6- sulfonic acid; ASC, ascorbate; APX, ascorbate peroxidase; BSA, bovine serum albumin; CAT, catalase; DHA, dehydroascorbate; DHAR, dehydroascorbate reductase; EDTA, Ethylene diamine tetraacetic acid; GR, glutathione reductase; GSH, glutathione; La, lanthanum; MDA, malondialdehyde; MDHA-R, monodehydroascorbate reductase; POD, peroxidase; REEs, rare earth elements; RGR, relative growth rate; ROS, reactive oxygen species; TBA, thiobarbituric acid; TCA, trichloroacetic acid; TMB, 3,3',5,5'-Tetramethylbenzidine

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were observed by Diatloff et al. (2008) in mung bean and genotoxic effects were also reported in soybean by Lopez-Moreno et al. (2010b), that also found that application of Ce nanoparticles decreased seed germination in maize and root growth in alfalfa and tomato (Lopez-Moreno et al., 2010a). An increase in reactive species of oxygen (ROS) and inhibition of several antioxidants following Ce treatments was observed in rice (Xu and Chen, 2011) and impairment in macronutrient metabolism has been reported in horseradish (Guo et al., 2007). Increased ROS production and apoptosis induced by Ce were also reported in *Taxus tricuspidata* plants (Yang et al., 2009) and cells (Ge et al., 2006). Toxicity of REEs at micromolar concentration was reported for the alga *Skeletonema costatum* (Tai et al., 2010). Significant accumulation of REEs has been detected in different organisms and even in bacteria and lichens (Bayer and Bayer, 1991; Paoli et al., 2014). Even if REEs are today considered as new and emerging contaminants in the ecosystems (Pagano et al., 2015a, 2015b; Xu et al., 2017), little information about their concentration in aquatic environments is so far available (Kulaksız and Bau, 2011; Protano and Riccobono, 2002) and, consequently, no regulatory thresholds for REEs levels and emissions to the environment have been so far indicated. Few information is also available about the responses of aquatic plants to REEs treatment (Tommasi and d'Aquino, 2017). For example, Xu et al. (2017) recently reported toxic effects in *Spirodela polyrhiza* treated with Ce at micromolar concentrations.

The aim of this work was to clarify the potential toxicity of Ce in aquatic environment collecting information about Ce effects in common duckweed *Lemna minor* L., an aquatic species widely studied for biomonitoring and bioremediation pollutants in freshwater (Basiglini et al., 2018; Forni and Tommasi, 2016). The effect of Ce supply on growth, pigment content, lipid peroxidation levels, ROS levels and antioxidant metabolism were investigated under laboratory conditions up to millimolar concentrations, considered as threshold levels for polluted water sites (Zhu et al., 2012), since lipid peroxidation, pigment alteration, ROS and antioxidant levels were proposed as toxicity markers (Ippolito et al., 2010). We also investigated the effect of pH on Ce plant responses and tested Ce uptake to determine if duckweed can be used for biomonitoring of Ce-polluted freshwater.

2. Materials and methods

2.1. Chemicals

Ce(III) nitrate, Ce(III) chloride and Ce(IV) sulphate solutions were prepared dissolving commercial reagents (analytical grade, Sigma Aldrich, 99% purity) in Knop nutrient solution (Knop, 1865) containing: 8.4 mM CaN_2O_6 $4H_2O$, 9.89 mM KNO_3, 8.31 mM $MgSO_4$ $7H_2O$, 7.35 mM H_2KO_4P and 0.36 mM $FeSO_4$ $7H_2O$. Solutions were filter sterilized and stored at room temperature prior to use.

2.2. Plant material and treatments

A strain of *L. minor,* registered at the *Herbarium Horti Barensis* (BI) n. 41980, was obtained from the collection of the Botanical Garden of the University Aldo Moro in Bari and botanical identification was confirmed on a phenotypic basis following Pignatti et al. (2017).

The plants were surface sterilized in 10–20% (v/v) bleach for 30 s, rinsed with sterile distilled water twice and then acclimatized in Knop nutrient solution in a growth chamber for 15 days. At the beginning of the acclimatization phase Cefotaxine 100 μg/ml was added to the culture media to prevent bacterial contamination. Knop nutrient solution was added every 3 days to maintain the solution level. Growing conditions were 24 ± 2 °C, 90 μmol m^{-2} s^{-1} white light and 16 h/8 h light/dark photoperiod. For the growth tests, plants with two fronds were transferred to culture multiwell plates (15 ml and 34 mm of diameter) each containing Ce nitrate at concentrations 0, 2.5, 5, 10, 20 μM and 0.1, 0.5, 1 mM in Knop's nutrient solution pH solution 5.5 or 4. The growth was measured after 3, 5, 7, 12 and 15 days as described below. To test the effects of different Ce forms, treatments with Ce chloride, Ce sulphate 0.5 and 1 mM for 7 days at pH 5.5 were also carried out as described above. For other experiments, plants were incubated in Petri dishes with Knop solution (pH 5.5) supplemented with Ce nitrate at concentrations 0, 2.5, 5, 10, 20 μM and 0.1, 0.5, 1 mM and were harvested after 5, 7 and 12 days growing. Knop nutrient solution and Knops nutrient solution (as described above) supplemented with Ca nitrate concentrations were used as controls in order to exclude nitrate effects. All treatments were applied with five replicates.

2.3. Plant growth

The plant growth was determined as relative growth rate (RGR) a parameter as suggested by the Iso/Dis 20079 (2004) protocol and calculated as follows:

$$RGR = (\ln N_t - \ln N_0) / t$$

In which N_0 is the number of fronds at the beginning of the experiment, N_t is the number of fronds at the selected exposure time and t is the exposure time (0, 3, 5, 7, 12 and 15 days).

2.4. Chlorophyll and carotenoids content

Plant samples (0.2 g) were homogenized at 4 °C with 80% acetone 1:15 w/v and the homogenates were centrifuged at 14,000*g* for 15 min. The absorbance at λ. 663.2, λ. 646.8 and λ. 470 nm was determined on the supernatant using a Beckmann DU-600 spectrophotometer and the pigment contents were determined as reported by Lichtenthaler (1987).

2.5. Hydrogen peroxide content

Plant samples (0.3 g) were homogenized in 5% TCA 1:6 w/v and the homogenates were centrifuged at 14,000*g* for 20 min. Hydrogen peroxide content was determined in the supernatant using POD type I as described by Konigshofer et al. (2008).

2.6. Lipid peroxidation levels

Plant samples (0.4 g) were homogenized with 0.1% TCA 1:6 w/v and the homogenates were centrifuged at 12,000*g* for 15 min. The supernatant (0.5 ml) was mixed with 20% TCA 1:1 v/v containing 0.5% TBA w/v. Lipid peroxidation was determined by measuring the MDA amount obtained by the reaction with TBA, as reported by Ippolito et al. (2010).

2.7. ASC and GSH content

Plant samples (0.4 g) were homogenized with 5% metaphosphoric acid 1:2 w/v at 4 °C and the homogenates were centrifuged at 20,000*g* for 15 min. Total ASC and GSH content was determined on the supernatant as described by Paradiso et al. (2008).

2.8. Total antioxidant activity

Plant samples (0.2 g) were homogenized with 85% ethanol 1:6 w/v and the homogenates were centrifuged at 20,000*g* for 15 min. The total antioxidant activity was determined as reported by Teow et al. (2007) using ABTS as radical reacting with the different antioxidant molecules. The ABTS absorbance at λ 730 nm was determined after 1 min reaction.

2.9. Enzymatic assays

Plant samples (0.5 g) were homogenized in 50 mM Tris-Cl pH 7.8 containing 0.3 mM mannitol, 1 mM EDTA, 0.05% cysteine and 10 mM magnesium chloride 1:2 w/v. The homogenates were centrifuged at