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Calcium availability but not its content modulates metal toxicity in *Scenedesmus quadricauda*

Jozef Kováčik[a,*], Sławomir Dresler[b]

[a] Department of Biology, University of Trnava, Priemyselná 4, 918 43 Trnava, Slovak Republic
[b] Department of Plant Physiology, Maria Curie-Skłodowska University, Akademicka 19, 20-033 Lublin, Poland

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ABSTRACT

Impact of calcium nutrition (pre-culture on solid medium with standard or elevated Ca dose, i. e. 0.17 and 4.40 mM marked as low and high Ca) on acute metal toxicity (Cd, Mn and Pb, 24 h of exposure to 10 μM) in freshwater green alga *Scenedesmus quadricauda* was studied. Surprisingly, Ca content differed only slightly between low and high Ca samples and applied metals rather suppressed its amount. Na content was higher in metal-exposed high Ca samples, indicating that Ca/Na ratio may affect accumulation of metals. Content of heavy metals increased in order Cd < Mn < Pb and high Ca samples contained less metal than low Ca samples at least in absorbed fraction. Accumulation of ascorbic acid and thiols (GSH – glutathione and PC2 – phytochelatin 2) was affected mainly by Cd, GSH also by Mn and PC2 by Pb with often significant differences between low Ca and high Ca samples. Calcium nutrition also affected responses of algae to metals at the level of antioxidative enzyme activities (SOD, APX, and CAT) and elevated values were typically found in high Ca samples while ROS (hydrogen peroxide and superoxide radical) were mainly depleted in Mn treatment. These data confirm that Ca nutrition affects accumulation of metals in algae and metabolic parameters as observed in vascular plants but, unlike them, rather Ca/Na ratio than absolute Ca content seems to regulate the uptake of metals.

1. Introduction

Elevated contamination of the environment by heavy metals is mainly evoked by numerous anthropogenic activities including their deposition in the water bodies. Among them, cadmium (Cd) and lead (Pb) have no physiological role in plants while manganese (Mn) is an essential micronutrient. Irrespective of whether toxic or beneficial, elevated levels of metals have negative impact on physiology and alter reactive oxygen species (ROS) formation or antioxidative enzyme activities in vascular or non-vascular plants (Farzadfar et al., 2013; Kováčik et al., 2014; Hamed et al., 2017; Piovár et al., 2017). Subsequent metabolic responses to metal excess involve mainly an alteration of ascorbic acid (AsA) and thiol (glutathione and phytochelatins) biosynthesis both in vascular plants and algae (Balestri et al., 2014; Kováčik et al., 2016; Romano et al., 2017).

Mineral nutrition is one of key factors affecting metal uptake and toxicity through ionic strength and competitive cations sharing common transporters with toxic metals. Among major metallic nutrients, calcium (Ca) was mainly studied owing to good solubility, low toxicity at high concentrations and beneficial effect on many aspects of plant physiology (Huang et al., 2017). Its impact was mainly studied in vascular plants under the excess of Cd where elevated Ca availability in the culture medium typically leads to higher endogenous Ca content and suppresses uptake of Cd and ameliorates Cd-induced oxidative stress as reported in chamomile (Farzadfar et al., 2013), *Arabidopsis* (Zeng et al., 2017), *Sedum* (Tian et al., 2011) or shrub species (Eller and Brix, 2015). In algae, protective role of Ca has only rarely been studied (EL-Naggar and EL-Sheekh, 1998) and recent work provided evidence that heavy metals such as Pb and essential metals including Ca and Cu may share common transporters (Sánchez-Marín et al., 2014). Detailed metabolic responses to Ca nutrition and metallic stress as well as indirect impact of Ca nutrition (pre-culture on Ca-enriched media) on metal toxicity were not studied.

Unicellular green algae of the genus *Scenedesmus* (now commonly accepted as three categories *Acutodesmus*, *Desmodesmus*, and *Scenedesmus*) are widely distributed freshwater species. They are considerably tolerant to metallic excess and accumulate various metals including Cd, Ni (Pokora et al., 2014; Kováčik et al., 2016), Pb (Piotrowska-Niczyporuk et al., 2015) or Zn (Hamed et al., 2017) but comparison of the impact of various metals in the same study is rather rare (Kováčik et al., 2016).

In this study, metal uptake and metabolic responses (24 h of

* Corresponding author.
E-mail address: jozkovacik@yahoo.com (J. Kováčik).

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exposure) to Cd, Mn or Pb (10 μM) in *Scenedesmus quadricauda* pre-cultured on solid medium with standard or elevated Ca dose (i. e. 0.17 and 4.40 mM, marked as low and high Ca) were monitored. Calcium and sodium content along with the total and intracellular content of applied metals were also investigated. To our knowledge, this is first study reporting indirect impact of Ca nutrition on metals toxicity and perspectives for further studies are also suggested.

2. Material and methods

2.1. Algal culture, experimental design and statistics

Scenedesmus quadricauda (Turp.) Bréb. (Chlorophyta, Chlorophyceae), strain UTEX 76 (originated from The University of Texas, Austin) was cultured under sterile conditions on Petri dishes (9 cm diameter, 25 ml of medium per dish closed with the strip of Parafilm® M) in cultivation room (25/20 °C day/night) at PAR ~30 μmol m^{-2} s^{-1} with 'Milieu Bristol' medium containing inorganic salts (in mg/L: 750 $NaNO_3$, 175 KH_2PO_4, 75 K_2HPO_4, 75 $MgSO_4·7H_2O$, 25 $CaCl_2·2H_2O$, 20 Fe-EDTA, 20 NaCl, 2.86H_3BO_3, 1.81 $MnCl_2·4H_2O$, 0.22 $ZnSO_4·7H_2O$, 0.1 $CoCl_2·6H_2O$, 0.08 $CuSO_4·5H_2O$ and 0.052 $Na_2MoO_4·2H_2O$), glucose (20 g/L; Duchefa Biochemie, prod. no. G0802.1000), casein hydrolysate (10 g/L; Duchefa Biochemie, prod. no. C1301.0500) and solidified with agar (10 g/L; Agar Agar 700, 60 mesh, REMI M. B. s. r. o., Jablonec nad Nisou, Czech Republic). The pH was corrected to 6.5 with 2 M HCl (Kováčik et al., 2015a, 2016, 2017b). This medium contains 0.17 mM Ca and ca. 9.17 mM Na (Mo was added as sodium salt in both media and is not included in this value) and is marked as "low Ca" medium for the purpose of this study.

Ca-modified medium was prepared by substitution of $NaNO_3$ and $CaCl_2·2H_2O$ with $Ca(NO_3)_2·4H_2O$ in order to maintain equimolar nitrogen content (8.80 mM). This medium contains 4.40 mM Ca and dropped Na concentration 0.35 mM and is marked as "high Ca" medium for the purpose of this study. Ca concentration was therefore ca. 26-times higher but Na concentration ca. 26-times lower in "high Ca" than in "low Ca" medium.

Algae were inoculated on the medium and cultured over 30 days. Thereafter, they were collected from the surface of the cultivation medium, weighed and resuspended in 5 mM HEPES buffer (pH 6.5) to achieve 0.2 g fresh weight (FW) algal biomass/50 ml of buffer with the addition of 10 μM Cd, Mn or Pb (as chlorides). After 24 h of exposure to these treatments, samples were centrifuged (5 min, 4500 rpm), washed twice with HEPES buffer and algal pellet was extracted with respective solvents mentioned below. Processing of fresh samples for the estimation of enzymes and metabolites involved cold mortar and pestle with the addition of small amount of inert so-called sea sand (Penta s. r. o., Prague, Czech Republic) to achieve complete cell disruption.

One-way ANOVA followed by a Tukey's test (MINITAB Release 11, Minitab Inc., State College, Pennsylvania, USA) was used to evaluate the significance of differences ($P < 0.05$) between treatments and Student's *t*-test was used to evaluate differences between low Ca and high Ca samples in the given treatment. Three individual 50-ml tubes were assessed for each treatment and parameter (then $n = 3$). Independent repetition was also tested in order to check reproducibility.

2.2. Quantification of metals

Samples were prepared by mineralization of dry material in the mixture of concentrated HNO_3 and water (3 + 3 ml) using microwave decomposition (Ethos Sel Microwave Extraction Labstation, Milestone Inc.) at 200 °C over 1 h. Resulting clear solution was quantitatively placed to inert plastic flasks and diluted to a final volume of 10 ml. All measurements were carried out using an absorption spectrometer Perkin-Elmer Model 1100 (Perkin-Elmer, Inc.; USA) and the air-acetylene flame. For the quantification of absorbed Cd, Mn and Pb, samples were rinsed for 20 min in 5 mM Na_2-EDTA to remove surface-bound metals and subsequently in deionised water to remove excess of Na_2-EDTA. Blank (mixture of HNO_3 and water) was checked to ensure the correctness of metal quantifications including reference plant material (Štork et al., 2013; Kováčik et al., 2015a, 2017b).

2.3. Quantification of ascorbic acid and thiols

For the assay of ascorbic acid (exactly reduced ascorbic acid), samples were prepared by homogenization of fresh material in 0.1 M HCl (0.1 g FW/ml). Samples were centrifuged at 14,000 g for 15 min at 4 °C. The reaction mixture contained 0.2 ml of sample, 0.2 ml of 0.1 M HCl, 0.2 ml of ethanol, 0.1 ml of 0.4% H_3PO_4-ethanol, 0.2 ml of 0.5% bathophenanthroline-ethanol and 0.1 ml of 0.03% $FeCl_3$-ethanol in a final volume of 1 ml (last three reagents were freshly prepared). The mixture was incubated at 30 °C for 90 min, after which the absorbance was recorded at 534 nm. The ascorbic acid content was determined using a standard curve (Kováčik et al., 2017a, b, c).

Reduced glutathione (GSH) and phytochelatin 2 (PC2) were quantified in homogenates prepared with 0.1 M HCl following the method described by Perez-Rama et al. (2005). The thiol peptides derivatized with monobromobimane were measured using capillary electrophoresis set coupled with diode-array detector (UV–VIS/DAD, 190–600) (Agilent 7100, Agilent Technologies, Santa Clara, CA, USA). Detection was performed at signal wavelengths of 390 nm. Freshly prepared standard solutions were used for the identification and quantification of GSH (Sigma-Aldrich, St. Louis, MO, USA) and PC2 (Anaspec Inc., San Jose, CA, USA).

2.4. Assay of antioxidative enzymes and ROS

To detect enzymatic activities, fresh material (0.2 g FW/ml) was homogenized in 50 mM potassium phosphate buffer containing 1% insoluble PVPP (pH 7.0). Ascorbate peroxidase (APX, EC 1.11.1.11) and catalase (CAT, EC 1.11.1.6) activities were measured as the oxidation of ascorbate at 290 nm and decomposition of H_2O_2 at 240 nm, respectively. Activity of superoxide dismutase (SOD) was assayed using SOD assay kit (catalogue number 19160, Sigma-Aldrich) according to manufacturer's instructions and calculated as U mg^{-1} protein. Proteins were quantified according to Bradford's method with BSA as standard at 595 nm and enzymatic activities as noted earlier (Kováčik et al., 2015b, 2017a, b).

Reactive oxygen species (ROS) hydrogen peroxide and superoxide radical were measured using $TiCl_4$ and hydroxylamine method as reported previously (Kováčik et al., 2014).

3. Results and discussion

3.1. Quantitative changes of Ca and Na

Alteration of solid medium used for pre-culture (elevation of Ca and depletion of Na content) did not affect appearance of colonies and their chlorophyll content or viability (data not shown).

It was surprising to find that despite elevated Ca and depleted Na availability in the culture medium, control samples from high Ca medium contained slightly but significantly less Ca than those from low Ca (0.141 vs. 0.167 mg/g DW; Fig. 1). At the same time, Na content did not differ (31 and 32 μg/g DW; Fig. 1). Considering that algae were inoculated directly on the surface of solid medium (see Petri dishes with colonies in Kováčik et al., 2016) and they can therefore freely absorb nutrients, our data indicate that algal cells actively regulate mineral nutrition which is not only a simple function of available concentration. This is an interesting finding because vascular plants accumulate Ca when present in elevated available doses, e.g. *Matricaria chamomilla* cultured with added 0.1 – 5 mM Ca accumulated more Ca in root and shoot tissue than control (Farzadfar et al., 2013) and ca. 3-times higher