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Comparison of vascular and non-vascular aquatic plant as indicators of cadmium toxicity

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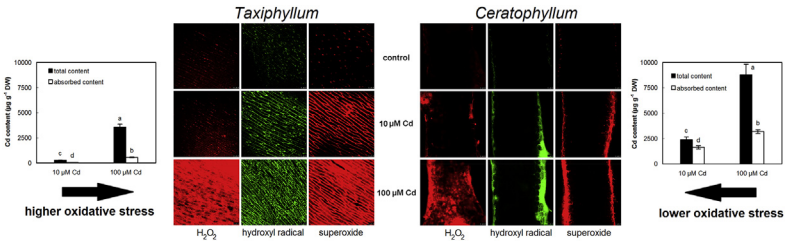
HIGHLIGHTS

- Responses of moss *Taxiphyllum* and vascular plant *Ceratophyllum* to Cd were compared.
- *Ceratophyllum* accumulated more Cd (8.80 mg/g DW) than *Taxiphyllum* (3.59 mg/g DW).
- *Taxiphyllum* showed stronger oxidative stress as detected by confocal microscopy.
- Ascorbic acid and non-protein thiols were more abundant in *Ceratophyllum*.
- Activities of SOD, CAT and APX were stimulated by Cd only in *Ceratophyllum*.

GRAPHICAL ABSTRACT



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ABSTRACT

Antioxidative and microscopic responses in non-vascular (moss *Taxiphyllum barbieri*) and vascular (*Ceratophyllum demersum*) aquatic plants exposed to short-term (24 h) cadmium (Cd) excess (10 and 100 μM) were compared. *Ceratophyllum* considerably accumulated Cd but less pronounced symptoms of oxidative stress were detected by confocal microscopy (at the level of general ROS, hydrogen peroxide, hydroxyl radical/peroxynitrite and superoxide) that could be related to enhanced activities of antioxidative enzymes (SOD, CAT, APX). Amounts of ascorbic acid and non-protein thiols were higher in *Ceratophyllum* than in *Taxiphyllum* and increased with increasing Cd dose, which may help to better regulate circulation of free metal ions in *Ceratophyllum* mainly. Besides, it was observed that citric acid increased in *Ceratophyllum* while malic acid in *Taxiphyllum* in response to Cd which may also contribute to Cd chelation. Our data indicate that *Ceratophyllum* is a suitable species for Cd bioaccumulation while *Taxiphyllum* is more sensitive to Cd excess and thus suitable as indicator species. It was also proven that sensitive microscopic techniques allow the visualization of Cd-induced changes in aquatic plants even after short-term exposure when no morphological signs of damage are visible.

1. Introduction

Cadmium (Cd) is a widespread pollutant with negative impact

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on plants and biota generally because it is readily absorbed by organisms (Kováčik, 2013; Habib et al., 2016). Its influence on aquatic plants is more pronounced in comparison with land plants owing to whole body being exposed to solution (Andresen et al., 2013; Oláh et al., 2015; Das and Mazumdar, 2016). Cd, such as other heavy metals, stimulates the formation of reactive oxygen species (ROS, Balestri et al., 2014; Kováčik et al., 2015b). Imbalance between generation and removal of ROS, called oxidative stress, leads to damage and depletion of vitality (Kováčik et al., 2014a).

Plants have developed an array of mechanisms to protect against ROS excess including synthesis of antioxidative molecules such as ascorbic acid (AsA) or phenolic metabolites and metal chelators such as phytochelatins or organic acids (Singh et al., 2006; Dresler et al., 2014). Cd variously affects their accumulation depending on the ontogenetic stage and applied concentration (Aravind and Prasad, 2005; Kováčik et al., 2014b). To our knowledge, aquatic mosses were not studied in this sense but some mosses are good accumulators of metals and respond to metals by an alteration of AsA or thiols (Choudhury and Panda, 2005; Chen et al., 2010; Sun et al., 2010). On the contrary, vascular aquatic plant *Ceratophyllum demersum*, a free floating macrophyte, has been partially studied in terms of metal accumulation and oxidative stress (Aravind and Prasad, 2005; Mishra et al., 2008a,b).

Antioxidative enzymes are frequently monitored in metal-stressed tissues including ROS-decomposing enzymes mainly. Among them, superoxide dismutase, catalase and various peroxidases remove superoxide and subsequently formed hydrogen peroxide, respectively. These enzymes had sometimes been reported in mosses or in *Ceratophyllum* (Choudhury and Panda, 2005; Mishra et al., 2008a,b; Sun et al., 2010), but comparison in vascular vs. non-vascular plant under identical exposure conditions along with the fluorescence microscopic detection of ROS formation is not known.

In this study, aquatic moss *Taxiphyllum barbieri* (commonly known as Java moss) and aquatic vascular species *Ceratophyllum demersum* were exposed to short-term Cd (24 h) aimed at comparing antioxidative responses and Cd accumulation. Confocal microscopy using various ROS and non-ROS staining reagents was involved in order to see microscopic changes. Data are compared with similar studies (involving mosses, *Ceratophyllum* or aquatic macrophytes) or with algal species in terms of basic values of metabolites.

2. Material and methods

2.1. Plant culture, experimental design and statistics

Aquatic moss (*Taxiphyllum barbieri*) and aquatic plant (*Ceratophyllum demersum*), frequently aquarium-cultured plants, were purchased from the Superzoo shop in Bratislava (Slovak Republic). Because they previously grew in the same aquarium, basal level of nutrients is considered to be species-specific (see Results). Plants were maintained in tap water (24 h) to remove eventual surface-bound pollutants prior to the start of the experiments. Thereafter, fully developed thalli of *Taxiphyllum* (5–7 cm) and subapical adult parts of *Ceratophyllum* (4–5 leaf stages) with ca. 0.2 g fresh weight were exposed to 10 or 100 μM Cd (applied as $CdCl_2 \cdot 2½H_2O$, Lachema Brno, Czech Republic) in 50 ml solutions prepared with distilled water (pH 6.0) is screw-cap tubes (Sarstedt, Germany) and maintained over 24 h in cultivation room with PAR ~400 μmol m^{-2} s^{-1} (~25/20 °C, 12 h/12 h day/night, Kováčik et al., 2014a,b). Control treatment was maintained in distilled water only under identical conditions as Cd-exposed samples. Samples were then washed with distilled water, carefully dried with filter paper, weighed and extracted with respective solvents mentioned below. In parallel, fresh and dry masses (dried at 75 °C to constant weight) were measured to determine water content [% = 100 − (dry mass × 100/fresh mass)] allowing recalculation of parameters measured in fresh samples.

Four tubes were analyzed for each treatment ($n = 4$). ANOVA followed by a Tukey's test (MINITAB Release 11, Minitab Inc., State College, Pennsylvania, USA) was used to evaluate the significance of differences ($P < 0.05$). Two independent repetitions of the whole experiment were performed in order to check reproducibility.

2.2. Quantification of cadmium and minerals

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 atomic absorption spectrometer AA30 (Varian Ltd.; Mulgrave, Australia) and the air-acetylene flame (Štork et al., 2013; Kováčik et al., 2015a,b). For the quantification of absorbed Cd, 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 (Virginia tobacco leaves CTA-VTL-2).

2.3. Quantification of metabolites

Organic acids (citric and malic acids) we monitored in extracts prepared by homogenization of fresh material in 0.1 M HCl (0.1 g FW ml^{-1}) using 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 tissue disruption. Samples were centrifuged at 14 000 g for 15 min at 4 °C. Quantification was done by enzymatic methods as described in detail previously (Delhaize et al., 1993) and basal values were verified by LC-MS/MS (Kováčik et al., 2015b).

For the assay of ascorbic acid (exactly reduced ascorbic acid), above-mentioned 0.1 M HCl were used. 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 (Wang et al., 1991).

Non-protein thiols (or reduced thiols) were quantified using 5,5'-dithiobis-(2-nitrobenzoic acid) known as Ellman's reagent (DTNB, Zezulka et al., 2013) in homogenates prepared with 0.1 M HCl as mentioned above. Reaction mixture contained 0.1 ml of sample, 0.8 ml of 50 mM potassium phosphate buffer (pH 7.0) and 0.1 ml of DTNB from freshly prepared stock solution (0.6 mM in 50 mM potassium phosphate buffer, pH 7.0) in a final volume of 1 ml. After incubation at laboratory temperature during 15 min, absorbance was detected at 412 nm. Cysteine was used for calibration instead of glutathione (GSH, which gives ca. 2-times lower content).

Total soluble phenols were extracted with 80% methanol (0.1 g FW ml^{-1}) and quantified using Folin-Ciocalteu method with gallic acid as standard and detection at 750 nm as described previously (Kováčik and Bačkor, 2007).