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

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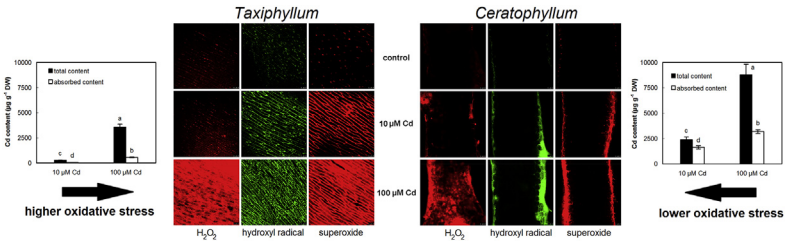
HIGHLIGHTS

- 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).

2.4. Assay of antioxidative enzymes

To detect enzymatic activities, fresh material (0.2 g FW ml^{-1}) was homogenized in potassium phosphate buffer containing 1% insoluble polyvinylpolypyrrolidone (pH 7.0) 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. Ascorbate peroxidase (APX) and catalase (CAT) activities were measured as the oxidation of ascorbic acid and decomposition of H_2O_2 at 290 and 240 nm, respectively (μmol min^{-1} mg^{-1} protein for CAT and nmol min^{-1} mg^{-1} protein for APX). Activity of superoxide dismutase 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 (Bradford, 1976) with BSA as standard at 595 nm (Kováčik et al., 2015a,b).

2.5. Confocal microscopy

The cell viability was assayed using fluorescein diacetate (FDA, Sigma-Aldrich) and propidium iodide (PI, Sigma-Aldrich): PI, a nucleic acid stain, penetrates through damaged cell membranes and intercalates the DNA of the cell, so PI positive cells are dead or dying. Living cells metabolize FDA to fluorescein, which emits green light upon excitation (Kováčik et al., 2015a).

Reactive oxygen species were stained using general ROS indicator CellROX Deep Red Reagent (Life Technologies), hydrogen peroxide indicator Amplex UltraRed (Life Technologies), hydroxyl radical/peroxynitrite indicator aminophenyl fluorescein (APF, Sigma-Aldrich) and superoxide indicator dihydroethidium (DHE, Life Technologies) according to earlier works and manufacturer's instructions (Kováčik et al., 2014b; Poborilova et al., 2015).

Nitric oxide (NO) was stained using 4,5-diaminofluorescein diacetate (DAF-2DA, Life Technologies) that is a cell-permeable sensitive indicator (Kováčik et al., 2014b). Confocal microscope Leica TCS SP8 X (Leica, Germany) was used for all observations.

3. Results and discussion

3.1. Ceratophyllum absorbs more Cd but mineral nutrients and organic acids differed Amount of Cd was higher in *Ceratophyllum* than in *Taxiphyllum* in total and absorbed fraction at both Cd doses (Fig. 1). On the contrary, Cd was more adsorbed (calculated as 100 − % of absorbed from total Cd) on *Taxiphyllum* thalli (82 and 84%) than on *Ceratophyllum* (31 and 64%), indicating various accumulation mechanisms in the moss and vascular plant. Low Cd dose (1 μM) was also tested and confirmed inter-specific difference (average total Cd 37.6 and 321.9 μg g^{-1} DW in *Taxiphyllum* and *Ceratophyllum*, respectively). Our value in 10 μM Cd-exposed *Ceratophyllum* (2376 μg g^{-1} DW) is higher than in previously reported 10 μM Cd-exposed samples over 24 h where ca. 500 μg g^{-1} DW was found (Mishra et al., 2008a). In other study, moss *Taxithelium* exposed to

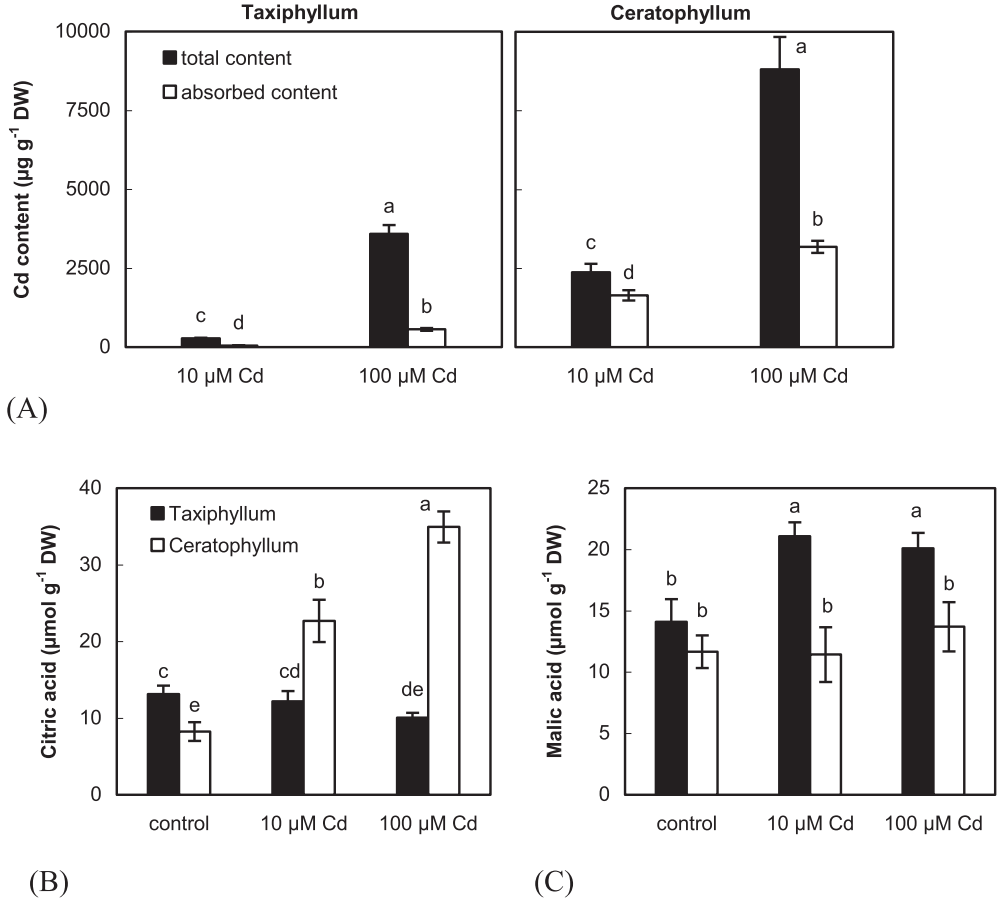

Fig. 1. Accumulation of cadmium (Cd) in the moss *Taxiphyllum barbieri* or *Ceratophyllum demersum* (vascular plant) exposed to given Cd concentrations over 24 h (A) and quantitative changes of citric acid and malic acid in both species (B and C). Note that control contained 1.59 ± 0.18 and 1.37 ± 0.21 μg Cd g^{-1} DW in *Taxiphyllum* and *Ceratophyllum*, respectively. Data are means ± SDs shown as bars ($n = 4$). Values within individual graphs, followed by the same letter, are not significantly different according to Tukey's test ($P < 0.05$).

100 μM Pb or Cr accumulated ca. 1000 μg g^{-1} DW (Choudhury and Panda, 2005). It is clear that exposure conditions mainly affect metal accumulation but *Ceratophyllum* is more potent accumulator of Cd than *Taxiphyllum*. However, field study showed that another *Taxiphyllum* species (*T. taxirameum*) had higher accumulation capacity than other species (Chen et al., 2010).

Organic acids are potential chelators of metals in plants (Dresler et al., 2014). Citric acid was elevated by both Cd doses in *Ceratophyllum* (Fig. 1B) and its molar ratio to absorbed Cd reached ca. 1.5:1 and 1.2:1, respectively (cf. Fig. 1A and B). In *Taxiphyllum*, malic acid significantly increased in response to Cd (Fig. 1C) and its molar ratio to absorbed Cd (at 100 μM Cd) was ca. 4:1 (cf. Fig. 1A and C). It seems that mentioned acids may contribute to intracellular Cd management and detailed time dynamics under prolonged exposure to Cd could clarify this assumption.

At the level of mineral nutrients, amount of K was not altered by Cd excess but was basically higher in *Ceratophyllum* (Fig. 2). On the contrary, Ca content was basically higher in *Taxiphyllum* followed by a decrease in both species under high Cd dose and more pronouncedly in *Ceratophyllum*: this may be related to higher accumulation of Cd in *Ceratophyllum* as Cd may enter plant cell through calcium channels. Amount of zinc was roughly similar in the species and significantly decreased in *Ceratophyllum* in response to higher Cd dose (Fig. 2). Zinc homeostasis may be essential for tolerance to Cd (owing to competition between these elements in various organic molecules) and redistribution of zinc in *Ceratophyllum* under low Cd doses (20–200 nM) was reported (Andresen et al., 2013). At the same time, Cd sequestration into epidermis and vein protects photosynthetic tissues (Andresen et al., 2013) and storage of Cd in the cell walls to protect cellular machinery has also been confirmed in macrophyte *Potamogeton crispus* (Xu et al., 2012).

3.1. Ascorbic acid, non-protein thiols and phenols are more accumulated in Ceratophyllum

Ascorbic acid (AsA) is a basic antioxidant produced by plant cells. Its accumulation depends on the applied metal, exposure time as well as plant species but, generally, strong oxidative stress and subsequent visible damage is related to ascorbate depletion (Kováčik et al., 2014a). Present data showed that AsA content increased in both species after exposure to 100 μM Cd mainly (Fig. 3). In agreement with our data, Pb or Cr (100 or 1000 μM) stimulated increase in AsA amount in the moss *Taxithelium* after 24 h of exposure (Choudhury and Panda, 2005) and Ni (10 or 100 μM) evoked the same in the moss *Hypnum* (Sun et al., 2010). On the contrary to our finding, Aravind and Prasad (2005) detected 10 μM Cd-induced depletion of AsA in *Ceratophyllum* after 7 days of exposure, indicating various responses in terms of time dynamics. Basal amount of AsA should also be briefly commented. As shown in Fig. 3, *Ceratophyllum* contained ca. 92-times more AsA than *Taxiphyllum*. This is in accordance with taxonomic differences between non-vascular and vascular species (Gest et al., 2013): typical AsA amount in Bryophytes and algae is ca. 0.1–0.6 μmol g^{-1} FW but >5 μmol g^{-1} FW in higher/vascular plants. Our values are close to this range and AsA content in control *Taxiphyllum* sample was 28.8 μg g^{-1} DW (= 7.59 μg g^{-1} FW = 0.043 μmol g^{-1} FW). Control AsA amount in other moss species was ca. 0.1 μmol g^{-1} FW in *Hypnum* (Sun et al., 2010) but ca. 4 μmol g^{-1} FW in *Taxithelium* (Choudhury and Panda, 2005). For comparison, LC-MS/MS detection of ascorbic acid revealed ca. 3 μg g^{-1} FW (0.017 μmol g^{-1} FW) in common algal species *Scenedesmus* (Kováčik et al., 2016), confirming similarity between green algae and water moss we analyzed. Control *Ceratophyllum* contained 2669.4 μg AsA g^{-1} DW (= 173.5 μg g^{-1} FW = 0.98 μmol g^{-1} FW) though Aravind and Prasad (2005) detected ca. 9 mg g^{-1} FW (50 μmol g^{-1} FW) in this species which seems to be too high value even in terrestrial plants such as chamomile leaves (24.5–230.2 μg g^{-1} FW; Kováčik et al., 2014a). However, differences between non-vascular and vascular plant species are clearly visible.

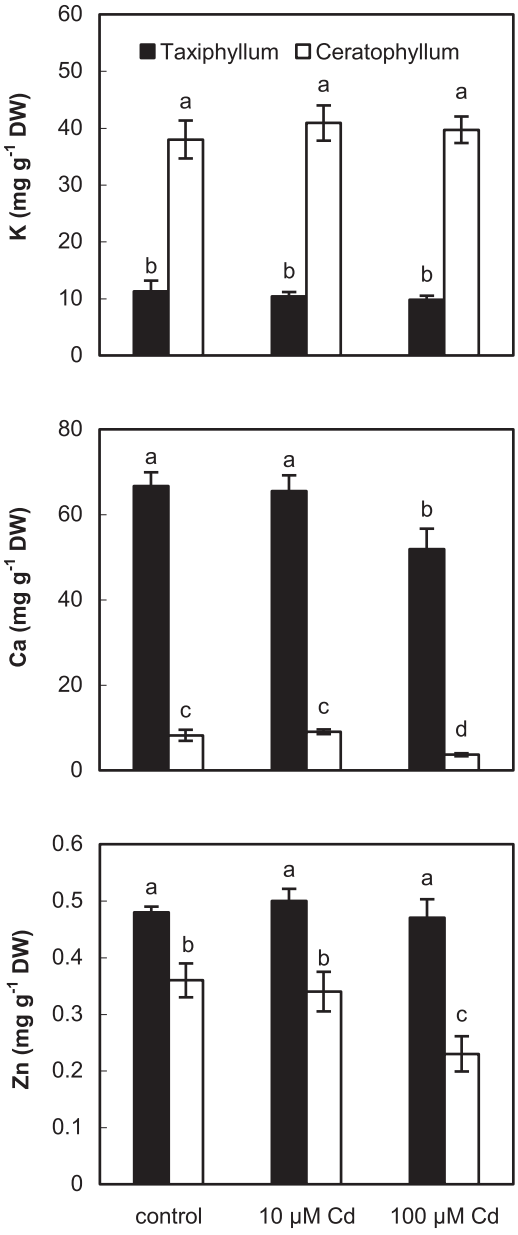

Fig. 2. Accumulation of selected mineral nutrients in *Taxiphyllum barbieri* or *Ceratophyllum demersum* exposed to given Cd concentrations over 24 h. Data are means ± SDs ($n = 4$). Values within individual graphs, followed by the same letter, are not significantly different according to Tukey's test ($P < 0.05$).

Non-protein thiols (NPT) include mainly reduced glutathione (GSH) which acts as antioxidant in ascorbate-glutathione cycle of ROS removal and phytochelatins, serving as metal chelators. Their accumulation in plants is strongly metal concentration- and time-dependently affected (Balestri et al., 2014; Kováčik et al., 2015b). Amount of NPT increased in both species and quantitative difference between *Ceratophyllum* (5336.9 μg g^{-1} DW = 346.9 μg g^{-1} FW = 2.86 μmol g^{-1} FW) and *Taxiphyllum* (179.5 μg g^{-1} DW = 47.3 μg g^{-1} FW = 0.39 μmol g^{-1} FW) control samples was

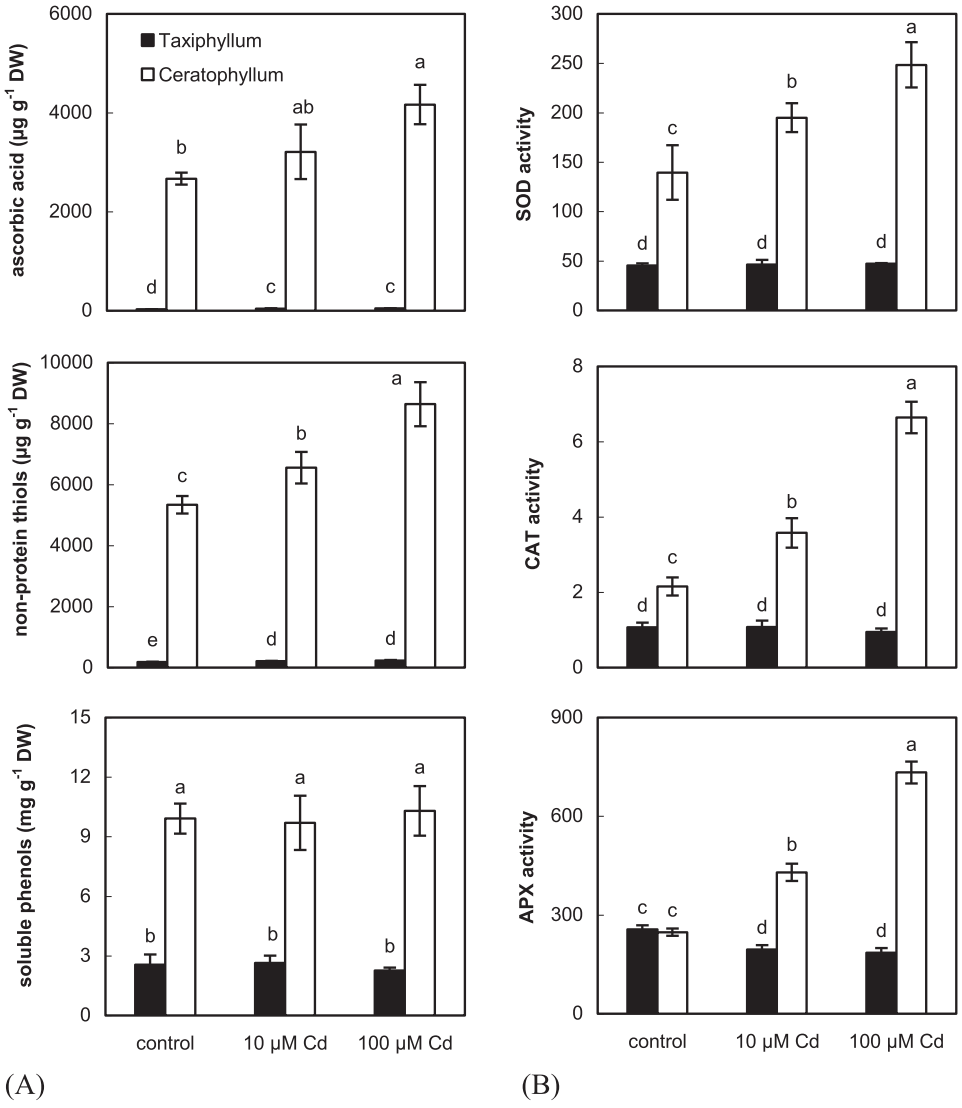

Fig. 3. Accumulation of non-enzymatic compounds (A) and enzymatic activities (B) in *Taxiphyllum barbieri* or *Ceratophyllum demersum* exposed to given Cd concentrations over 24 h. Data are means ± SDs shown as bars ($n = 4$). Values within individual graphs, followed by the same letter, are not significantly different according to Tukey's test ($P < 0.05$). SOD – superoxide dismutase (U mg^{-1} protein), CAT – catalase (μmol min^{-1} mg^{-1} protein). APX – ascorbate peroxidase (nmol min^{-1} mg^{-1} protein). Note that some data are also mentioned per g FW in Discussion allowing easier comparison with the cited literature. Values of ascorbic acid are 28.8–44.7 μg g^{-1} DW and non-protein thiols 179.5–232.9 μg g^{-1} DW in *Taxiphyllum*.

again visible (Fig. 3). Our values are in agreement with earlier studies where ca. 1–1.5 μmol NPT g^{-1} FW was observed in control *Ceratophyllum* and longer exposure to 10 μM Cd decreased (Aravind and Prasad, 2005) while prolonged exposure to As increased their accumulation (Mishra et al., 2008b). In the moss *Physcomitrella*, 3 days of exposure to 10 μM Cd increased GSH level almost 4-fold (from initial value ca. 0.2 μmol g^{-1} FW; Hermsen et al., 2010) and the same was detected in *Taxithelium* exposed to 100 or 1000 μM Pb or Cr over 24 h (but GSH level in control was even 25 μmol g^{-1} FW; Choudhury and Panda, 2005). Preliminary LC-MS/MS identification revealed elevation of phytochelatins in responses to Cd in *Ceratophyllum* (data not shown) which certainly contributes to increase in non-protein thiols we found. It is concluded that aquatic plants including mosses synthesize thiol metabolites in response to metal excess and further more complex analytical comparison among moss species is needed.

Phenolic metabolites also contribute to plant protection against oxidative stress (Kováčik and Bačkor, 2007). Total soluble phenols were not affected by Cd (Fig. 3), indicating probably that short exposure time was insufficient to stimulate their synthesis (24 h). It is therefore clear that AsA and NPT play a more prominent role in short-term tolerance to Cd. Absolute quantitative value in control was ca. 4-times higher in *Ceratophyllum*. Lower value in *Taxiphyllum* (2.26–2.64 mg g^{-1} DW) is in agreement with data from several moss species from Malaysia, containing 0.80–1.45 mg g^{-1} DW (Karim et al., 2010) assayed by identical method (and extracted with 80% methanol).

3.2. Confocal microscopy revealed higher oxidative stress in *Taxiphyllum*

Viability staining was performed using double reagent involving a cell permeable fluorescein diacetate (FDA) that is hydrolyzed by intracellular esterases to highly fluorescent product fluorescein (indicating vital cells) and propidium iodide (PI) that is a nucleic acid stain penetrating cells with damaged plasma membrane

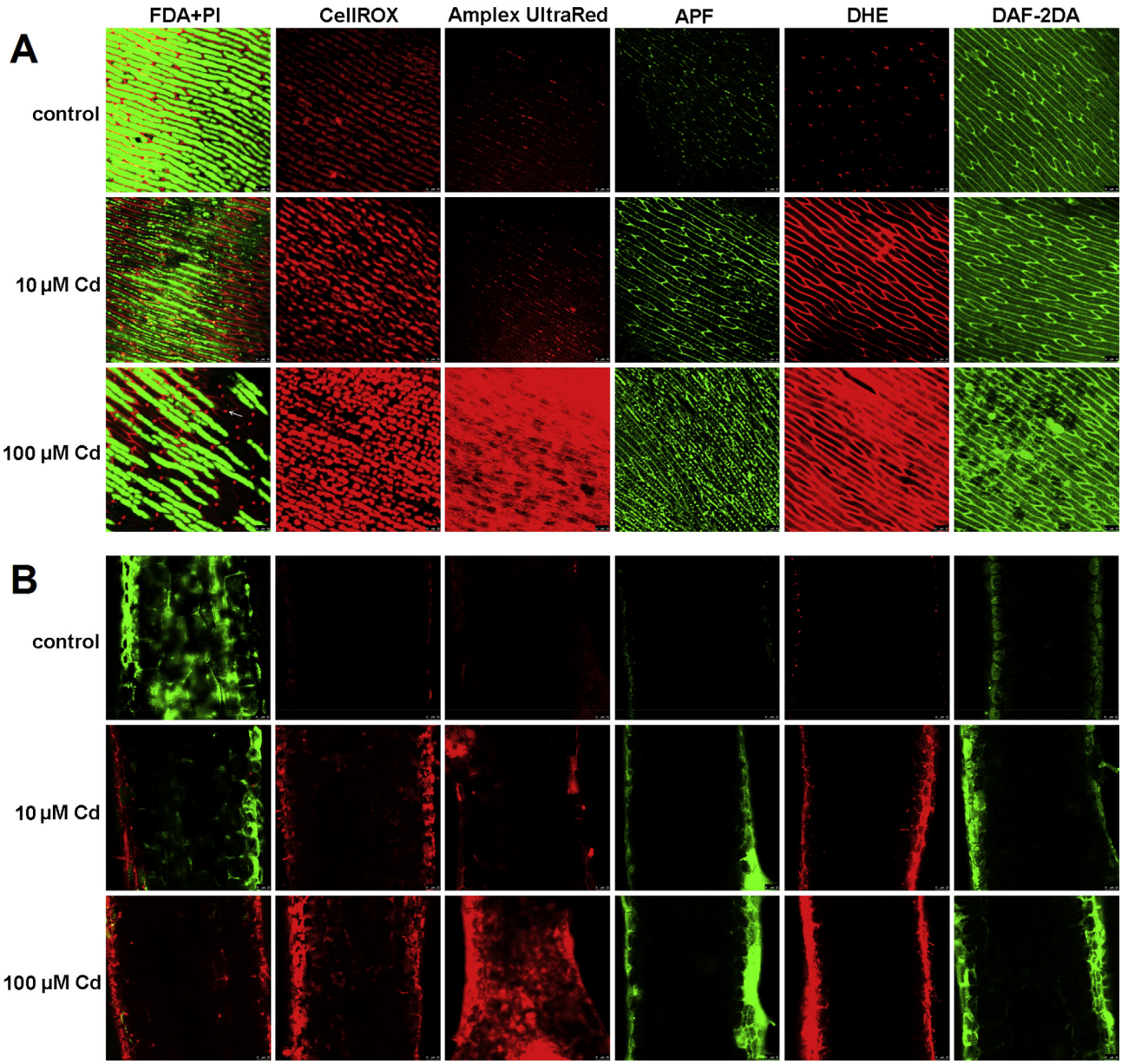

Fig. 4. Confocal microscopy in *Taxiphyllum barbieri* (A) or *Ceratophyllum demersum* (B) exposed to given Cd concentrations over 24 h. Several individual leaves were stained and representative photos are shown. Staining reagents: viability visualization with fluorescein diacetate + propidium iodide (FDA + PI, arrow indicates PI-positive nucleus), reactive oxygen species indicators: CellROX Deep Red Reagent (general ROS), Amplex UltraRed (hydrogen peroxide), APF (aminophenyl fluorescein, hydroxyl radical/peroxynitrite) and DHE (dihydroethidium, superoxide indicator). Nitric oxide (NO) was stained with DAF-2DA (4,5-diaminofluorescein diacetate). Bars indicate 10 and 25 μm for *Taxiphyllum* and *Ceratophyllum*, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(indicating dying or dead cells). Red spots indicating PI bound to nucleus were more visible in *Taxiphyllum* than in *Ceratophyllum* under 100 μM Cd (Fig. 4) and highlight higher sensitivity of the moss species despite lower accumulation of Cd. Depletion of FDA signal also indicates general depletion of viability in response to increasing Cd dose in both species, but, surprisingly, almost no signal was visible in *Ceratophyllum* under 100 μM Cd (Fig. 4B). Various surface of leaves certainly affect uptake of dye between species and PI staining seems therefore be more accurate viability method. However, difference between Cd doses in *Taxiphyllum* lead to conclusion that, under higher Cd concentration, some cells are clearly viable while some dead (red spots) and active regulation of Cd distribution within leaf could play a role (Fig. 4A).

Visualization of ROS by general indicator (CellROX) showed slight difference between 10 and 100 μM Cd while H_2O_2 signal (Amplex UltraRed) differed more pronouncedly (Fig. 4). Detection of hydroxyl radical/peroxynitrite with APF and superoxide radical with DHE also confirmed stimulation by Cd treatments in both species but higher difference between 10 and 100 μM Cd in *Taxiphyllum* than in *Ceratophyllum* (Fig. 4). All these data confirm stronger appearance of oxidative stress in *Taxiphyllum* despite lower content of Cd. Choudhury and Panda (2005) reported stronger increase in superoxide and H_2O_2 in the moss *Taxithelium* exposed to 100 μM Pb or Cr though metal content was lower compared to Cd excess tested here. It is expected that less visible Cd-induced symptoms in *Ceratophyllum* are due to higher absolute amount of AsA and NPT (Fig. 3), allowing more precise regulation of oxidative stress at subcellular level. Besides, comparison of

enzymatic activities revealed Cd-induced stimulation of SOD, CAT and APX in *Ceratophyllum* but stagnation or depletion in the moss (Fig. 3). In agreement, some ROS-decomposing enzymes were stimulated in *Ceratophyllum* by Cd or As excess while H_2O_2/superoxide quantification did not reveal extensive changes (Mishra et al., 2008a,b). Alteration of antioxidative enzymes activities in responses to Cd (10–200 μM) has also been reported in aquatic plant *Bacopa monnieri* (Singh et al., 2006). It is clear that confocal microscopy allows detection of altered oxidative status even when no visible symptoms are observable. Nitric oxide staining (DAF-2DA) showed slight increase in both species (Fig. 4) as previously observed in vascular plants and its role in the amelioration of Cd-induced ROS formation is possible (Kováčik et al., 2014b).

4. Conclusions

It was confirmed that vascular plant *Ceratophyllum* readily absorbed cadmium but showed less visible symptoms of oxidative stress (presence of reactive oxygen species and PI-stained cells): this may be related to basically higher amount of ascorbic acid and thiols leading to lower circulation of free metal ions. On the other hand, aquatic moss *Taxiphyllum* seems to be suitable indicator species for metal toxicity as it revealed clear sensitivity at microscopic level.

Disclosure statement

The authors declare that there are no conflicts of interest.

Role of the funding source

Sponsor had no involvement in the present study.

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