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Original Paper

Cadmium-induced response of protein profile and antioxidant enzymes in aquatic macrophytes *Myriophyllum spicatum* and *Ceratophyllum demersum*

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Abstract

The aquatic species *Ceratophyllum demersum* and *Myriophyllum spicatum* were grown in a hydroponic system to analyze the activity of antioxidant enzymes and protein response under different cadmium concentrations. The behavior of the studied antioxidant enzymes (APX and POD) was affected by Cd-exposure and exhibited to some extent different activity. In comparison with control, the activity of APX and POD varied significantly (P<0.01) among Cd-treatments, where the increase in Cd-concentration coupled with an initial increase in the activity of both antioxidant enzymes and subsequent Cd-treatments caused a decline in the activity. Generally, the activity of APX found to be more pronounced in *M. spicatum* (4-8 fold of control) in comparison to *C. demersum.* The treated plants exhibited different protein patterns depending on the plant species, Cd- concentration and time of exposure. Protein synthesis appears to be more sensitive to cadmium in *C. demersum*, than other plants where, appearance of high molecular weight (138, 127, 112, 109 and 100 kDa) protein at 25mg/L-Cd, disappearance of 38 kDa proteins was recorded in all sets of experiment.

Keywords: Protein pattern, POD, APX, biomarker, metal toxicity

Introduction

Heavy metal tolerant plants have efficient mechanisms for restricting excessive metal concentrations in metabolically active compartments of the cells (Salt *et al*., 1999; Wójcik *et al*., 2005; Ma *et al*., 2005; Ueno *et al*., 2005). However, some metal ions are likely to remain in the cytoplasm and induce oxidative stress via generation of reactive oxygen species (ROS), which will hinder cell metabolism (Foyer *et al*.,1994). To protect themselves against these toxic oxygen intermediates, plant cells and its organelles like chloroplast, mitochondria and peroxisomes employ antioxidant defense systems. A great deal of research has established that the induction of the cellular antioxidant machinery is important for protection against various stresses (Tuteja, 2007; Khan and Singh, 2008; Singh *et al*., 2008; Gill *et al*., 2011). Prevention of oxidative stress by scavenging ROS is actively performed by antioxidative systems, which comprise antioxidant enzymes, such as catalase, peroxidases, superoxide dismutase and glutathione reductase (GR), as well as non-enzymatic antioxidants, such as glutathione (GSx) (El-Khatib *et al*., 2004; Vitória *et al*., 2001; Gratão *et al*., 2005; Tamás *et al*., 2008). Evidence exists that antioxidative defense plays an important role also in hyperaccumulators, as changes in the activities of different antioxidative enzymes were observed in hyperaccumulating plants in response to heavy metals treatments (Boominathan and Doran, 2003; Wójcik *et al*., 2006). In addition, heavy metal phytotoxicity is controlled by a number of factors, including the element's uptake site, bioavalability, competition for binding sites and ionic speciation (Ralph and Burchett, 1998; Panda and Choudhury, 2005).

Cadmium (Cd) is a widespread non-essential toxic heavy metal (HM), mainly released into the environment by power stations, heating systems, metal-working industries, waste incinerators, urban traffic, cement factories and as a by-product of phosphate fertilizers (Sanita` di Toppi and Gabbrielli, 1999). Cadmium emitted into the environment can have significant biological and ecological effects on higher plants,

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which they protect themselves from Cd stress by a number of additive and/or synergic response mechanisms (Sanita` di Toppi and Gabbrielli, 1999). A frequent outcome following exposure to Cd pollution is the overproduction of reactive oxygen species (ROS), potentially causing oxidative damage in plant cells and thus requiring the intervention of antioxidant defense systems (Sandalio *et al*., 2001).

Aquatic macrophytes have proved to possess high metal binding capacities (Schiewer and Volesky, 2000). due to the presence of polysaccharides, proteins or lipid on the surface of their cell walls containing some functional groups such as amino, hydroxyl, carboxyl and sulphate, which can act as binding sites for metals (Holan and Volesky, 1994; Yu *et al.,* 1999). Hence, aquatic plants are often the first link in relation to metal contents of aquatic environments. Myriophyllum spicatum L. (family Haloragidaceae) and Ceratophyllum demersum L. (family Ceratophyllaceae) are two perennial aquatic macrophytes spread all over the world and considered noxious and extremely invasive for freshwater environments. These species carry out their entire life cycle completely submerged. Both the above species take up metals from water, producing an internal concentration several folds greater than their surroundings and showing much higher metal-accumulating capacity and rate than non-hyperaccumulating terrestrial plants (El-Khatib and El-Sawaf, 2001). Despite the intensive work on aquatic macrophytes for their potential use in phytoremediation, the relative contribution of the diverse mechanisms leading to metal detoxification and tolerance, as well as the interspecific differences in defense strategies, have been given scanty consideration so far. For this reason, the present work was planned to test the hypothesis that induction of antioxidant enzymes APX and POD, as well as changes in protein profiles represent compensatory mechanisms developed by the studied species due to Cd detoxification.

Material and Methods

Plant material, growth conditions and cadmium treatment

The two studied species *(C. demersum* and *M.spicatum*) were taken from the main stream of the River Nile bank. They exposed to different cadmium concentrations under controlled conditions of hydroponic cultures that similar to those of their original environment. One hundred gram from each healthy bi-distilled water pre rinsing species was transported onto the hydroponic cultures. They were kept for two weeks in Hoagland nutrient solution (Cowgill *et al*., 1989). before enriched with 25, 50, and 75 mg/L concentration of cadmium ($Cd(NO_3)_2$, Sigma, St. Louis, MO). All the concentrations were calculated based on the individual element versus their compound form. Plant sampling was carried out on 1, 3, 5 and 7 days intervals.

Antioxidant enzymes (APX & POD)

The activities of the tested antioxidant enzymes were assayed using the method of (Nakano and Asada, 1981). (*EC 1.11.1.11*) for APX, and those of (Wakamatsu and Takahama, 1993). (*EC 1.11.1.7*) for POD. In reference to control, the relative activity of the two enzymes calculated using the extinction coefficient 26.6 mM^{-1} cm^{-1} and 2.8 mM^{-1} cm^{-1} for APX and POD, respectively.

Protein Electrophoresis (SDS-PAGE)

SDS-PAGE of total proteins in control and treated plant samples was carried out according to the method of (Laemmli, 1970). The resulting gels were scanned using Olympus camera model No C-7070. Protein bands were assigned in reference to protein marker (Fermentas, PageRuler ^{TM} Unstained protein Ladder #SM0661, 10 kDa to 200 kDa), and analyzed to determine their molecular weight using BioDoc Analyze, Biometra 2006 program Version 2.49.8.1. Statistical analysis

Two-way ANOVA of SPSS 15.0 computer program was used to test the significant difference among means of the triplicate tested parameters at $P<0.01$, 0.001. Regression coefficient computed to determine the relationships between the relative activity of antioxidant enzymes and both Cd-concentration and exposure time.

Results

The relative activity of antioxidant enzymes (APX, POD) of studied plant species was comparatively lower in controlled conditions as compared to those grown in the presence of Cd^{+2}. Under conditions of the present study, short

duration of Cd^{+2}-exposure and low Cd^{+2} concentration induced increase in activities of the two-antioxidant enzymes, Meanwhile with the long duration and high Cd^{+2} and vice versa (Fig. 1). The interactions between Cd-concentration and time of exposure appeared to have significant effect ($P< 0.01$) on the relative activities of the two-antioxidant enzymes. POD attained its maximum relative activity when the studied species treated with 50 mg/L-Cd^{+2} at 5-days exposure. It is being 1125% and 657.4% for *C. demersum and M. spicatum*, respectively (Fig 1a). After 7-day exposure to 75 mg/L-Cd^{+2}, the POD activity reached its minimum value in *C. demersum (*932.44%*) and M. spicatum (*355.55 %*)*. APX enzyme (Fig 1b) showed its maximum relative activity value of 440% at 5-day exposure and 25 mg/L-Cd^{+2} in *C. demersum*, and 848.48% at 3-day exposure and 50 mg/L- Cd^{+2} in *M. spicatum*. On the other side, the relative activity of APX started to decrease at 50 mg/L-Cd^{+2}, exhibiting significantly different patterns ($P < 0.001$) in relation to time of exposure and plant species. It attained its minimum value of 40% in *C. demersum* and 381.81% in *M. spicatum* at 7-day exposure.

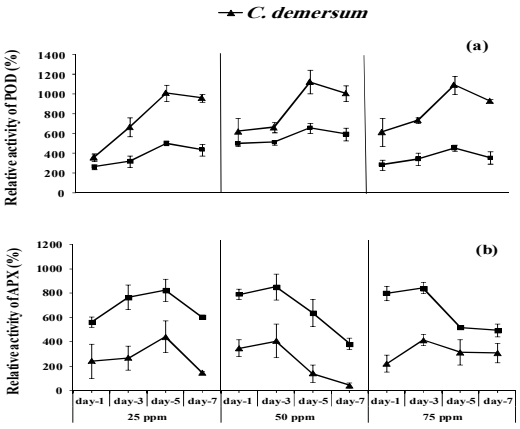

Figure 1: Changes in relative activity of antioxidant enzymes, (a) POD and (b) APX in *C. demersum* and *M. spicatum* treated with different Cd concentrations during the experiment duration.

The electrophoretic pattern of protein analysis in *C. demersum* is shown in Figure (2a). Compared to the control, the low Cd^{+2}-concentration (25 mg/L) was found to simulate the synthesis of new polypeptides of high molecular weight (138, 127, 112, 109 and 100 kDa), which they disappeared on the 5-day of Cd^{+2}-exposure. In addition, new lower molecular weight polypeptides (67, 37, 32, 16 and 15 kDa) appeared since the first day in all Cd^{+2}-concentrations and disappeared on the seventh day of exposure. The noticeable feature was the absence of 38 kDa band in the profiles of all treated plants, which coupled with the appearance of chlorosis, and stem and leaf disintegration (Fig 3).

M. spicatum electrophoretic pattern (Fig 2b) exhibits close similarity between control and treated plants in their contents of proteins. All the detected polypeptides were of the low molecular weights that include 67, 64, 61, 57, 39 and 34 kDa polypeptides and appeared on the day 3 and disappeared on the day 7 of Cd^{+2}-exposure in all set of experiment. Generally, in both macrophytes studies, increasing of Cd^{+2} -concentration influenced the density of protein bands, while long exposure time induced the disappearance of these bands as compared to control.

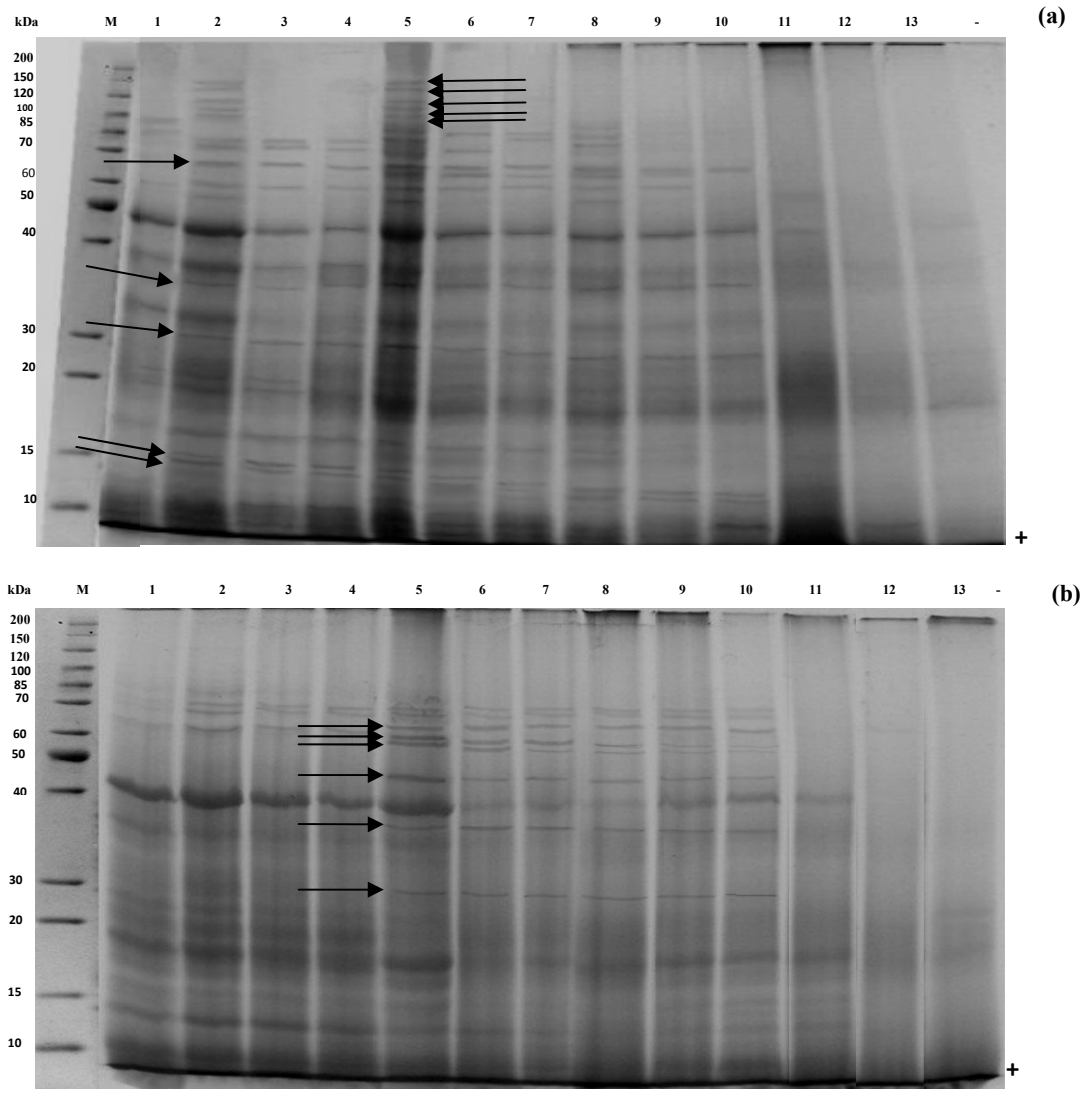

Figure 2: Protein banding patterns of *C. demersum* a) and *M. spicatum* b) under different Cd-concentrations and exposure time. M = markers, Lane 1: control. Lane 2, 5, 8, &11 represent protein banding pattern at 1, 3, 5 &7 days of 25 mg/L Cd treatment, respectively. Lane 3, 6, 9 &12 represent protein banding pattern at 1, 3, 5 &7 days of 25 mg/L Cd treatment, respectively.

Lane 4, 7, 10 &13 represent protein banding pattern at 1, 3, 5 &7 days of 50 mg/L Cd treatment, respectively. The arrows indicate appearance of new bands.

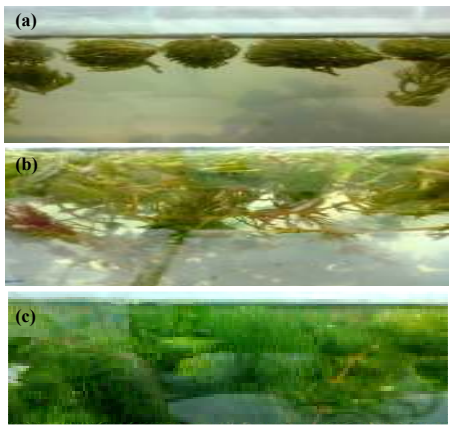

Figure. 3: Morphological changes in *C. demersum* induced by Cd stress: (a) disintegration, (b) chlorosis, and (c) control.

Discussion

The results of present study clearly revealed an induction of the antioxidant enzymes (POD& APX) upon exposure to different Cd^{+2} concentrations. The protective mechanisms by plants to scavenge free radicals include several antioxidative enzymes as glutathione reductase, peroxidase and ascorbic acid oxidase (Chen *et al*., 2000). The increase of POD activity as indicator of heavy metal stress was reported by many studies (MacFarlance and Burchett, 2001; Markkola *et al*., 2002; Baycu *et al*., 2006; Wang *et al*., 2010). suggests its role in the detoxification of H_2O_2. APX found to be more pronounced in *M. spicatum* (4-8 fold of control) in comparison to *C. demersum.* (Van Assche and Clijsters, 1990), have reported such increase in the relative activity and

concluded that the extent of increase varied with metal ion, metal concentration, the enzyme tested and plant species. In this work, the changes in antioxidant enzyme activities in response to heavy metal stress appeared to be dependent on metal concentration. Activities of these enzymes might increase in order to cope with the oxidative stress imposed by heavy metals on plants, as was repeatedly found in other experiments (Thomas *et al*., 1999; Radotic *et al*., 2000; Shah *et al*., 2001; Ianelli *et al*., 2002; El-Khatib *et al*., 2004; Baycu, *et al*., 2006). Alternatively, they might be diminished if the toxic effects of higher concentrations of heavy metals were greater than can be tolerated and combated by the antioxidant enzymes, as is the case in the present experiment. The decreases in antioxidant enzyme activities may result in the accumulation of reactive oxygen species, which can cause severe damage to plants; thus leading to the recorded stem disintegration in *C. demesum* with all Cd-treatments on the 3-day exposure. the activity of the tested antioxidant enzymes in the studied aquatic species varied significantly (P<0.01) among Cd-treatments, where increase in Cd- concentration coupled with an initial increase of their activities and subsequent Cd-treatments caused a decline in the activity. This suggesting that the generated H_2O_2 increased to some extent that exceeds the elimination ability of these enzymes, the H_2O_2 as well as other oxyradicals can inversely insult the enzymes, making them inactivated and suffering oxidative stress.

The present study explained that Cd-exposure induced the synthesis of a considerable number of stress proteins of different molecular mass in the two studied species. The density of protein bands in both studied species appears to be affected by Cd-concentration and time of exposure. In comparison with control, the treated plants exhibited protein patterns of their owner depending on the plant species and Cd-concentration. The disappearance of some polypeptides and the *de novo* synthesis of others, in response to Cd^{+2} treatments, indicated that such treatments are highly effective in causing a major re-shuffle of the protein profiles of the two studied species. Protein synthesis in *C. demersum* appears to be more sensitive to cadmium exposure than that of *M. spicatum*. As cited in (UniProtKB/Swiss-Prot database, 2011), 38-kDa polypeptide (Plastid-encoding protein) play role in PSII as electron donor and affect the formation of chlorophyll and pheophytin. Thus, its disappearance may be the reason for the observed chlorosis in the treated plants. In agreement with our results, (Prasad *et al*., 2009). found that *C. demersum* plants showed leaf and tissue disintegration, chlorosis, and survived for 7 days, when they exposed to Cd. The detected lower molecular weight proteins in *M. spicatum* under Cd stress were not reported in the literatures. While polypeptides of 15, 16, 32 kDa in *C. demersum* were reported previously as photosystem Q(B) protein family (UniProtKB/Swiss-Prot database, 2011), polypeptides of 37 and 67 kDa is the first time to be reported here in the present study. Therefore, further studies are needed to reveal the mechanisms of these polypeptides at molecular levels for regulating the Cd-induced stress in these macrophytes.

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الملخص العربى:

قد تم زراعة نوعين من النباتات المائية نبات السيراتوفيلم (Ceratophyllum demersum) ونبات الميروفيلم (Myriophyllum spicatum) في نظام الزراعة المائية لتحليل الانزيمات المضادة للأكسدة، وتحليل طرزالبروتين تحت تركيزات مختلفة من عنصر الكادميوم. اظهرت نتائج الدراسة أن نشاط الانزيمات المضادة للأكسدة (APX وPOD) تتاثر بزيادة التعرض للكادميوم.كما اظهرت النتائج الاختلافات المعنوية بين نشاط الانزيمات المضادة للاكسدة وبين التركيزات المختلفة من الكادميوم (P <٠,٠١) وذلك مقارنة بالكنترول، حيث ان الزيادة في تركيز الكادميوم تتسب فى خفض نشاط الانزيمات، وعموما وجد ان نشاط انزيم APX اعلى في M spicatum. (٤-٨ أضعاف) مقارنة ب Ceratophyllum، كما اظهر تحليل طرز البروتين تاثر النباتات بالتركيزات المختلفة من الكادميوم وايضا تتاثر بزمن التعرض لها حيث وجد ان نبات Ceratophyllum اكثر حساسية للتعرض لعنصر الكادميوم حيث تم تسجيل ظهور مجموعة جديدة من البروتينات ذات الوزن الجزئى العالى فى تركيز 25 mg/L Cd بالاضافة لاختفاء بروتين 38kDa من جميع المعالجات.