CORE
Metadata, citation and similar papers at core.ac.uk
Provided by Archivio della Ricerca - Università di Pisa

Acta Physiologiae Plantarum

Response of Pteris vittata to different cadmium treatments

--Manuscript Draft--

Manuscript Number:	ACPP-D-13-00418R3
Full Title:	Response of Pteris vittata to different cadmium treatments
Article Type:	Original Research
Corresponding Author:	Carmelina Spanò ITALY
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	
Corresponding Author's Secondary Institution:	
First Author:	Mirko Balestri
First Author Secondary Information:	
Order of Authors:	Mirko Balestri
	Stefania Bottega
	Carmelina Spanò
Order of Authors Secondary Information:	
Abstract:	Pteris vittata is known as an arsenic hyperaccumulator but there is little information about its tolerance to cadmium and on its ability to accumulate this heavy metal. Our aim was to analyse the accumulation capacity, oxidative stress and antioxidant response of this fern after cadmium treatments. Cadmium content, main markers of oxidative stress and antioxidant response were detected in leaves of plants grown in hydroponics for both short- (5 days) and long- (15 days) term exposure to 0 (control) 60 and 100 μM CdCl2. In leaves the concentration of cadmium and oxidative stress were parallel with the increase of cadmium exposure. In the short-term exposure antioxidant response was sufficient to contrast cadmium phytotoxicity only in 60 μM cadmium treated plants. In the long-term exposure all treated plants, in spite of the increase in activity of some peroxide-scavenging enzymes, showed a significant increase in oxidative damage. As in the long-term stress markers were comparable in all treated plants, with no clear correlation with hydrogen peroxide content, at least part of cadmium-induced oxidative injury seems not mediated by H2O2. Based on our studies, Pteris vittata, able to uptake relatively high concentrations of cadmium, is only partially tolerant to this heavy metal.

Powered by Editorial Manager® and ProduXion Manager® from Aries Systems Corporation

*Authors' Response to Reviewers' Comments
Click here to download Authors' Response to Reviewers' Comments: Response to the reviewers.doc

Reviewers' comments:

I accept scientific revision. In terms of graphics, please submit graphs with better resolution (simply made using Excel).

We have increased the quality of our graphs starting from Excel.

*Manuscript
Click here to download Manuscript: Manuscript.doc
Click here to view linked References

Mirko Balestri · Stefania Bottega · Carmelina Spanò

Response of *Pteris vittata* to different cadmium treatments

Department of Biology, University of Pisa, Via L. Ghini 13, 56126 (PI) (Italy)

Corresponding author: C. Spanò

E-mail: cspano@biologia.unipi.it, Tel. (+39) 0502211335; Fax:(+39) 502211309

Abstract

Pteris vittata is known as an arsenic hyperaccumulator but there is little information about its tolerance to cadmium and on its ability to accumulate this heavy metal. Our aim was to analyse the accumulation capacity, oxidative stress and antioxidant response of this fern after cadmium treatments. Cadmium content, main markers of oxidative stress and antioxidant response were detected in leaves of plants grown in hydroponics for both short- (5 days) and long- (15 days) term exposure to 0 (control) 60 and 100 µM $CdCl_2$. In leaves the concentration of cadmium and oxidative stress were parallel with the increase of cadmium exposure. In the short-term exposure antioxidant response was sufficient to contrast cadmium phytotoxicity only in 60 µM cadmium treated plants. In the long-term exposure all treated plants, in spite of the increase in activity of some peroxide-scavenging enzymes, showed a significant increase in oxidative damage. As in the long-term stress markers were comparable in all treated plants, with no clear correlation with hydrogen peroxide content, at least part of cadmium-induced oxidative injury seems not mediated by H_2O_2. Based on our studies, *Pteris vittata,* able to uptake relatively high concentrations of cadmium, is only partially tolerant to this heavy metal.

Keywords Antioxidant response · Cadmium · Leaves · Oxidative stress · *Pteris vittata* · Tolerance

Introduction

Cadmium is a non-essential heavy metal, widespread in the environment due to natural sources and human activities (Pál et al. 2006). It is considered one of the most dangerous pollutants because of its high water solubility and toxicity for most living organisms, plants included (Romero-Puertas et al. 2004). In fact this element can be absorbed by plants from soil or water and it is toxic to plant cells even at low concentrations (Gallego et al. 2012). Cadmium can cause several symptoms of toxicity such as leaf chlorosis, growth and photosynthesis inhibition (Yu et al. 2013), disturbance in water balance (Aghaz et al. 2013). Similar to other heavy metals, it is known to induce oxidative

stress (Wang et al. 2008). It has been suggested that cadmium induces oxidative stress mainly through indirect mechanisms (Wang et al. 2008) such as the interaction with the antioxidant machinery (Chaoui et al. 1997), inhibition of electron transport chain (Smeets et al. 2005) and activation of lipoxygenase with resulting lipid peroxidation (Somashenkaraiah et al. 1992). In addition, cadmium, non-redox reactive metal, is able to induce indirectly reactive oxygen species (ROS) production (Gallego et al. 2012) for example, through activation of NADPH oxidase in membranes (Gallego et al. 2012).

Reactive oxygen species, such as superoxide, hydrogen peroxide and hydroxyl radicals produced in a controlled-manner in physiological conditions, can be extremely harmful to organisms at high concentration; ROS can oxidise proteins, lipids and nucleic acids leading to alteration in cell structures and mutagenesis (Benavides et al. 2005). Apart from the damage they cause, Cd-induced ROS also exert a positive role. ROS accumulation, in fact, has been suggested as part of the signalling cascade leading to stress protection, acting as an elicitor of common stress response in plants (Mittler 2002). To counteract the damaging effects of ROS, plants have evolved an antioxidant defence machinery, including both enzymatic and non-enzymatic mechanisms. Compatible solutes, such as proline, contribute to protect plants from stress through the detoxification of reactive oxygen species, cellular osmotic adjustment, protection of membrane integrity and stabilization of enzymes/proteins, playing functions also in stress signalling (Szabados and Savouré 2009). Low molecular weight antioxidants, such as ascorbate and glutathione, can cooperate with the enzymes ascorbate peroxidase (APX) and glutathione reductase (GR) in scavenging ROS through the ascorbate-glutathione cycle (Lenher et al. 2006). Glutahione peroxidase (GPX), guaiacol peroxidase (POD) and catalase (CAT) are other important enzymes in hydrogen peroxide detoxification (Mittler 2002).

Pteris vittata is a perennial and fast-growing fern species, known as an arsenic hyperaccumulator (Drava et al. 2012). In As contaminated soil and water often higher contents of heavy metals such as Cd co-exist. The use of *P. vittata* for remediation or revegetation of these polluted soils depends

also on its Cd tolerance and accumulation (Xiao et al. 2008). Gupta and Devi (1994) described damage caused by Cd at different levels in the entire life cycle of *P. vittata*; in addition, Drava et al. (2012) reported the accumulation of cadmium by this fern resulting in a negative impact on plant growth. In contrast, Fayiga et al. (2004) recorded a Cd-induced stimulation of plant growth. Finally, Xiao et al. (2008) found ecotypical differences with Cd-tolerant ecotypes able to grow in polluted sites co-contaminated with Cd and As. Based on the few and contrasting findings available on the tolerance of this fern and on its ability of accumulation of Cd (Drava et al. 2012), the aim of this work was to analyse the phytotoxicity of cadmium to *P. vittata* and the tolerance of this fern to this heavy metal. As oxidative stress play a major role in metal toxicity (Sharma and Dietz 2009), oxidative damage and antioxidant defence response were studied after both short- (5 days) and long- (15 days) term exposure to different concentrations of cadmium.

Materials and Metods

Plant material

Spores of *Pteris vittata*, collected from mature fertile fronds, germinated within two weeks period by sowing on a mixture of potting soil (75%), and sand (25%). After four weeks, most of the germinated spores developed into gametophyte stage, fourteen weeks were needed for the development of sporophytes. The plants were watered on a daily basis or as necessary. During fern growth, the temperature in the greenhouse was 23 °C with 16/8 h light/dark photoperiod. Healthy and uniform *Pteris vittata* plants with 8 - 9 fronds were selected for the experiments. Randomly selected pinnae, hereinafter referred to as leaves, **w**ere collected along the length of fronds and immediately used or fixed in liquid nitrogen and stored at -20 °C until use.

Cadmium treatments

The plants were acclimatized in a hydroponic system in 0.2 strength Hoagland nutrient solution for two weeks, 5 plants of *P. vittata* for each treatment were transferred into hydroponic pots supplied

with 0 μM (control), 60 μM, 100 μM of $CdCl_2$ and maintained in a greenhouse for five and fifteen days. Air supply during hydroponic cultivation was provided by an aquarium air pump in the medium solution.

Cadmium content determination

Fronds from five control plants and five plants treated with Cd after 5 and 15 days were, oven-dried at 60 °C for 4 days, then were digested with nitric-perchloric acid (3:1, v/v) at 100 °C. A microwave oven MLS-1200 Mega (Milestone-FKV, USA) equipped with a UV generation device was used to eliminate the interfering organic substances. Then, the measurements of Cd content were made by an atomic absorption spectrophotometer (Analyst 300; Perkin Elmer, Überlingen, Germany). For each treatment, measurements were made in triplicate.

Determination of water content and of relative water content

Calculations of leaf fresh weight, dry weight and moisture content were based on weights determined before and after oven drying (100 °C) of leaf samples to constant weight. Water content percentage was estimated on the fresh weight basis. Leaf relative water content (RWC; Turner 1981 with minor modifications) was calculated with the formula:

$$RWC = [(FW-DW)/(TW-DW)] \times 100$$

FW is fresh weight, DW is dry weight and TW is turgid weight.

Fresh weight was obtained by weighing the fresh leaves. The leaves were then immersed in water over night, blotted dry and then weighed to get the turgid weight. The leaves were then dried in an oven at 100 °C to constant weight and reweighed to obtain the dry weight.

Chlorophyll determination

Chlorophylls (*a, b* and total) and carotenoids were extracted in 80% acetone and determined according to Hassanzadeh et al. (2009) and to Lichtenthaler (1987) respectively. Fresh leaves were homogenised and after centrifugation, for 10 min at 6,000*g* at 4 °C, the extracts were centrifuged

and the supernatants were collected. The pellets were resuspended and extracted with 80% acetone until they resulted colourless. The collected supernatants were read spectrophotometrically at 645, 663 and 470 nm and pigment contents were expressed as mg g^{-1} DW.

Hydrogen peroxide and lipid peroxidation

Hydrogen peroxide content of leaves was determined according to Jana and Choudhuri (1982) spectrophotometrically at 410 nm, using titanium chloride in H_2SO_4 for peroxide detection. The amount of H_2O_2 in the extracts, calculated from a standard curve, was expressed as μmol g^{-1} DW.

The amount of lipid peroxidation products in leaves was estimated by determining the malonyldialdehyde (MDA) content in the leaves according to Hartley-Whitaker et al. (2001) with minor modifications as in Spanò et al. (2007). Freeze-dried leaves were powdered and mixed with TBA reagent (10% w/v trichloroacetic acid + 0.25% w/v thiobarbituric acid), heated (95 °C), cooled and centrifuged. Malonyldialdehyde(155 mM^{-1} cm^{-1} extinction coefficient) content was measured as specific absorbance at 532 nm and by subtracting the non-specific absorbance at 600 nm (De Vos et al. 1989) and expressed as TBARS (TBA-reactive materials) in nmol g^{-1} DW.

Electrolytic conductivity method for membrane damage estimation

Membrane damage was estimated as in Spanò et al. (2002) with minor modifications as in Spanò et al. (2013). Segments of leaves (0.5 cm, cut 0.5 cm from the tip), after a short washing, were incubated in deionised water, evacuated for 30 min, and allowed stirring for 22 h at 4 °C. The conductivity of the aqueous solution was measured with a Jeenway 4310 Conductivity Meter at 25 °C. Conductivity was also detected at 25 °C after boiling the test tube in a water bath for 1 h. The percentage injury was calculated according to as a percentage of membrane damage using the formula:

$$(C_1-C_w) / (C_2-C_W) \times 100$$

C_1 is electro-conductance value of samples at the first measurement, C_2 is electro-conductance value after boiling and C_W is electro-conductance value of deionised water.

Extraction and determination of proline, ascorbate, and glutathione

Proline concentration was determined according to the method of Bates et al. (1973) with minor modifications, as in Spanò et al. (2013). Leaf tissue was homogenized with 3% sulfosalicylic acid. The supernatant was incubated with glacial acetic acid and ninhydrin reagent (1:1:1) and boiled in a water bath at 100 °C for 60 min. After cooling the reaction mixture, toluene was added, and the absorbance of toluene phase was read at 520 nm. Calculations were made on the base of a standard curve and proline content was expressed as μmol g^{-1} DW.

Ascorbate (AsA) and dehydroascorbate (DHA) extraction and determination were performed according to Kampfenkel et al. (1995) with minor modifications. Total ascorbate (AsA + DHA) was determined at 525 nm after reduction of DHA to AsA by dithiothreitol. Dehydroascorbate content was estimated on the basis of the difference between total ascorbate and AsA value. Calculations were made on the base of a standard curve and ascorbate content was expressed as mg g^{-1} DW.

Glutathione was extracted and determined according to Gossett et al. (1994). Total glutathione (GSH + GSSG) was determined by the 5,5'-dithio-bis-nitrobenzoic acid (DTNB)-glutathione reductase recycling procedure. The reaction was monitored as the rate of change in absorbance at 412 nm and GSSG was determined after removal of GSH from the sample extract by 2-vinylpyridine derivatisation. Calculations were made on the basis of a standard curve and content was expressed as nmol g^{-1} DW.

Enzyme extraction and assays

Leaves were ground in liquid nitrogen with a mortar and pestle. Extraction was made according to Spanò et al. (2013) at 4 °C. For APX, 2 mM ascorbate was added to the extraction medium, and for GR the supernatant was desalted on a Sephadex G-25 column. The extracts were stored in liquid

nitrogen until use.

Ascorbate peroxidase (APX, EC 1.11.1.11) activity was measured according to Nakano and Asada (1981) with modifications. Enzyme activity was assayed from the decrease in absorbance at 290 nm (absorbance coefficient of 2.8 mM^{-1} cm^{-1}) as ascorbate was oxidised, one unit of the enzyme oxidising 1.0 µmole of -ascorbate to dehydroascorbate per min. Enzymatic extract contained 25 µg protein ml^{-1}. Correction was made for the low, non enzymatic oxidation of ascorbate by hydrogen peroxide (blank).

Glutathione reductase (GR, EC 1.6.4.2) activity was determined as described by Rao et al. (1995) following the oxidation of NADPH at 340 nm (extinction coefficient 6.2 mM^{-1} cm^{-1}), one unit reducing 1.0 µmole of oxidised glutathione per min. Enzymatic extract contained 25 µg protein ml^{-1}. A correction for the non-enzymatic oxidation of NADPH was carried out in the absence of enzyme extract (blank).

Glutathione peroxidase (GPX, EC 1.11.1.9) activity was determined according to Navari-Izzo et al. (1997) by coupling its reaction with that of GR, using as substrate 0.45 mM H_2O_2. The activity was determined by following the oxidation of NADPH at 340 nm (extinction coefficient 6.2 mM^{-1} cm^{-1}). Enzymatic extract contained 25 µg protein ml^{-1}.

Catalase (CAT, EC 1.11.1.6) activity was determined as described by Aebi (1984). Enzymatic extract contained 25 µg protein ml^{-1}. A blank containing only the enzymatic solution was made. Specific activity was calculated from the 23.5 mM^{-1} cm^{-1} extinction coefficient, one unit decomposing 1.0 µmole of hydrogen peroxide to oxygen and water per min.

Guaiacol peroxidase (POD, EC 1.11.1.7) activity was determined as described by Arezky et al. (2001) using as substrate 1% guaiacol. Enzymatic extract contained 25 µg protein ml^{-1}. Enzymatic activity was determined following guaiacol oxidation by H_2O_2 (extinction coefficient 26.6 mM^{-1} cm^{-1}) at 470 nm, one unit oxidising 1.0 µmole guaiacol per min.

All enzymatic activities were determined at 25 °C and expressed as enzyme units (U) per g soluble protein. The total soluble protein content was measured according to Bradford (1976), using

BSA as standard.

Statistical analysis

The data were based on three replicates from three independent experiments. All data were analysed by one-way analysis of variance (ANOVA) and the mean differences were compared using the Student Newman Keuls test.

Results

Cadmium content

In leaves the accumulation of cadmium was parallel with the increase of cadmium concentration in growth medium and of the time of treatment (Table 1). After 5 days only plants treated with the higher concentration showed a concentration of this heavy metal in the range 5-10 μg g^{-1} DW. By day 15, in leaves from plants treated with 100 μM cadmium, the concentration of this toxic metal reached values higher than 150 ppm.

Water status and chlorophyll content

Water content was not significantly different in all samples (Table 1). All treated samples showed a significant decrease in RWC value at 15 days of treatment. The ratio of fresh weight to dry weight, not significantly different among the samples at 5 days (about 6), decreased at 15 days in Cd-treated samples reaching the minimum value in 100 μM Cd-treated plants (4,88). While the content of pigments (Table 1) was not significantly different at 5 days in all samples, after 15 days only total chlorophyll was significantly lower in Cd-treated samples. Chl*a*/Chl*b* ratio was significantly lower only in 100 μM Cd-treated plants.

Oxidative stress evaluation

ROS production was monitored by measuring the concentration of hydrogen peroxide. The content

of this signalling molecule (Fig. 1) was significantly higher in the plants treated with 100 μM Cd than in other samples both at 5 days and at 15 days. Lipid peroxidation (TBARS, Fig. 1) was consistently higher in treated plants than in control plants both after 5 and after 15 days. However, after 5 days membrane damage assessed by the electrolytic conductivity method (Fig. 1) was significantly higher only in the maximum Cd concentration, while after 15 days of treatment all treated plants showed the damage value significantly higher than in control plants.

Proline and non enzymatic antioxidants

Proline content (Table 2) was significantly higher in Cd-treated than in control plants, both after 5 and after 15 days of treatment. There was a strong decrease in its content in Cd-treated plants from 5 to 15 days of treatment. Antioxidant content was assessed by monitoring variations in ascorbate and glutathione. Total ascorbate (AsA + DHA, (a reduced form + an oxidised form, respectively), Table 2) was significantly higher in treated plants after 5 days, while at 15 days in 60 μM Cd-treated plants there was a strong reduction (to about one half) of the content of this low molecular weight antioxidant. Interestingly, just these plants showed the highest reducing power of AsA/DHA couple (Table 2). Total glutathione (GSH + GSSG, (a reduced form + an oxidised form, respectively), Table 2) was higher in 5 days treated plants than in control ones and the highest value was detected in 100 μM Cd plants while after 15 days it was not significantly different between treated and control plants. Reducing power of GSH/GSSG couple was significantly lower in 100 μM Cd plants after 5 days, while at 15 days both treated samples had a reducing power that was significantly lower than in control plants.

Antioxidant enzymes

Results of enzymatic assays are shown in Fig. 2. After 5 days of Cd treatment the activities of ascorbate peroxidase (APX), guaiacol peroxidase (POD) and catalase (CAT) were significantly lower in Cd-treated plants. In particular POD activity reached the minimum value in 100 μM Cd-

treated plants. Unlike than other enzymes the glutathione peroxidase (GPX) showed significantly higher activities in stressed plants. After 15 days of treatment APX and CAT activities were significantly lower in 100 μM Cd plants. Instead of that the activity of POD was higher in stressed than in control plants with a maximum in 60 μM Cd plants. Glutathione peroxidase activity was not significantly different among the three samples. GR activity did not differ significantly after 5 days nor after 15 days among control and stressed plants.

Discussion

Pteris vittata has been reported as a natural hyperaccumulator plant for arsenic (Ma et al. 2001), but not for heavy metals such as mercury (Chen et al. 2009). Our focus was to assess performance and antioxidant response of this plant to cadmium stress. Cadmium able to cause several symptoms of phytotoxicity (Sanità di Toppi and Gabbrielli 1999), can induce oxidative stress by the generation of ROS able to cause damage to macromolecules and cell structures.

Cadmium content of leaf and oxidative stress

In *Pteris vittata* after five days of treatment only leaves of plants treated with the highest Cd concentration showed a content of this heavy metal in the range 5-10 μg g^{-1} DW, considered toxic to most plants (White and Brown 2010). In this condition leaching and lipid peroxidation (indirectly indicating the membrane damage), the content of hydrogen peroxide and proline, stress markers, were significantly higher than in control plants. Despite the leaf cadmium content was below the threshold of toxicity, also the plants treated with the lower concentration of this metal showed some signs of stress, such as high lipid peroxidation and proline content, probably due to the presence of higher concentration of Cd in the underground part of the plant (data not shown) whose effects can be overtaken on the rest of the plant. In 100 μM Cd plants only lipid peroxidation further increased after 15 days of treatment, showing that the other stress parameters reached their maximum value already after the first period of treatment. On the contrary in 60 μM Cd plants both leaching and

lipid peroxidation further increased after 15 days, reaching values not significantly different from plants treated with the maximum Cd concentration. Interestingly, there was not a correlation between the hydrogen peroxide content and the membrane damage level assessed as lipid peroxidation and this seems to confirm that cadmium can cause damage by an indirect induction of oxidative stress, not ROS-mediated. As a matter of fact, a lipid peroxidation induced by the Cd-mediated activation of lipoxygenase has been reported (Chaoui et al. 1997).

Relative water content and pigments

Despite some signs of stress were evident after 5 days of treatment, a significant decrease in RWC and in FW/DW ratio, generally accepted as an indicator of phytotoxicity of heavy metal stress (Su et al. 2005, Jócsák et al. 2008), was recorded in treated plants after 15 days. In Cd-treated plants disturbation of water balance, already reported in literature (Benavides et al. 2005), was associated with a significant decrease in total chlorophyll content, another effect of Cd toxicity (Sanità di Toppi and Gabbrielli, 1999). However a significant decrease in Chl*a*/Chl*b* ratio, another stress indicator (Rout and Shaw 2001), was only recorded in 100 μM Cd plants, underlining the different effects of used Cd concentrations on *P. vittata*. Carotenoids, often found increased under stress condition (Munne-Bosch and Alegre 2000), did not significantly differ among the different treatments, in accordance to a minor effect of cadmium on this pigment group (Pál et al. 2006).

Proline and antioxidant molecules

Changes in proline content, characterised by a strong decrease in treated samples from 5 to 15 days, are rather interesting. As commonly accepted, cadmium has a serious negative impact on nitrogen uptake and distribution (Pál et al. 2006), what could impair the accumulation of nitrogen-containing defence molecules, such as proline. The decrease in proline content in 15-day treated plants could be considered as a way to recover nitrogen for the synthesis of other amino acids (Dandekar and Uratsu 1988). As it is commonly accepted that proline plays a protective role against oxidative stress induced by Cd (Hossain et al. 2010), the decrease of its content in the long period can be

considered a symptom of the only partial tolerance of *P. vittata* to this heavy metal.

Ascorbate is one of the low molecular weight antioxidants involved in plant defence system (Mittler 2002) and in the short period *P. vittata* was able to efficiently regulate the pool of this molecule. In the long period however, in accordance with data on mercury exposure (Chen et al. 2009), ascorbate pool significantly decreased in 60 μM Cd-treated plants. On the other hand there was a concentration-dependent increase in AsA/DHA ratio, generally reported as an indicator of a low sensitivity to stress (Gupta et al. 1999). This ratio was always significantly higher in treated plants than in control plants, in particular after 15 days of treatment, and it was a signal of a good capacity of *P. vittata* to maintain a high reducing power under cadmium treatment. The strong increase in glutathione pool after 5 days strictly depended on cadmium concentration and is a further confirmation of the importance of this sulphur-containing molecule in stress response (Gallego et al. 2012). The significant depletion of glutathione concentration in the long-term treatment, particularly at the expense of the reduced form, could be in part explained considering the increase in production of phytochelatins in the presence of high concentrations of cadmium (Lux et al. 2011) and the role of GSH in phytochelatin biosynthesis (Ederli et al. 2004). In fact these molecules have been given a role in detoxification of elements like arsenic and cadmium in Indian mustard (Pickering et al. 2000; Zhu et al. 1999) and cupper in bean (Gupta et al. 1998). The decrease in reducing power of GSH/GSSG couple in treated samples was generally proportional to the increase in reducing power of AsA/DHA couple, showing the importance of glutathione in AsA-GSH cycle, the major pathway in protection against cadmium stress (Smeets et al. 2009).

Antioxidant enzymes

Our results clearly indicated an involvement of antioxidant enzymes in stress defence. In the short-term treatment, when enzymatic activity was generally lower in Cd-treated than in control plants, among the enzymes studied, GPX seems to contribute in hydrogen peroxide detoxification, that only in 60 μM Cd-treated plants was not significantly different from control plants. The decrease in

GPX after 15 days treatment, is in 60 μM Cd-treated plants partially compensated by the increase in activity of CAT and APX reaching values of activity of control plants. In particular POD showed a significant stimulation in the long-term Cd treatment, as it was already reported for *Phragmites australis* (Ederli et al. 2004).

Conclusions

In conclusion, cadmium induced oxidative damage in *Pteris vittata* that only in the short-term was able to tolerate this heavy metal by activation of an adequate antioxidant response based mainly on proline, ascorbate, glutathione and the scavenging contribution of GPX. This also when the Cd content was in the range considered as toxic to most plants. In the long-term treatment, the 100 μM Cd-treated plants had in leaves high content of cadmium, however significantly lower than those detected in Cd hyperaccumulators (Kováčik, 2013), but showed clear signs of damage accompained by an increase of all tested markers of oxidative damage and a failure to relieve the inhibition of most of the antioxidant enzymes. As in the long-term treatment the values of stress markers were comparable in all treated plants, with no clear correlation with hydrogen peroxide content, we must conclude that at least part of the oxidative injury induced by cadmium is not mediated by hydrogen peroxide.

Author contribution All the authors conceived, designed and performed the experiments and wrote the paper.

References

Aebi H (1984) Catalase *in Vitro*. Method Enzymol 105: 121-125

Aghaz M, Bandehagh A, Aghazade E, Toorchi M, Ghassemi-Gholezani K (2013) Effects of cadmium stress on some growth and physiological characteristics in dill (*Anethum graveolens*) ecotypes. Int J Agric; Res Rev 3: 409-413

Arezki O, Boxus P, Kevrs C, Gaspar T (2001) Changes in peroxidase activity and level compounds during light-induced plantlet regeneration from *Eucalyptus camaldulensis* Dehn. nodes in vitro. Plant Growth Regul 33: 215-219

Bates LS, Waldren RP, Teare ID (1973) Rapid determination of proline for water stress studies. Plant Soil 39: 205-207

Benavides MP, Gallego SM, Tomaro ML (2005) Cadmium toxicity in plants. Braz J Plant Physiol 17: 21-34

Bradford M (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248-254

Chaoui A, Mazhoudi S, Ghorbal MH, Ferjani EE (1997) Cadmium and zinc induction of lipid peroxidation and effect on antioxidant enzyme activities in bean (*Phaseolus vulgaris* L.). Plant Sci 127: 139-147

Chen J, Shiyab S, Han FX, Monts DL, Waggoner CA, Yang Z, Su Y (2009) Bioaccumulation and physiological effects of mercury in *Pteris vittata* and *Nephrolepis exaltata*. Ecotoxicol 18: 110-121

Dandekar AM, Uratsu SL (1988) A single base pair change in proline biosynthesis genes causes osmotic stress tolerance. J Bacteriol 170: 5943-5945

De Vos CHR, Vooijs R, Schat H, Ernst WHO (1989) Copper-induced damage to the permeability barrier in roots of *Silene cucubalus*. J Plant Physiol 135: 165-169

Drava G, Roccotiello E, Minganti V, Manfredi A, Cornara L (2012) Effects of cadmium and arsenic on *Pteris vittata* under hydroponic conditions. Environ Toxicol Chem 31: 1375-1380

Ederli L, Reale L, Ferranti F, Pasqualini S (2004) Responces induced by high concentration of cadmium in *Phragmites australis* roots. Physiol Plant 121:66-74

Fayiga AO, Ma LQ, Cao X, Rathinasabapathi B (2004) Effects of heavy metals on growth and arsenic accumulation in the arsenic hyperaccumulator *Pteris vittata* L. Environ Pollut 132: 289-296

Gallego SM, Pena LB, Barcia RA, Azpilicueta CE, Iannone MF, Rosales EP, Zawoznik MS, Groppa MD, Benavides MP (2012) Unravelling cadmium toxicity and tolerance in plants: insight into regulatory mechanisms. Environ Exp Bot 83: 33-46

Gossett DR, Millhollon EP, Lucas MC (1994) Antioxidant response to NaCl stress in salt-tolerant and salt-sensitive cultivars of cotton. Crop Sci 34: 706-714

Gupta M, Cuypers A, Vangronsveld H, Clijsters H (1999) Copper afects the enzymes of the ascorbate-glutathione cycle and its related metabolites in the roots of *Phaseolus vulgaris*. Physiol Plant 106: 262-267

Gupta M, Devi S (1994) Chronic toxicity of cadmium in *Pteris vittata*, a roadside fern. Ecotoxicol 3: 235-247

Gupta M, Tripathi RD, Rai UN, Chandra P (1998) Role of glutathione and phytochelatin in *Hydrilla verticillata* (l.f.) Royle and *Valusneria spiralis* L. under mercury stress. Chemosphere 37: 785-800

Hartley-Whitaker J, Ainsworth G, Vooijs R, Bookum WT, Schat H, Merag AA (2001) Phytochelatins are involved in differential arsenate tolerance in *Holcus lanatus.* Plant Physiol 126: 299-306

Hassanzadeh M, Ebadi A, Panahyan-e-Kivi M, Eshghi AG, Jamaati-e-Somarin Sh, Saeidi M, Zabihi-e-Mahmoodabad R (2009) Evaluation of drought stress on relative water content and chlorophyll content of Sesame (*Sesamum indicum* L.) genotypes at early flowering stage. Res J Environ Sci 3: 345-350

Hossain MA, Hassanuzzaman M, Fujita M (2010) Up-regulation of antioxidant and glyoxalase

systems by exogenous glycinebetaine and proline in mung bean confer tolerance to cadmium stress. Physiol Mol Biol Plants 16: 259-272

Kampfenkel K, Montagu MV, Inzé D (1995) Extraction and determination of ascorbate and dehydroascorbate from plant tissue. Anal Biochem 225: 165-167

Kováčik J (2013) Hyperaccumulation of cadmium in *Matricaria chamomilla*: a never-ending story? Acta Physiol Plant 35: 1721-1725

Jana S, Choudhuri MA (1982) Glycolate metabolism of three submerged aquatic angiosperm during aging. Aquat Bot 12: 345-354

Jócsák I, Villányi V, Rabnecz G, Droppa M (2008) Investigation of nickel stress induction in terms of metal accumulation and antioxidative enzyme activity in barley seedlings. Acta Biol Szeg 52: 167-171

Lichtenthaler HK (1987) Chlorophylls and carotenoids: Pigments of photosynthetic biomembranes. Method Enzymol 148: 350-382

Lenher A, Bailly C, Flechel B, Poels P, Côme D, Corbineau F (2006) Changes in wheat seed germination ability, soluble carbohydrate and antioxidant enzymes activities in the embryo during the desiccation phase of maturation. J Cereal Sci 43: 175-182

Lux A, Martinka M, Vaculik M, White PJ (2011) Root responses to cadmium in the rhizosphere: a review. J Exp Bot 62: 21-37

Ma LQ, Komar KM, Tu C, Zhang WH, Cai Y, Kenneley ED (2001) A fern that hyperaccumulates arsenic: a hardy versatile, fast growing plant helps to remove arsenic from contaminated soils. Nature 409: 579

Mittler R (2002) Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci 7: 405-410

Munne-Bosch S, Alegre L (2000) Changes in carotenoids, toco-pherols and diterpenes during drought and recovery, and the biological significance of chlorophyll loss in *Rosmarinus officinalis* plants. Planta 210: 925-931

Nakano Y, Asada K (1981) Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in

spinach chloroplasts. Plant Cell Physiol 22: 867-880

Navari-Izzo F, Meneguzzo S, Loggini B, Vazzana C, Sgherri CLM (1997) The role of the glutathione system during dehydration of *Boea hygroscopica.* Physiol Plant 99: 23-30

Pál M, Horváth E, Janda T, Páldi E, Szalai G. (2006) Physiological changes and defense mechanisms induced by cadmium stress in maize. J Plant Nutr Soil Sci 169: 239-246

Pickering IJ, Prince RC, George MJ, Smith RD, George GN, Salt DE (2000) Reduction and coordination of arsenic in Indian mustard. Plant Physiol 122: 1171-1177

Rao MV, Beverley AH, Ormrod DP (1995) Amelioration of ozone-induced oxidative damage in wheat plants grown under high carbon dioxide. Role of antioxidant enzymes. Plant Physiol 109: 421-432

Romero-Puertas MC, Rodríguez-Serrano M, Corpas FJ, Gómez M, del Rio LA, Sandalio LM (2004) Cadmium-induced subcellular accumulation of $O_2^{\cdot -}$ and H_2O_2 in pea leaves. Plant Cell Environ 27:1122-1134

Rout NP, Shaw BP (2001) Salt tolerance in aquatic macrophytes: possible involvement of the antioxidative enzymes. Plant Sci 160: 415-423

Sanità di Toppi L, Gabbrielli R (1999) Response to cadmium in higher plants. Environ Exp Bot 41: 105-130

Sharma SS, Dietz KJ (2009) The relationship between metal toxicity and cellular redox imbalance. Trends Plant Sci 14: 43-50

Smeets K, Cuypers A, Lambrechts A, Semane B, Hoet P, Van Laere A, Vangronsveld J (2005) Induction of oxidative stress and antioxidative mechanisms in *Phaseolus vulgaris* after Cd application. Plant Physiol Biochem 43: 437-444

Smeets K, Opdenakker K, Remans T, Van Sanden S, Van Belleghem F, Semane B, Horemans N, Guisez Y, Vangronsveld J, Cuypers A (2009) Oxidative stress-related responses at transcriptional and enzymatic levels after expousure to Cd or Cu in a multipollution context. J Plant Physiol 166: 1982-1992

Somashenkaraiah BV, Padmaja K, Prasad ARK (1992) Phytotoxicity of cadmium ions on germination seedling of mung bean (*Phaseolus vulgaris*) involvement of lipid peroxides in chlorophyll degradation. Physiol Plant 85: 85-89

Spanò C, Bruno M, Bottega S (2013) *Calystegia soldanella*: dune versus laboratory plants to highlight key adaptive physiological traits. Acta Physiol Plant 35: 1329-1336

Spanò C, Buselli R, Ruffini Castiglione M, Bottega S, Grilli I (2007) RNases and nucleases in embryos and endosperms from naturally aged wheat seeds stored in different conditions. J Plant Physiol 164: 487-495

Spanò C, Crosatti C, Pacchini R, Meletti P, Grilli I (2002) Ribonucleases during cold acclimation in winter and spring wheats. Plant Sci 162: 809-815

Su Y, Han FX, Sridhar BBM, Monts DL (2005) Phytotoxicity and phytoaccumulation of trivalent and hexavalent chromium in brake fern. Environ Toxicol Chem 24: 2019-2026

Szabados L, Savouré A (2009) Proline: a multifunctional amino acid. Trends Plant Sci 15: 89-97

Turner NC (1981) Techniques and experimental approaches for the measurement of plant water status. Plant Soil 58: 339-366

Wang Z, Zhang Y, Huang Z, Huang L (2008) Antioxidative response of metal-accumulator and non-accumulator plants under cadmium stress. Plant Soil 310: 137-149

White PJ, Brown PH (2010) Plant nutrition for sustainable development and global health. Ann Bot 105: 1073-1080

Xiao X, Chen T, An Z, Lei M, Huang Z, Liao X, Liu Y (2008) Potential of *Pteris vittata* L. for phytoremediation of sites co-contaminated with cadmium and arsenic: the tolerance and accumulation. J Environ Sci 20: 62-67

Yu L, Gao R, Shi Q, Wang X, Wei M, Yang F (2013) Exogenous application of sodium nitroprusside alleviated cadmium induced chlorosis, photosynthesis inhibition and oxidative stress in cucumber. Pak J Bot 45: 813-819

Zhu YL, Pilon-Smits EAH, Tarun AS, Weber SU, Jouanin L, Terry N (1999) Cadmium tolerance

and accumulation in Indian mustard is enhanced by overexpressing γ-glutamylcysteine synthetase. Plant Physiol 121:1169-1177

Figure captions

Fig. 1 Hydrogen peroxide content, a; lipid peroxidation measured by determining thiobarbituric acid reactive substances (TBARS), b; membrane damage assessed by electrolytic conductivity method, c in leaves of *P. vittata* plants treated with 0 (control), 60 and 100 μM Cd for 5 and 15 days. Values are means of triplicate and vertical bars indicate SE. Different letters denote significant differences at $P < 0.05$.

Fig. 2 Activity of ascorbate peroxidase (APX), a; glutathione reductase (GR), b; glutathione peroxidase (GPX), c; catalase (CAT), d; guaiacol peroxidase (POD), e, in leaves of *P. vittata* plants treated with 0 (control), 60 and 100 μM Cd for 5 and 15 days. Values are means of triplicate and vertical bars indicate SE. Different letters denote significant differences at $P < 0.05$.

Figure 1
Click here to download high resolution image

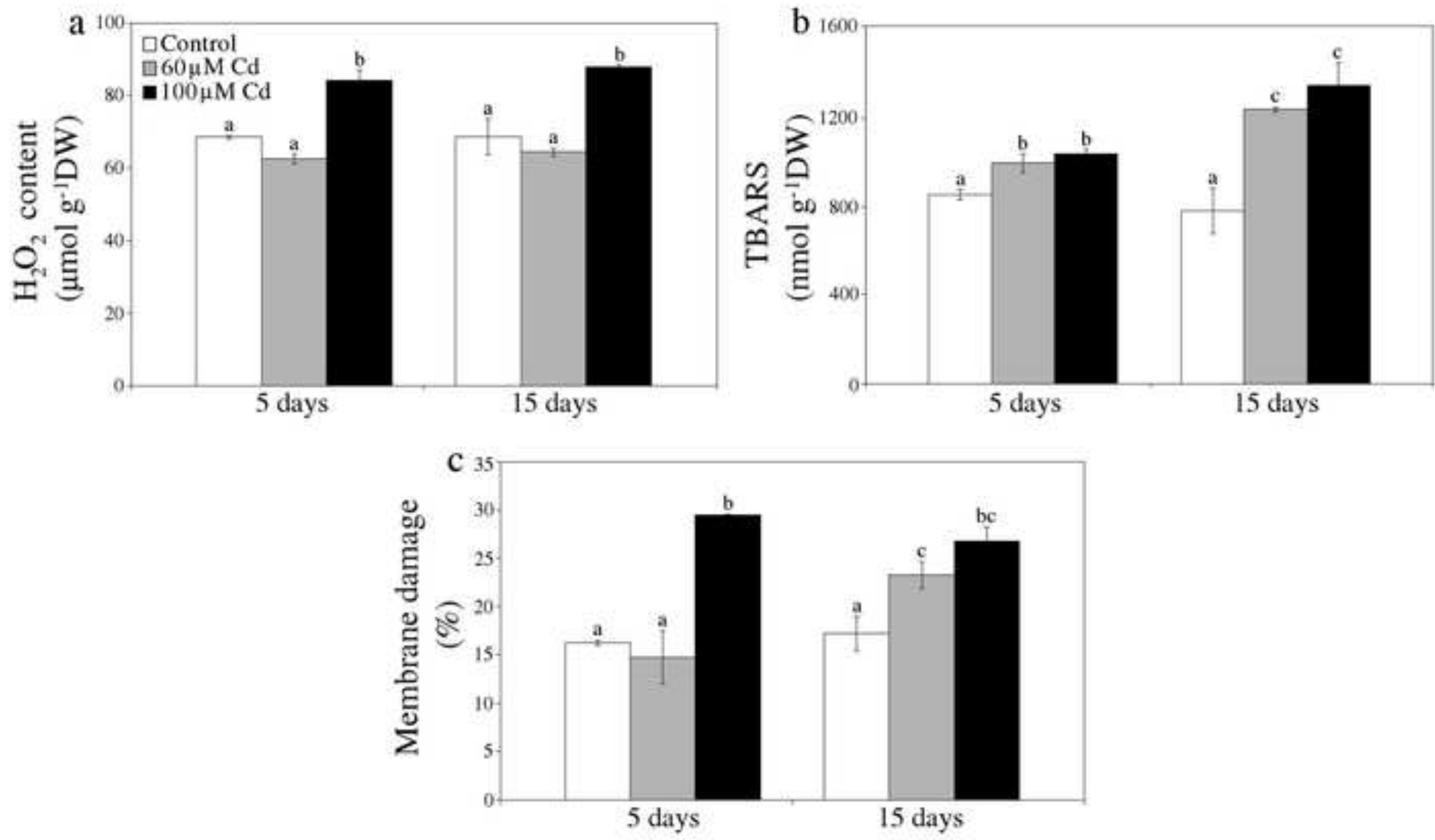

Figure 2

Click here to download high resolution image

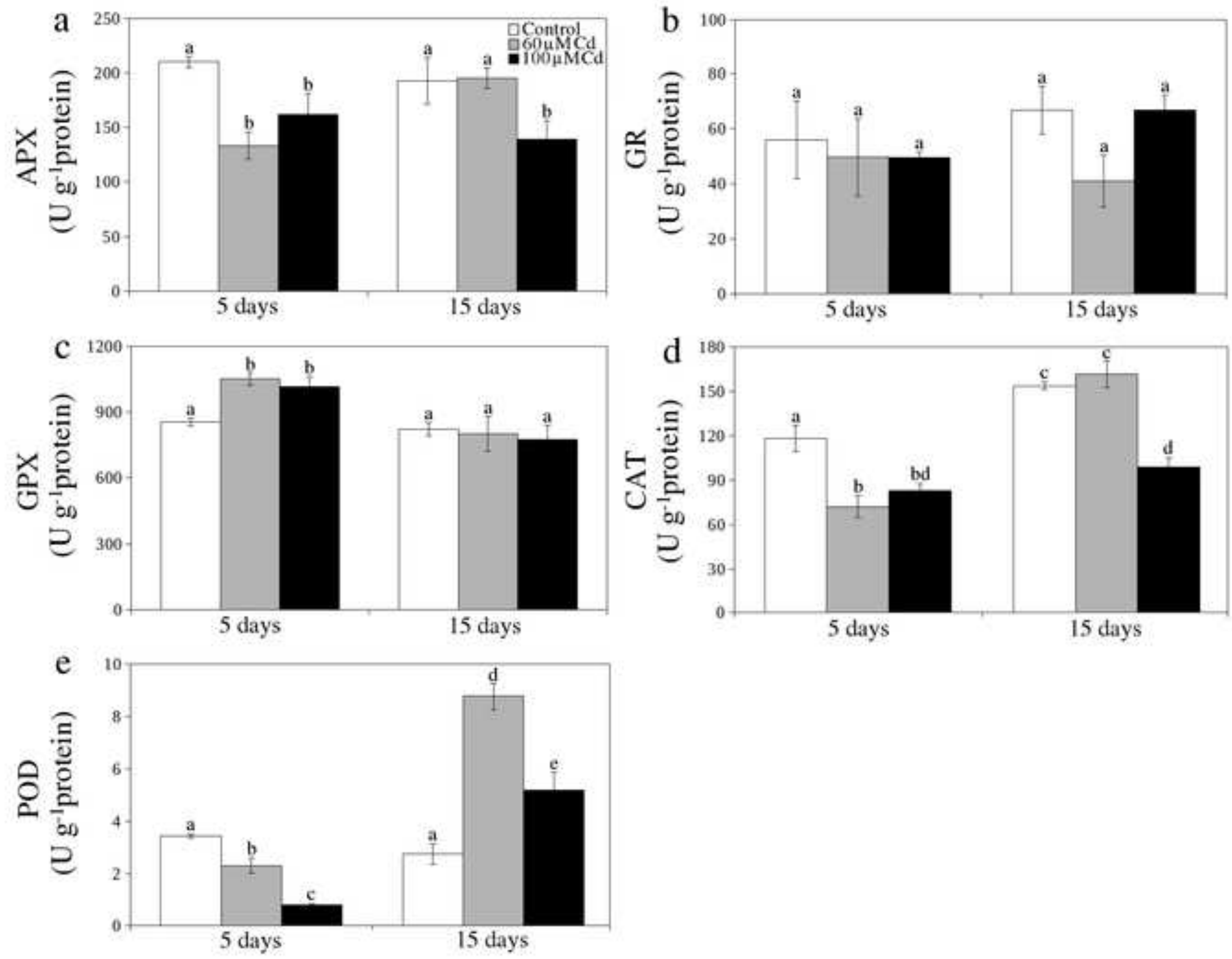

Table 1
Click here to download Table: Tab1.doc

Table 1 Cadmium content, water content, relative water content (RWC) and pigment content of leaves of *P. vittata* plants treated with 0 (control), 60 and 100 µM Cd for 5 and 15 days.

	5 days			**15 days**		
	Control	**60 µM Cd**	**100 µM Cd**	**Control**	**60 µM Cd**	**100 µM Cd**
Cd content of leaf (µg g^{-1} DW)	0.016±0.001e	0.326±0.002d	7.998±0.007c	0.014±0.001e	72.040±0.002b	159.150±0.001a
RWC (%)	90.83±1.01a	87.00±2.45a	89.00±0.33a	87.00±1.20a	77.50±3.67b	75.90±1.80b
Total chlorophyll (mg g^{-1} DW)	18.03±0.53a	15.20±2.03a	15.75±2.40a	19.01±0.25a	13.03±1.37b	13.90±1.62b
Chl*a*/Chl*b*	2.18±0.01a	2.18±0.01a	2.11±0.00a	2.22±0.02a	2.02±0.02a	1.67±0.00b
Carotenoids (mg g^{-1} DW)	2.54±0.40a	2.27±0.19a	2.47±0.29a	2.59±0.16a	2.03±0.25a	2.07±0.13a

Values are means of triplicate ± SE. Different letters denote significant differences at $P < 0.05$

Table 2
Click here to download Table: Tab2.doc

Table 2 Contents of proline, ascorbate: total, reduced (AsA) and oxidised (DHA), glutathione: total reduced (GSH) and oxidised (GSSG) in leaves of *P. vittata* plants treated with 0 (control), 60 and 100 µM Cd for 5 and 15 days.

	5 days			**15 days**		
	Control	**60 µM Cd**	**100 µM Cd**	**Control**	**60 µM Cd**	**100 µM Cd**
Proline (µmol g^{-1} DW)	7.60±1.11d	23.43±0.35a	20.13±0.97b	6.66±1.33d	13.88±0.01c	15.61±0.01c
AsA (mg g^{-1} DW)	0.31±0.01d	1.16±0.01a	0.82±0.03b	0.61±0.06c	0.62±0.06c	1.08±0.02a
DHA (mg g^{-1} DW)	0.54±0.02ab	0.54±0.01ab	0.65±0.04a	0.65±0.01a	0.21±0.06c	0.42±0.03b
Total ascorbate (mg g^{-1} DW)	0.85±0.01d	1.70±0.02a	1.47±0.04b	1.26±0.04c	0.83±0.01d	1.50±0.01b
AsA/DHA	0.57±0.00e	2.15±0.03b	1.26±0.00c	0.94±0.01d	2.95±0.01a	2.57±0.00b
GSH (nmol g^{-1} DW)	53.65±2.39c	101.52±8.08b	203.33±19.84a	89.11±4.92bc	53.88±3.81c	51.93±8.74c
GSSG (nmol g^{-1} DW)	10.02±1.19d	20.74±3.43c	89.29±2.35a	26.15±3.37c	27.27±0.67c	42.28±1.68b
Total glutathione (nmol g^{-1} DW)	73.69±0.00c	143.00±3.17b	381.92±15.16a	141.42±11.68b	108.42±4.74b	136.5±22.86b
GSH/GSSG	5.35±0.03a	4.89±0.01b	2.28±0.01d	3.41±0.02c	1.97±0.03d	1.23±0.00e

Values are means of triplicate ± SE. Different letters denote significant differences at $P < 0.05$

Author contribution

All the authors of this manuscript equally contributed to conceive, design and perform the experiments and to wrote the paper.