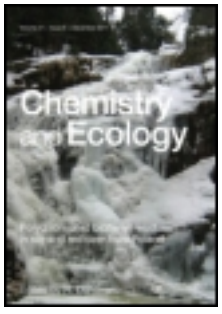

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Metabolic responses of *Azolla pinnata* to cadmium stress: photosynthesis, antioxidative system and phytoremediation

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This present study deals with the growth, photosynthesis, oxidative stress and phytoremediation character of *Azolla pinnata* L. exposed to different levels (0.05, 0.1, 0.5, 1.0, 1.5 and 2.0 $mg \cdot L^{-1}$) of cadmium (Cd). Significant accumulation of Cd in *Azolla* fronds was noticed after 24 and 96 h of exposure and the accumulation rate was dose and time dependent. Growth of *A. pinnata* increased significantly after both exposure times with and without metal. At lower Cd doses (0.05 and 0.1 $mg \cdot L^{-1}$), growth and photosynthesis of *A. pinnata* showed a marginal increase over the respective control, however, at higher Cd doses (0.5, 1.0, 1.5 and 2.0 $mg \cdot L^{-1}$), a decreasing trend was noticed. At lower doses, *Azolla* fronds could counterbalance the negative effect of enhanced levels of superoxide radicals (SOR) and hydrogen peroxide (H_2O_2) through the greater activity of antioxidative enzymes. The decaresing trends in catalase and peroxidase activity at higher Cd doses suggest that *Azolla* fronds were not able to mitigate the negative effects of H_2O_2, hence an increase in malondialdehyde content was noticed. The study concludes that up to 0.1 ,$mg \cdot L^{-1}$ Cd, *A. pinnata* can flourish and be used as biofertiliser and for phytoremedial purposes in Cd-contaminated fields; beyond this concentration poor growth may restrict its application.

Keywords: *Azolla*; heavy metal; growth; photosynthesis; oxidative stress; antioxidants

1. Introduction

The accumulation of high amounts of heavy metal in soil and aquatic bodies is one of the most common environmental problems [1,2]. Irrigation using waste water, and the use of pesticide and fertilisers increase heavy metal accumulation in crop fields. Casova et al. [3] showed that repeated application of sewage sludge led to a risk of Cd accumulation in soils. Among the different heavy metals, Cd is one of the most toxic, and because of its high availability in soil and water, it can easily enter into the food chain [4]. Metal is translocated from the root to the shoot with the help of two processes: root pressure and leaf transpiration. Metals are accumulated in roots, probably due to some physiological barriers against metal transport to aerial parts. There exists a mechanism in roots that could detoxify heavy metals or transfer them to aerial parts.

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The localization of Cd in vacuoles, synthesis of phytochelatins (PCs) and their binding to heavy metals occur mainly in roots. These are mechanisms to deal with an excess of heavy metal in the growth medium [5]. There are variations in accumulation patterns of metals in plants. Soltan and Rashed [6] treated water hyacinth with several heavy metals (Cd, Co, Cr, Cu, Mn, Ni, Pb and Zn) and concluded that it accumulated higher concentrations of heavy metal in the roots than in the aerial parts. Garbisu and Alkorta [7] observed a higher accumulation of Cd in shoots than in roots In addition, Sela et al. [8] observed a similar Cd content in roots and fronds of *Azolla filiculoides* grown in Cd-contaminated growth medium.

When heavy metal enters into a plant, it leads to the generation of oxygen free radicals [9]. These free radicals affect biological membranes, protein cleavage, enzyme inactivation and DNA strand breakage [9]. A high rate of tissue auto-oxidation is generally indicated by the lipid peroxidation of cellular membranes and this effect is further stimulated by endogenously generated active oxygen radicals [9]. The main effect of heavy metal stress observed in most plant species is the inhibition of photosynthesis. This has been attributed to an indirect action of Cd on plant water balance, stomatal conductance and CO_2 availability, or to a more direct effect on chloroplast organisation, chlorophyll biosynthesis, electron transport and the enzymes involved in photosynthetic carbon metabolism [10]. Studies investigating Cd toxicity on chloroplast functionality and electron transport have suggested that this heavy metal exhibits its toxic effect by damaging PS II [11].

Plants generate an array of enzymatic and non-enzymatic antioxidants to scavenge active oxygen species (H_2O_2, $O_2^{-\bullet}$, $^{\bullet}OH$) in order to survive under stress conditions [9]. Superoxide dismutase (SOD) is an important enzyme of the antioxidant system and is considered as the first line of defence. SOD is a metallo-enzyme containing Cu, Zn or Mn which catalyses the dismutation reaction of the superoxide anion ($O_2^{-\bullet}$) into H_2O_2 and O_2. An excessive accumulation of H_2O_2 is harmful for the plant so among different the antioxidative enzyme peroxidases are monomeric haemoproteins that catalyse the oxidation of a range of substrates by H_2O_2 Catalase, a heam-containing tetrameric enzyme, uses H_2O_2 as a substrate and converts it into H_2O and O_2, thus protecting cells against the damaging effects of H_2O_2 accumulation [12].

Azolla, an aquatic fern, has a symbiotic association with the heterocystous cynobacterium *Anabaena azollae* and is used as a biofertiliser in rice fields due to its nitrogen-fixation capability [13]. *Azolla* contains very high levels of protein and fat, and may be effective in helping developing countries to establish a more sustainable agriculture, without risking long-term soil fertility, soil productivity and environmental issues, when compared with chemical fertilisers [14]. Further, due to its metal-binding capacity, *Azolla* may also be considered as the best option for heavy metal remediation from contaminated systems [15,16]. Rai and Tripathi [17] used *A. pinnata* to ameliorate industrial effluents (thermal power, chlor-alkali and coal mine effluent) contaminated with Hg. Remediation of heavy metals using plants is better in terms of ecology and economy than chemical treatment [18].

The use of living aquatic plants may be a viable alternative process for removing metals. Rai [19] extensively reviewed the use of macrophytes in heavy-metal-polluted industrial effluents, and many studies have shown that wetland plants can accumulate heavy metals in their tissues [20–25].

The addition of heavy metal to the environment also affects *Azolla* sp. and reduces its economic importance, therefore this study was aimed at investigating the impact of Cd on *A. pinnata* and the extent to which the plant can endure Cd stress. Heavy metal accumulation, growth, chlorophyll and carotenoid content, photosynthetic and respiratory activities were estimated after 24 and 96 h of Cd treatment. *Azolla pinnata* plants were analysed under different levels of Cd following 24 and 96 h of exposure to understand the level of toxicity, oxidative stress and antioxidant system.

2. Materials and methods

2.1. *Plant material, metal treatment and growth conditions*

Azolla pinnata was collected from ponds located in Roxberg Garden, Botany Department, University of Allahabad, India. The plants were washed and cleaned of contaminating organisms. Further, the plants were surface sterilised by keeping them in a solution of 0.1% mercuric chloride for 30 s and then dipped into a large volume of sterile distilled water. Healthy plants were picked up gently and transferred in Espinase and Watanabe medium [26] for 24 h under laboratory conditions for acclimatisation. Further, plants (400 mg) were kept in 200 mL of Espinase and Watanabe medium containing different levels of Cd (0.05, 0.1, 0.5, 1.0 and 2.0 $mg \cdot L^{-1}$) for 24 and 96 h at $26 \pm 2\,^{\circ}C$ under a light (PAR; 200 $\mu mol \cdot photon^{-2} \cdot s^{-1}$) and dark period of 16:8 h. Parameters were analysed after 24 and 96 h of exposure.

2.2. *Growth and photosynthetic pigment analysis*

Plant fresh mass was determined by measuring the mass of *Azolla* fronds after 24 and 96 h of metal treatment. Chlorophyll (Chl) and carotenoids (Car) were extracted with 80% acetone and their contents estimated spectrophotometrically using the method described by Lichtenthaler [27].

2.3. *Measurement of photosynthesis and respiration*

Photosynthetic O_2 yield was measured in *Azolla* fronds using a Clark-type oxygen electrode (Rank Brothers, UK) in the presence of 5 mL of 50 mM HEPES–NaOH buffer (pH 7.6) containing 20 mM $NaHCO_3$, as described by Kurra-Hotta et al. [28]. Treated and untreated frond samples were sliced into small pieces in a Petri dish containing 10 mL of 0.5 mM $CaSO_4$. The pieces were washed and transferred to the temperature-controlled air-tight reaction vessel of an oxygen electrode. O_2 consumption during respiration in darkness and evolution during photosynthesis in the presence of light (400 $\mu mol \cdot m^{-2} \cdot s^{-1}$, PAR) were estimated at 28 °C.

2.4. *Determination of reactive oxygen species and indices of oxidative damage*

Superoxide radical (SOR; $O_2^{-\bullet}$) was estimated using the method of Elstner and Heupel [29] by monitoring nitrite formation from hydroxylamine in the presence of $O_2^{-\bullet}$ contained in the homogenates supernatant. For H_2O_2 estimation, fronds were homogenised in 3.5 mL of 5% trichloroacetic acid (w/v) and after centrifugation at 10,000 ***g*** for 15 min, the total peroxide in the supernatant was estimated following the method described by Velikova et al. [30]. Indices of oxidative damage were determined for a test sample by estimating thiobarbituric-acid-reactive malondialdehyde (MDA), a product of lipid peroxidation, following the method of Heath and Packer [31].

2.5. *Measurement of antioxidative enzymes*

For catalase (CAT; EC 1.11.1.6) activity, fronds were homogenised in 100 mM potassium phosphate buffer (pH 7.0), whereas for peroxidase (POD; EC 1.11.1.7) and SOD (EC 1.15.1.1) activity, samples were extracted in 150 mM potassium phosphate buffer (pH 6.1) and 100 mM EDTA–phosphate buffer (pH 7.8), respectively. CAT activity in a reaction mixture (2 mL) containing 50 mM potassium phosphate buffer (pH 7.0), 1 mM EDTA, 10 mM H_2O_2 and enzyme extract was

recorded as a decrease in absorbance at 240 nm using a double-beam UV-B visible spectrophotometer (Shimadzu, Japan, UV-1700). Activity was calculated using an extinction coefficient of 39.4 $mM^{-1}\cdot cm^{-1}$ [32] and represented as H_2O_2 decomposed [n mol·(g FW)$^{-1}\cdot min^{-1}$]. POD activity was determined spectrophotometrically with guaiacol as the substrate, following the method described by Zhang [33]. The reaction mixture (3 mL) contained 50 mM potassium phosphate buffer (pH 6.1), 1% guaiacol, 0.4% H_2O_2 and enzyme extract, and an increase in the absorbance due to the oxidation of guaiacol ($E = 25.5\ mM^{-1}\cdot cm^{-1}$) was measured at 470 nm. SOD activity was assayed by using the photochemical Nitroblue tetrazolium (NBT) reduction method, as described by Giannopolitis and Reis [34]. The reaction mixture (3 mL) contained 50 mM potassium phosphate buffer (pH 7.8), 1.3 μM riboflavin, 0.1 mM EDTA, 13 mM methionine, 63 μM NBT, 0.05 M sodium carbonate (pH 10.2) and enzyme extract (100 μL). The reaction mixture was illuminated (200 $\mu mol\cdot m^{-2}\cdot s^{-1}$) for 10 min. Photoreduction of NBT (formation of purple formazone) was measured at 560 nm. One unit of SOD activity is defined as the amount of enzyme required to cause 50% inhibition in the reduction of NBT. The enzyme activity was represented as [unit (mg^{-1} protein)]. Protein content was estimated according to the method of Bradford [35].

2.6. *Digestion and analysis for heavy metal*

Fronds (0.5 mg) were digested by adding a tri-acid mixture (HNO_3, H_2SO_4 and $HClO_4$ in a 5:1:1 ratio) at 80 °C until a transparent solution was obtained [36]. Heavy metal concentrations in the sample filtrates were estimated using an atomic absorption spectrophotometer (Model 2380, Perkin-Elmer, Norwalk, CT, USA) fitted with a lamp specific for a particular metal and using the appropriate drift blank.

2.7. *Statistical analysis*

Statistical analysis was carried out using the SPSS program (version 11). The significance of differences in measured parameters among treated and untreated plants was assessed by conducting a one-way analysis of variance (ANOVA) followed by Duncan's multiple range tests at 5% level. A linear correlation was carried out to understand the relation between heavy metal accumulation and growth of *Azolla* fronds.

3. Results

3.1. *Heavy metal uptake*

With increases in its concentration in the medium, Cd accumulation increased in *Azolla* fronds (Figure 1). At lower concentrations (0.05–0.5 $mg\cdot L^{-1}$), the amount of Cd in *Azolla* fronds ranged from 6 to 10 $\mu g\cdot g^{-1}$ dry mass at 24 h of exposure and 10 to 15 $\mu g\cdot g^{-1}$ dry mass at 96 h of exposure. At the highest concentration (2.0 $mg\cdot L^{-1}$), Cd reached 18 and 22 $\mu g\cdot g^{-1}$ dry mass at 24 and 96 h of exposure, respectively (Figure 1).

3.2. *Growth of* Azolla

Azolla biomass increased by 35 and 94% over initial values under the control condition after 24 and 96 h of incubation, respectively (Figure 2a). Treatment with lower Cd doses for 24 h (0.1 mg $Cd\cdot mL^{-1}$) and 96 h (0.05 mg $Cd\cdot mL^{-1}$) did not decrease the growth of *Azolla*, and even showed a marginal increase (3–5%) in biomass accumulation over controls (Figure 2). In contrast to this, at

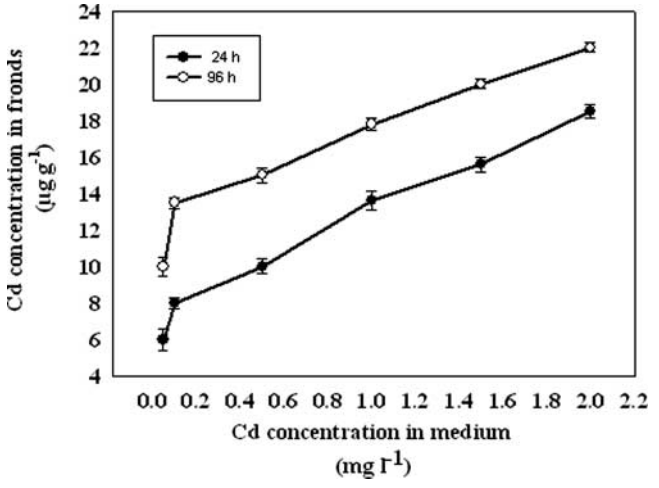

Figure 1. Heavy metal concentration in *Azolla* fronds at different concentrations of Cd (0.05, 0.1, 0.5, 1.0, 1.5 and 2.0 mg·L^{-1}) after 24 and 96 h of exposure.

higher Cd doses (0.5–2.0 mg Cd·L^{-1}), biomass accumulation decreased by 4–9% after 24 h and 12–18% after 96 h of treatment (Figure 2a).

3.3. *Photosynthetic pigments*

The increase in photosynthetic pigments over initial values is more significant after 96 h of growth than after 24 h (Table 1). Cd concentrations up to 0.1 mg·L^{-1} did not affect the Chl *a* and *b* content recorded after 24 and 96 h. However, above these concentrations (at 0.5–2.0 mg Cd·L^{-1}), there was an appreciable decrease in chlorophyll. At the high dose (2.0 mg·L^{-1}), Chl *a* and *b* contents decreased by 35 and 15% after 24 h, and by 42 and 38% after 96 h of metal exposure, respectively. In contrast to Chl *a* and *b* contents, carotenoid content increased significantly with increasing Cd concentration (0.05–2.0 mg·L^{-1}) after 24 and 96 h of exposure (Table 1).

3.4. *Photosynthesis and respiration*

The photosynthetic rate of the fronds increased marginally after 24 h of treatment at lower doses (0.05–0.1 mg·L^{-1}) of Cd and thereafter decreased at higher doses (0.5–2.0 mg·L^{-1}) (Figure 2b). With the increase in exposure time (96 h), even 0.1 mg Cd·L^{-1} was found to be inhibitory (Figure 2b). Cd treatment at 2.0 mg·L^{-1} caused $\sim$ 39% inhibition in the rate of photosynthesis after 24 h and 53% inhibition after 96 h compared with controls. In contrast to photosynthesis, the respiration rate showed no significant change at lower doses, but at higher doses there was a marginal increase over the control value after 24 h of exposure (Figure 2c). However, after 96 h, the respiration rate showed a 14–37% increase when the Cd concentration was incraesed from 0.1 to 2.0 mg·L^{-1} compared with controls (Figure 2c).

3.5. *Active oxygen species and oxidative damage*

Figure 3 shows that increasing doses of Cd (0.5–2.0 mg·L^{-1}) caused a significant increase in SOR and H_2O_2 in tissues. Similarly, these Cd doses enhanced the rate of lipid peroxidation, and an increased MDA content in *Azolla* fronds was therefore noticed (Figure 3). After 96 h of exposure,

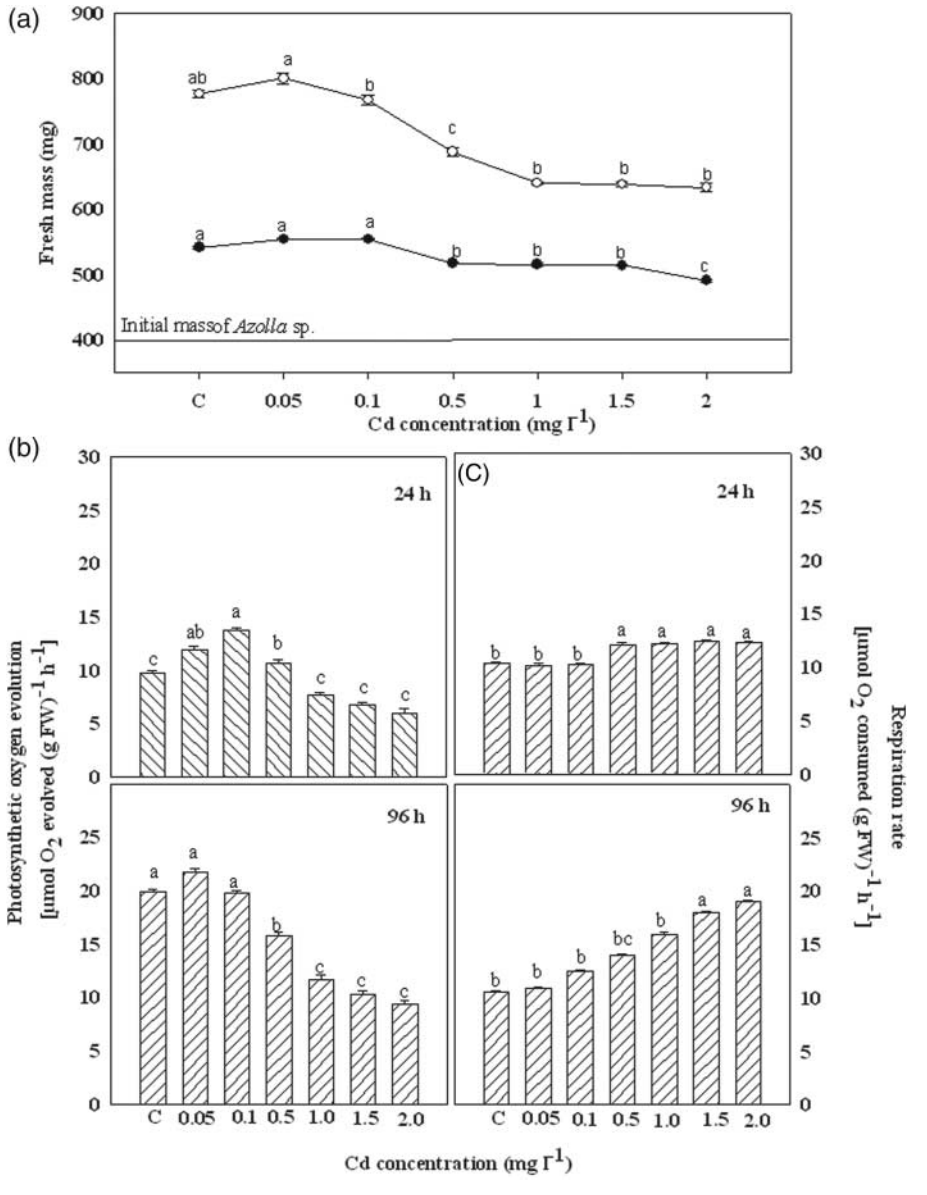

Figure 2. Effect of different concentrations of Cd (0.05, 0.1, 0.5, 1.0, 1.5 and 2.0 mg·L^{-1}) on (a) fresh weight of *Azolla* fronds, (b) photosynthetic activity and (c) respiration rate after 24 and 96 h of exposure ($n = 5$). Different letters on the line diagram show a significant difference at $p \leq 0.05$.

the degree of oxidative stress became more intense at higher doses of Cd. At lower doses (0.05–0.1 mg Cd·L^{-1}), SOR, H_2O_2 and MDA contents showed no significant increase after 24 and 96 h of treatment.

3.6. *Antioxidative enzymes*

Figure 4 shows the responses of SOD, CAT and POD to oxidative stress induced by varying doses of Cd after 24 and 96 h of treatment. As the duration of exposure increased, more antioxidative

Table 1. Chlorophyll *a*, *b* and carotenoid contents (mg·g^{-1}FW) in *Azolla* fronds under Cd stress.

	24 h			96 h		
Cd (mg·L^{-1})	Chl *a*	Chl *b*	Carotenoid	Chl *a*	Chl *b*	Carotenoid
Control	0.377 ± 0.001^{a}	0.184 ± 0.002^{a}	0.098 ± 0.02^{c}	0.471 ± 0.001^{a}	0.277 ± 0.001^{a}	0.181 ± 0.001^{c}
0.05	0.389 ± 0.01^{a}	0.189 ± 0.005^{a}	0.097 ± 0.003^{c}	0.474 ± 0.001^{a}	0.280 ± 0.001^{a}	0.181 ± 0.001^{c}
0.1	0.392 ± 0.01^{a}	0.190 ± 0.003^{a}	0.114 ± 0.001^{ab}	0.410 ± 0.001^{a}	0.277 ± 0.013^{a}	0.192 ± 0.001^{b}
0.5	0.303 ± 0.001^{b}	0.180 ± 0.002^{b}	0.110 ± 0.004^{b}	0.383 ± 0.02^{b}	0.243 ± 0.002^{b}	0.200 ± 0.001^{b}
1.0	0.277 ± 0.001^{c}	0.177 ± 0.001^{b}	0.116 ± 0.003^{a}	0.356 ± 0.006^{b}	0.225 ± 0.002^{c}	0.214 ± 0.005^{a}
1.5	0.272 ± 0.01^{c}	0.162 ± 0.04^{bc}	0.119 ± 0.001^{a}	0.345 ± 0.05^{bc}	0.184 ± 0.014^{d}	0.219 ± 0.005^{a}
2.0	0.244 ± 0.01^{d}	0.157 ± 0.01^{c}	0.121 ± 0.001^{a}	0.272 ± 0.005^{c}	0.169 ± 0.002^{d}	0.226 ± 0.001^{a}

Notes: Data represents the mean ±1SE. $n = 5$. Different letters in each column indicate a significant difference at $p \leq 0.05$. Chl, chlorophyll.

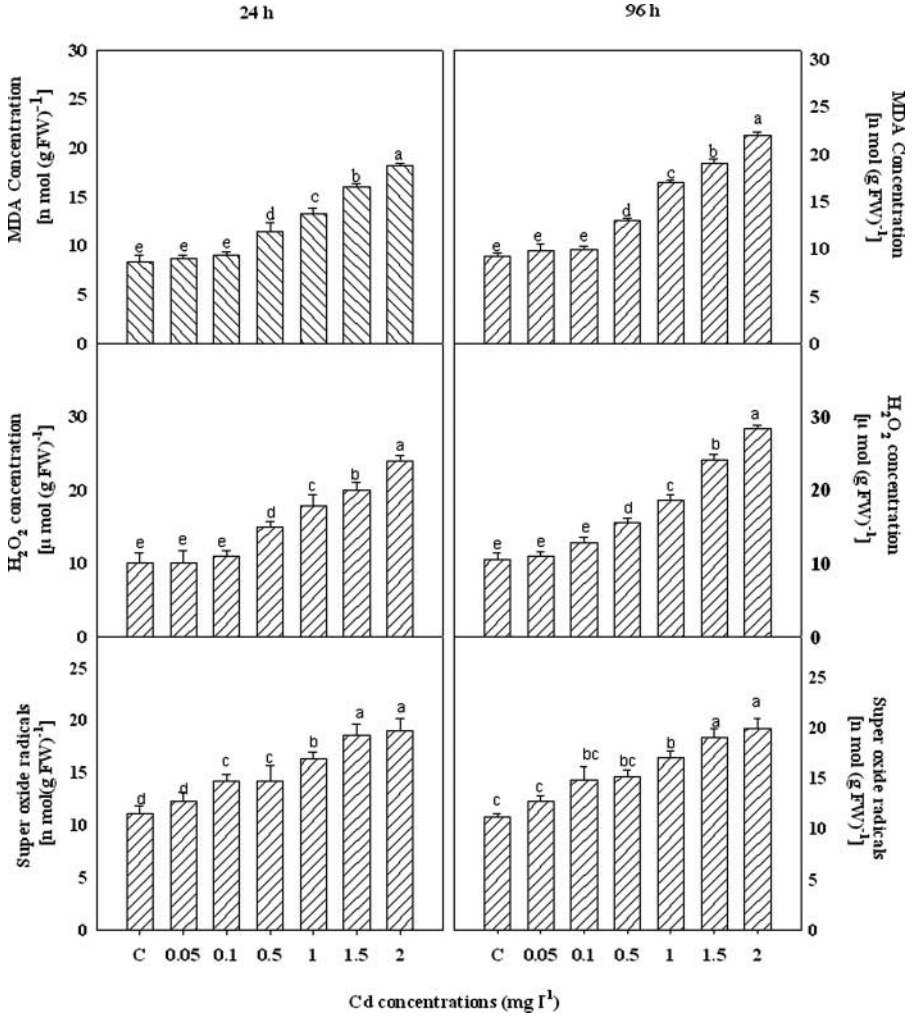

Figure 3. Effect of different concentrations of Cd (0.05, 0.1, 0.5, 1.0, 1.5 and 2.0 mg·L^{-1}) on SOR, H_2O_2 and MDA contents in *Azolla* fronds after 24 and 96 h of exposure ($n = 3$). Bars with different letters show a significant difference at $p \leq 0.05$.

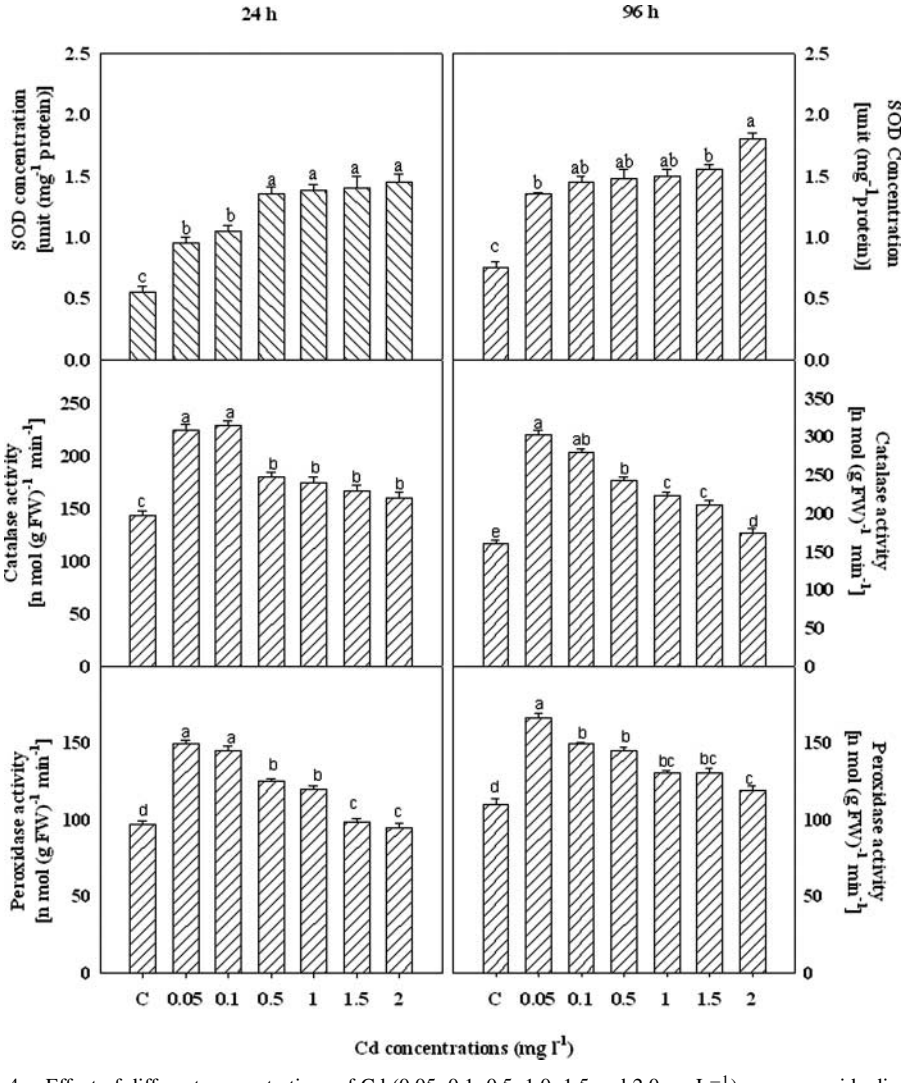

Figure 4. Effect of different concentrations of Cd (0.05, 0.1, 0.5, 1.0, 1.5 and 2.0 mg·L^{-1}) on superoxide dismutase, catalase and peroxidase activity in *Azolla* fronds after 24 and 96 h of exposure ($n = 3$). Bars with different letters show a significant difference at $p \leq 0.05$.

enzyme was produced. SOD activity increased by 72–163% at 24 h and by 66–140% at 96 h of exposure when the Cd concentration was increased from 0.05 to 2.0 mg·L^{-1}. CAT activity increased with 0.1 mg·L^{-1} Cd after 24 h and 0.05 mg·L^{-1} Cd after 96 h of metal exposure, but beyond these concentrations (at 0.5–2.0 mg·L^{-1}) there was a decrease in CAT activity, although values were still higher than in controls (Figure 4). A similar trend was seen in the case of POD activity with varying concentrations of Cd after 24 and 96 h of metal exposure.

4. Discussion

With an increase in the Cd concentration in the medium there was an increase in the accumulation of Cd in *Azolla* fronds. Khosravi et al. [37] also found a similar increasing trend. They

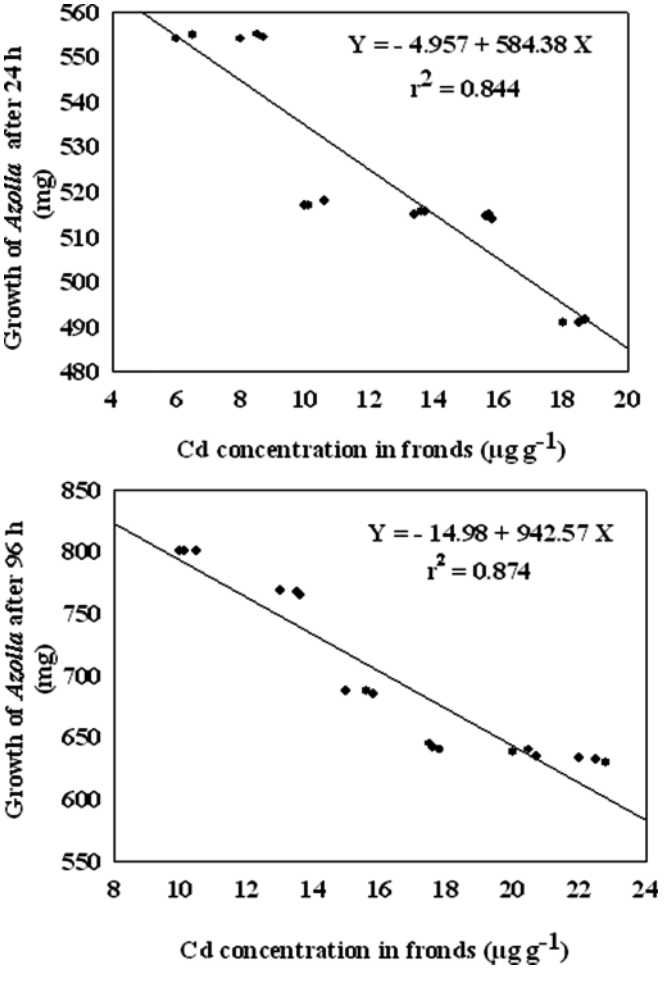

Figure 5. Linear correlation between heavy metal concentrations in *Azolla* fronds and their growth rate.

found that among concentrations of 0.25, 0.5, 0.75 and 1.0 mg $Cd \cdot L^{-1}$ in medium, the maximum accumulation of Cd in *Azolla filiculoides* was observed at 1.0 mg $Cd \cdot L^{-1}$ [37]. Singh et al. [38] found an increased concentration of Cd in plants with increasing concentrations of Cd in soil. Accumulation of Cd in *Azolla* fronds leads to disturbance in the metabolic activities of the plant that subsequently affect growth. Nagajyoti et al. [39] reported toxic effects of Cd, Pb, Zn, Mn, Ni, Cu, Cr and As on plants. Singh and Agrawal [40] also reported a reduction in the growth of *Beta vulgaris* L. in heavy-metal-contaminated soil. At lower Cd concentrations, the fresh mass of *A. pinnata* increased, but at higher concentrations it was found to be decreased at both 24 and 96 h of exposure. Similarly, Gomes-Junior et al. [41] found that at 0.05 mM $CdCl_2$, growth of coffee (*Coffea arabica L.*) was stimulated, but at 0.5 mM $CdCl_2$, the rate of growth reduced. Fronazier et al. [42] and Gratão et al. [43] also found a reduction in the growth rate of sugar cane and tomato plants, respectively, at higher Cd concentrations. Calabrese and Baldwin [44] have shown that low concentrations of toxic elements appear to stimulate growth.

At higher concentrations, excess accumulation of Cd in the plant tissues disrupted the metabolism and physiological activities of the plant and consequently decreased plant growth. Similar to our study, Garmash and Golovko [45] also found a reduction in the growth rate of

barley at higher Cd concentrations (30, 60 or 100 $\mu mol \cdot L^{-1}$). Khosravi et al. [37] reported a 42% decrease in *A. filiculoides* growth at 4 $mg\,Cd \cdot L^{-1}$ when compared with controls. There was a neative linear correlation between heavy metal accumulation and frond growth which suggests that with the increases in the Cd concentration in fronds there was significant decrease in plants growth after both 24 h ($r^2 = 0.844$) and 96 h ($r^2 = 0.874$) of exposure (Figure 5). Similar to our study, Geremias et al. [46] found an inverse relationship between copper concentrations and leaf growth in *Allium cepa*.

In this study, there was a significant increase in SOR, H_2O_2 and MDA content with the increase in Cd concentration after both 24 and 96 h of exposure. This is because Cd is a nonessential element that has a significant damaging effect on plant metabolism and induces oxidative stress at higher concentrations [9]. At lower Cd concentrations, free radicals generated in the plants act as a signal transducer and stimulate the activity of antioxidative enzymes, making the plants grow well. In contrast to this, at higher metal concentrations, these radicals damage the enzyme system and increase the permeability of the cell membrane [47]. With reduction of O_2, the SOR, $^{\bullet}OH$ radical and H_2O_2 are accumulated in the plant system. Hydrogen peroxide is also produced as a result of enzymatic dismutation of SOR. The SORs exacerbate H_2O_2 production in cellular compartments such as apoplasts and chloroplasts [48].

The increase in lipid peroxidation with an increase in Cd concentration is due to the generation of free radicals that distort the membrane architecture causing oxidative damage, as reported in other plants [49]. Peroxidation is measured in terms of thiobarbituric-acid-reactive substances, chiefly MDA, and an increase in MDA with the increasing Cd concentration, as observed in this study, is due to the accumulation of high concentrations of heavy metal. Panda and Upadhyay [50] reported an increase in MDA content in water lettuce (*Pistia stratiotes* L.) under Cu treatment that ranged from 0 to 100 μM for 12, 18 and 24 h. Among the ROS, the superoxide anion ($O_2^{-\bullet}$) plays a central role in the lipid peroxidation of polyunsaturated fatty acids present in the plasma membrane by the formation of more active species, such as hydroxyl radical and singlet oxygen. These active species react directly with unsaturated fatty acids to generate lipid peroxides. Peroxidation of fatty acids decreases the fluidity of the membrane, increases its leakiness, and causes secondary damage to plant membranes [51].

In this study, as the Cd concentration increased, there may have been disruption in enzymatic activities and enhanced generation of free radicals. Cd decreases the concentrations of photosynthetic pigment (Chl *a*, Chl *b*) and consequently reduces photosynthetic activity of the *Azolla* plant. The decrease in the photosynthetic rate under Cd stress might be of a different nature, such as disruption in the pigment apparatus, light reactions and biochemical reactions of the Calvin cycle. Exposure of plants to Cd causes fronds to roll, chlorosis and reduced growth, as well as inhibiting chlorophyll synthesis and various reactions in the Calvin cycle [52]. The reduction in chlorophyll pigment might be due to interference by Cd at the sulfydryl site of enzymes involved in chlorophyll biosynthesis [53]. Heavy metals also decrease chlorophyll content due to a reduction in chlorophylase activity that subsequently affects the Hill reaction in *Azolla* [54]. Carotenoid content increased with increase in Cd concentration. Carotenoids are responsible for scavenging free radicals by electron transfer to their double-bond structure. Carotenoids play a significant role in protecting chlorophyll pigment under stress conditions by quenching photodynamic reactions, replacing peroxidation and membrane collapse in chloroplasts [55].

There was a marginal increase in the respiration rate during metal treatment (0.5–2.0 $mg \cdot L^{-1}$) after 24 h of exposure, but after 96 h the increase in respiration rate at these Cd concentrations was considerable. A high concentration of Cd increases the energy demand for its active exclusion and sequestration, which consequently increases the respiration rate [56]. In addition, reduction in photophosphorylation in the chloroplasts enhances the demands for a mitochondria-based energy supply [57].

Plants show protective behaviour by scavenging free radicals via some antioxidative enzymes. SOD, CAT and POD are important enzymes for protecting the plant under stress conditions. The harmonious interactions of the three enzymes create a balance of free radicals to prevent injury to the cell. An increase in SOD activity with an increase in the Cd concentration indicated a higher production of H_2O_2 through dismutation of the superoxide anion. Isoenzymes of SOD such as Mn-SOD and Cu/Zn-SOD and Fe-SOD are located in different cell compartments [58]. Rodrígutz-Serrano et al. [59] observed that a high concentration (50 μmol) of Cd downregulated Mn-SOD and Cu/Zn-SOD, whereas the plastidic Fe-SOD was upregulated in pea leaf. Under similar conditions, the enhanced activity of total SOD in our study was correlated with the increased activity of chloroplastic Fe-SOD, hence the greater accumulation of H_2O_2 may have adversely affected photosynthetic pigments and activity. H_2O_2 accumulated in the *Azolla* fronds due to deactivation of the chief H_2O_2-detoxifying enzymes, catalase and peroxidase, which showed a decrease in the protection behaviour of the *Azolla* plant at a higher Cd concentration ($2.0\,mg{\cdot}L^{-1}$). A gradual increase in SOD activity with the simultaneous dcrease in CAT activity in *A. pinnata* under Cr stress has been also reported by Panda and Upadhyay [50]. Reduction in the catalase and peroxidase activity of *Azolla* may lead to accumulation of H_2O_2 in the plant which damages the membrane structure and physiological activity of plants. A Cd-induced decrease in catalase and guaiacol peroxidase activity has also been reported in pea plants [60]. Although antioxidative enzymes increased in plants after 96 h of exposure compared with 24 h, this may not be able to counterbalance the increased oxidative stress in the plant. Accumulation of H_2O_2 and generation of free radicals led to a decrease in plant growth with the increase Cd concentration, compared with controls.

5. Conclusion

It was concluded from this study that accumulation of a high concentration of Cd at higher doses in the growth medium may have a deleterious effect on plant metabolic activities. After 24 h of exposure, *Azolla* fronds showed an increase in fresh mass up to $0.1\,mg\,Cd{\cdot}L^{-1}$, whereas after 96 h of exposure, *Azolla* showed an incraese only up to $0.05\,mg\,Cd{\cdot}L^{-1}$, compared with controls. Above these concentrations, the plants showed a decrease in growth and photosynthetic rate. Up to $0.1\,mg\,Cd{\cdot}L^{-1}$, plants are able to withstand the metal stress condition, but beyond this limit there was imbalance in oxidative stress and antioxidative enzyme production that led to a decrease in growth and physiological activities in *Azolla*. A greater accumulation of Cd in plants led to a decrease in growth and disturbance in the metabolic activities. Therefore, *Azolla* can be used for the remediation of heavy metal to certain extent and as a sustainable technique to remove the heavy metal from contaminated fields. However, further study is needed at the subcellular and molecular levels to obatin deeper insights into the mechanism of Cd toxicity.

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