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EFFECTS OF SPERMIDINE ON THE PHYSIOLOGICAL ACTIVITIES OF *MARSILEA MINUTA* LINN. UNDER CADMIUM STRESS

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Summary: *Marsilea minuta* Linn., an aquatic fern species was grown in nutrient solution with varying concentrations (0, 50, 100 and 200 μM) of cadmium (Cd) along with 2 mM spermidine (Spd) as supplementation. The accumulation of Cd in the root tissues showed concentration-dependent relationship. Moreover, significant changes of tissue organization in petiole were also observed by scanning electron micrographs (SEM). Cd was identified using electron dispersive x-ray spectroscopy (EDXS) and found mostly in the cortical tissues as well as in the peripheral zones. Dissolution of the inner wall of cortical cells and distortion of xylem lumens were maximum at the highest Cd concentration (200 μM) as compared to control (0 μM). Treatment with Spd significantly mitigated those damages accompanied by lowering of Cd absorption. At the cellular level, a significant rise of proline accumulation was accompanied by an increase of γ-glutamyl kinase (γ-GK) and γ-glutamyl phosphate reductase (γ-GPR) activity. Spd also retrieved the proline accumulation by modulation of the enzyme activities involved in its biosynthesis. Nitrate reductase (NR) activity both in shoot and root showed a similar declining trend at all concentrations of Cd tested. The activity of NADH-dependent glutamate dehydrogenase (GDH) in shoot and root was quite discriminatory at the different Cd concentrations. For NADPH-dependent GDH, the activity remained subdued irrespective of shoots and roots. In that case, the application of Spd was significantly effective in up regulation of activities in root only, but not in shoot. The activity of glutamate synthase (GS) initially showed an increase and thereafter declined in both shoots and roots. Cellular responses of the *Marsilea* plants for metal hyper-accumulation are discussed with their evaluation as biomarkers under Cd toxicity in aquatic environment.

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Keywords: Cadmium toxicity; *Marsilea*; nitrogen metabolism; proline.

Abbreviations: EDXS – Electron Dispersive X-ray Spectroscopy; GDH – glutamate dehydrogenase; GS – glutamate synthase; NR – nitrate reductase; PAs – polyamines; PVP – polyvinylpyrrolidone; PVPP – polyvinylpolypyrrolidone; SEM – scanning electron micrograph; γ-GK – gamma glutamyl phosphate kinase; γ-GPR – gamma glutamyl phosphate reductase.

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INRODUCTION

Amongst the heavy metals, Cd, a divalent, non-redox cation has been implicated as most toxic to vegetation. Cd as a pollutant is serious with its diverse effects on vegetation and its toxicity is more furnished through contamination of food chains. As a non-nutrient mineral, uptake of Cd is manifested as a partial or complete retardation of cellular homoeostasis in plant-water relations, inhibition of root growth, acceleration of necrosis, retardation of shoot and leaf growth and finally drastic fall in yield (Roychoudhury et al., 2012). Cd induces oxidative stress in plants owing to its pro-oxidant nature. Reactive oxygen species (ROS), as a result are over expressed in different tissues inevitably causing oxidative damages of bio-molecules following lysis of entire tissues (Ghosh et al., 2012). Plant tissues are well adapted to a variety of chemical elicitors either endogenous or exogenous in origin and application to perceive the stress signal, transduction into nucleus for gene expression and finally modulation of the cellular reactions (Kumar et al., 2013). Amongst those polyamines (PAs) have been implicated in many facets of abiotic stresses with special reference to oxidative exposure in plants as well as to amend the antioxidation pathways. PAs are groups of low molecular weight, straight chain, aliphatic compounds ranging in minute concentration in tissues. They are involved in many ways in plant growth and development. Adjustment of cellular processes with the illustration of PAs metabolism and its application has been warranting a suitable measure for plants to ameliorate oxidative stress (Piterkova et al., 2012). However, application of PAs in other plants (besides the angiosperm crop species) is rather less studied. Apart from the latter crop species, a few non angiosperm plants have added their efficiency to accumulate heavy metals in excess without much restrain of growth. The common Chinese Brake fern (*Pteris vittata* Linn.) is supposedly the pioneer to mark the in-built tolerance for heavy metals and their profuse absorption with sustained growth (Ma et al., 2001). These are commonly referred to as hyper-accumulating plant species. Now, ferns or other pteridophytic species have been exploited in a number of ways as per recommendation for accumulation of toxic metals, particularly, from contaminated aquatic environments covering the water bodies, marshy waste lands with industrial effluents (Shuvaeva et al., 2013). Incidentally fern species, particularly the aquatic ones, are profusely grown without any significant deterioration of physiological activities under such conditions. This is found for many aquatic ferns like *Salvinia*, *Azolla, marsilea* with reference to heavy metals. (Mandal et al., 2013; Das et al., 2013). Thus, metal tolerance after application of PAs may supposedly be improved with reactions of more anti-oxidation (Takahashi and Kakehi, 2010). With this view we hypothesized that exogenously applied Spd (a triamine) might be helpful to support the pathways of metal tolerance even in *Marsilea minuta* Linn., an aquatic fern species. In the present study, we focused on the structural changes in tissues under elevated concentrations of Cd, accumulation of proline and its biosynthetic enzyme activities, glutamate dehydrogenase, glutamate synthase and

nitrate reductase. These physiological responses are discussed in view of being used as biomarkers as well as opting the *Marsilea* plant as an efficient hyper-accumulator species under Cd enriched water bodies.

MATERIALS AND METHODS

Plant material and experimental treatments

Marsilea minuta Linn. was collected from the industrial belts of Kalyani, Nadia, which are contaminated with various heavy and toxic metals with industrial effluents. Plants were grown for 7 days in Hoagland's solution and then supplemented with various concentrations (0, 50, 100 and 200 µM) of cadmium chloride ($CdCl_2$). Another set was done with 2 mM Spd with 200 µM of $CdCl_2$ solution. Plants were then grown for 7 days in a growth chamber at a temperature of 37±1°C, 85% relative humidity and 14h/10h light/dark (irradiance 72-80 $\mu M/m^2/s$). The nutrient solution was changed at every two alternate day. On completion of the incubation period, plants were sampled, separated into shoots and roots, frozen in liquid nitrogen following storage at -80°C for further biochemical assays.

Determination of Cd content

The root samples were dried completely into ash at 550°C, dissolved in tri-acid mixture of nitric acid, hydrochloric acid and perchloric acid in the ratio of 1:1:1. The acid digested product was used for Cd analysis with atomic absorption spectrophotometer (ICS-AES) according to Giannakoula et al., (2010).

Detection of Cd at the tissue level

For detection of Cd deposition in the tissues, the petiole sample was preserved in a fixative solution for 4 h and then microtome sections were coated with gold (5 mA, 200 Å gold coating) using IB2 ion coater EIKO engineering. The SEM photographs were taken using Hitachi S 530 scanning electron microscope attached with energy dispersed X-ray (EDX) Unit.

Determination of proline

1.0g of fresh tissue was extracted with 3% aqueous sulphosalicylic acid and treated with acid-ninhydrin solution (0.8 g stannous chloride in 500 ml of 0.2 M citrate buffer, pH 5.4 and 20.0 g ninhydrin in 500 ml of 2-methoxy ethanol) (Yemm and Cocking, 1955). The proline content was estimated as suggested by Bates et al. (1973).

Assay of γ-glutamyl kinase (γ-GK, EC 2.7.2.11) and γ-glutamyl phosphate reductase (γ-GPR, EC 1.2.1.41)

1.0g of plant tissue was extracted with 50 mM Tris-HCl buffer (pH 6.8). 0.1 ml extract was added to the reaction buffer containing 0.1 ml of 10X ATP and 1.8 ml of extract and incubated at 37°C for 30 min following addition of 2 ml of stop solution. γ-GK activity was measured spectrophotometrically at 535 nm as suggested by Jaleel et al., (2008). For γ-GPR activity, similar extraction buffer was used except for the addition of 0.1 M cysteine. The assay mixture contained 100 mM Tris-HCl (pH 7.2), 1.5 mM $MgCl_2$, 5 mM glutamine, 5mM ATP and 0.2 mM NADPH and 50 µg enzyme. The activity was measured according to Jaleel et al. (2008, 2009).

Assay of glutamate dehydrogenase (GDH)

1.0g of plant sample was homogenized in two volumes (w/v) of extraction buffer containing 50 mM Tris-HCl (pH 8.2), 5mM 2-mercaptoethanol, 1mM calcium chloride and 5% PVP. The supernatant was used for the amination reaction in 100 mM Tris-HCl (pH 7.0), containing 100 mM NH_4Cl, 10 mM 2-oxoglutarate, 0.16 mM NADH and 4 mM calcium chloride (for NADH-GDH, EC 1.4.1.2). The oxoglutarate-dependent oxidation of NADH was read at 340 nm as suggested by Loyola-Vargas and Jimenez (1984). The other reaction was monitored at 30°C in the same assay mixture (100 mM Tris-HCl, pH-7.8 and 0.16 mM NADPH).

Assay of glutamate synthase (GS, EC 1.4.1.13)

1.0g of plant sample was homogenized in 50 mM phosphate buffer (pH 7.5), containing 1 mM disodium EDTA, 1 mM DTT, 2 mM 2-oxoglutarate, 1 mM PMSF and 0.1% PVPP. GS activity was assayed according to Esposito et al. (2005) monitoring NADH oxidation at 340 nm in an assay mixture containing 50mM Tris-HCl (pH 7.6), 5mM glutamine, 5.0 mM 2-oxoglutarate, 0.25 mM NADPH).

Assay of nitrate reductase (NR, EC 1.6.6.1)

1.0g of sample was homogenized in 0.1M potassium phosphate buffer (pH 7.5). 0.2 ml of enzyme extract was added to 0.2 ml of potassium nitrate (0.1 M) solution and 0.4 ml of 2 mM NADH and incubated at 30°C for 15 min. The reaction was stopped by addition of 1.0 ml of 1% sulphanilamide followed by 1.0ml of 0.2% naphthyl ethylenediamine reagent and incubated for 30 min. Absorbance was read at 540nm and the enzyme activity was calculated according to Carelli and Fahl (2006).

Statistical Analysis

Observations were recorded taking three replications (n = 3).The statistical analysis was performed by one-way ANOVA analysis, taking $P \leq 0.05$ as significant. The data in the figures were presented as a mean value ±SE.

RESULTS

Accumulation of Cd in tissues is essentially related to the development of oxidative stress in the plants. Cd accumulation could affect the mechanical injuries in the tissues. Thus, we found a concomitant effect of internal tissue disorganization along in the petiole as revealed by the SEM micrographs coupled with EDXS study (data not shown). A typical concentration-dependent response of Cd acquisition was found in the petioles of the treated plants. Mostly, the affected tissues were demarcated in the endodermis following the stelar region and the cortical tissue to some extent (Fig. 1). It is interesting to note that the deposition of Cd was more scattered in the cortical zone extending periphery. The maximum disintegration of the tissues was recorded at 200 μM Cd and the lumens were more disorganized distorting the intercellular wall. Xylem lumens were found more dilated and distorted on their wall. The wall of the lumens showed a gradual breakage according to concentrations of Cd (50, 100 and 200 μM). However, plants treated with 2mM Spd appeared to be stable with respect to tissue integrity. Moreover, the

intercellular spaces and cortical regions were found with less deposition of Cd as detected by the EDXS study. The internal structure of plants treated with Cd also correlated with the linear increase in Cd accumulation and was recorded within a range of 112 µg mg^{-1} – 262 µg mg^{-1} under control (0 µM) and 200 µM Cd concentration, respectively (Table 1). Therefore, plants were maximally affected by the highest concentration of Cd and that was 134% over control. Interestingly, Cd accumulation was minimized by 16.74 % in the presence of 2 mM Spd. In perception of such osmotic imbalances plants have to adjust water relation by development of some compatible solutes, proline being the predominant one. The comparative accumulation of proline was assayed by determination of both proline content and its biosynthetic enzyme activities. Likewise, following Cd stress (50 µM, 100 µM and 200 µM), the content of proline increased proportionately (by 36%, 88.3% and 117%, respectively) (Fig. 2a). Interestingly, application of Spd had significantly ($p \leq 0.05$) down regulated the activity of proline maximally by 12.71 % against 200 µM Cd. The activity of two proline biosynthetic enzymes showed

Table 1. Accumulation of Cd [µg/mg DW] in roots of *Marsilea* under varying treatments (0 µM, 50 µM, 100 µM, 200 µM) of Cd concentrations and supplemented with Spermidine (200 µM + 2 mM Spd) for 7 days.

Treatments	Accumulation of Cd
C (0 µM)	112.0 ± 3.8
50 µM	176.2 ± 4.3
100 µM	196.8 ± 4.9
200 µM	262.1 ± 5.4
200 µM + 2mM Spd	218.2 ± 5.1

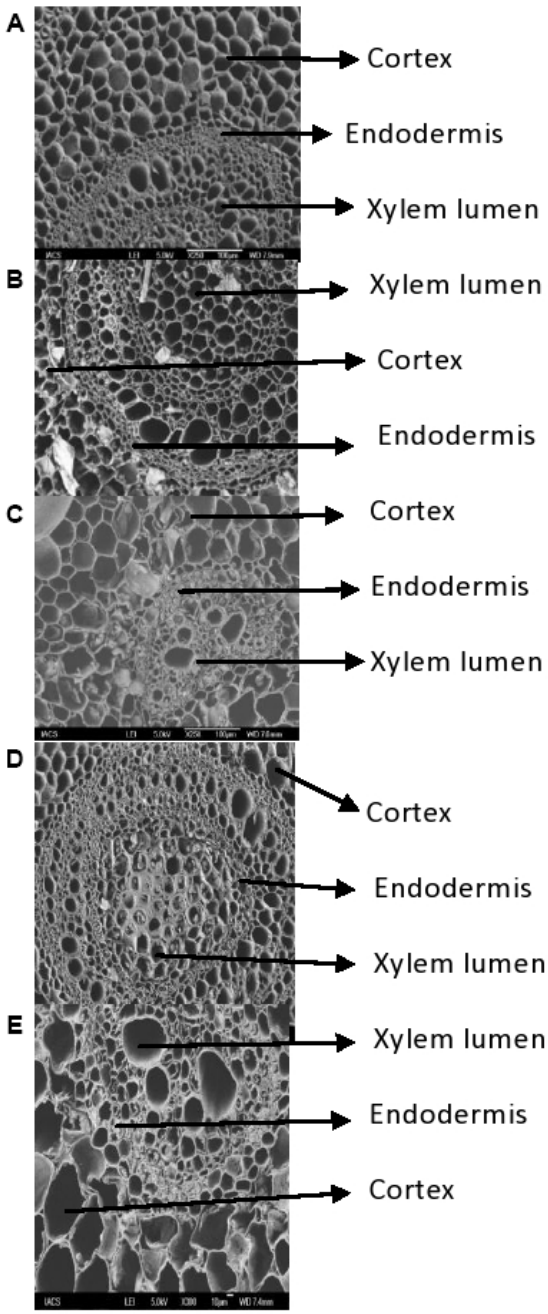

Figure 1. SEM micrograph of internal structures of petiole in *Marsilea* under varying treatments (0 µM, 50 µM, 100 µM, 200 µM) of Cd concentrations and supplemented with Spermidine (200 µM Cd + 2 mM Spd) for 7 days.

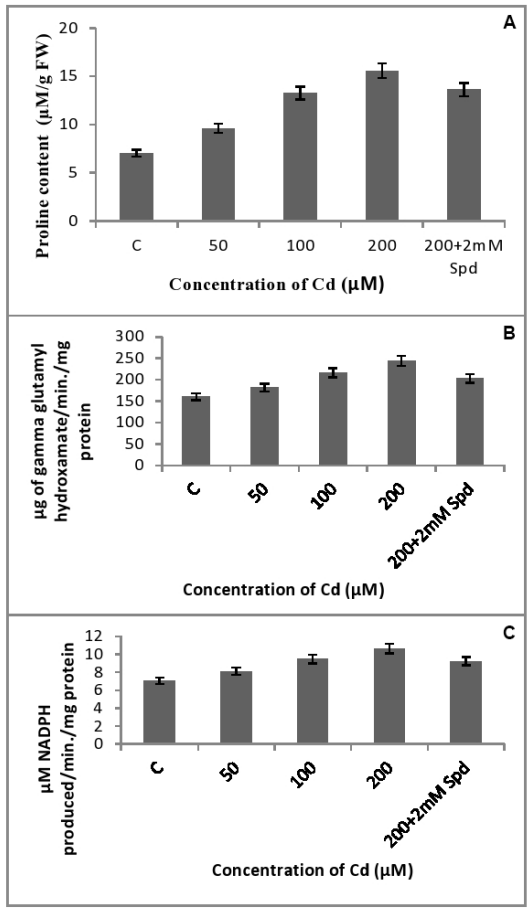

Figure 2. Proline content (A), activity of γ-glutamyl kinase (γ-GK) (B), activity of γ-glutamyl phosphate reductase (γ-GPR) (C) of shoot in *Marsilea* under varying treatments (0 µM, 50 µM, 100 µM, 200 µM) of Cd concentrations and supplemented with Spermidine (200 µM Cd + 2 mM Spd) for 7 days. The data represented as mean value of observations (n=3) ±SE and put by the vertical bars in each bar. Statistical differences ($p \leq 0.05$) have been compared with student's t test.

the similar trend in plants under Cd treatment as that of proline biosynthesis. These two enzymes were γ-glutamyl kinase (γ-GK) and γ-glutamyl phosphate reductase (γ-GPR). At the cellular level, the activities of these enzymes coordinate the phosphorylation and reduction respectively of glutamic acid in two consecutive steps. As a whole, activities of these enzymes constitute the overall activity of the rate-limiting steps in the biosynthesis of proline. In our present experiment, we assayed the activity of γ-GK and γ-GPR from plant extracts using varying substrates. The activity recorded up regulation through the concentrations of Cd being maximum at 200 µM. The extent of induction of γ-GK and γ-GPR was maximized by 52.5% and 51.2%, respectively as compared to control (Fig. 2b and Fig. 2c). However, a significant minimization of the enzyme was recorded when plants were fed with Spd.

Cd being a heavy metal, could interfere plant's nutritional status in a number of ways. Thus, nitrogen metabolism showed significant changes in the present experiment. In this context we have evaluated the sensitivity of some nitrogen metabolizing enzymes in both shoot and root of *Marsilea* plants under varying Cd concentrations. Differences in the activities of these enzymes were recorded in shoots and roots under the same conditions of Cd exposure. NADH-GDH activity in shoot showed a steeper decline at all Cd concentrations and a maximum decline under 200 µM with 74.28% less compared to control (Fig. 3). In the roots, a gradual increase was recorded which was maiximum by 65%. On the other hand, supplementation of Spd imposed differential regulation of NADH-GDH activity in shoots and roots. The activity in shoot resumed significantly ($p \leq 0.05$) by 158.9% after Spd supplementation. The activity in root was suppressed by 26.10% when Spd was added as compared to 200 µM of Cd. Another form that takes NADP as an electron donor is NADPH-GDH. In

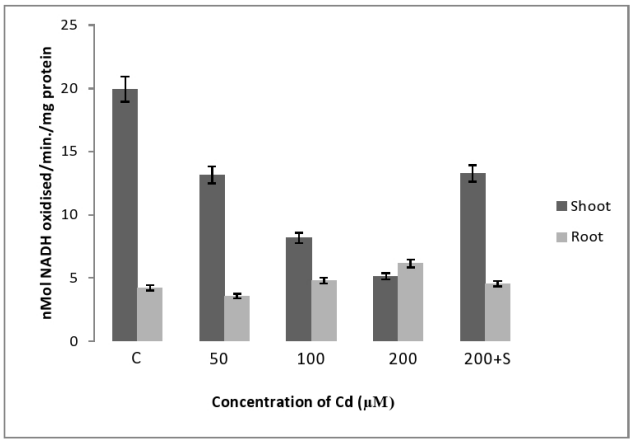

Figure 3. Activity of glutamate dehydrogenase (GDH) (NADH dependent) from shoot and root of *Marsilea* plant under varying treatments (0 µM, 50 µM, 100 µM, 200 µM) of Cd concentrations and supplemented with Spermidine (200 µM Cd + 2 mM Spd) for 7 days. The data represented as mean value of observations (n=3) ±SE and put by the vertical bars in each bar. Statistical differences ($p \leq 0.05$) have been compared with student's t test.

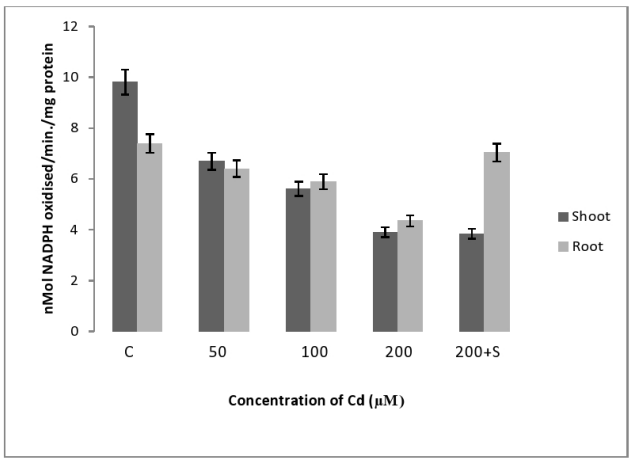

Figure 4. Activity of glutamate dehydrogenase (GDH) (NADPH dependent) from shoot and root of *Marsilea* plant under varying treatments (0 µM, 50 µM, 100 µM, 200 µM) of Cd concentrations and supplemented with Spermidine (200 µM Cd + 2 mM Spd) for 7 days. The data represented as mean value of observations (n=3) ±SE and put by the vertical bars in each bar. Statistical differences ($p \leq 0.05$) have been compared with student's t test.

fact, this isoform is exclusively seated in cytosol and vacuole and more sensitive to alteration of cytosolic pH. Thus, metal acquisition in excess altering the redox state of cytosol may be attributed by the activity of this isoenzyme. However, a similar declining trend of the activity of NADPH-GDH in shoot and root was observed (Fig. 4). The maximum decline was found to be by 60.15% and 41.12% in shoot and root, respectively as compared to control. Interestingly, Spd

supplementation had significantly different effects in resuming the activity in shoot and root. For shoot, it was insignificant in modulation of NADPH-GDH activity. A steeper retrieval by 61.5% was recorded in root in the presence of Spd. In nitrogen metabolism glutamate synthase (GS) is the most important enzyme that could replenish the reversible accumulation of glutamate and oxoglutarate in plants. It maintains homeostasis of amino acid and keto acid pool, particularly, in nitrogen depleted soil. In the present experiment, *Marsilea* plants responded differently

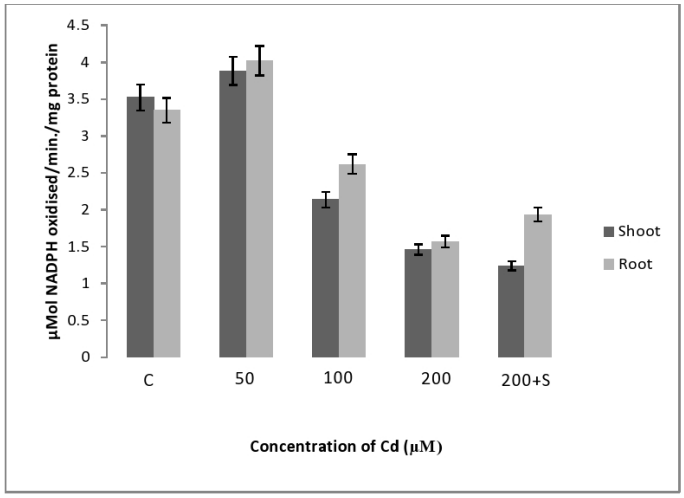

Figure 5. Activity of glutamate synthase (GS) from shoot and root of *Marsilea* plant under varying treatments (0 µM, 50 µM, 100 µM, 200 µM) of Cd concentrations and supplemented with Spermidine (200 µM Cd + 2 mM Spd) for 7 days. The data represented as mean value of observations (n=3) ±SE and put by the vertical bars in each bar. Statistical differences ($p \leq 0.05$) have been compared with student's t test.

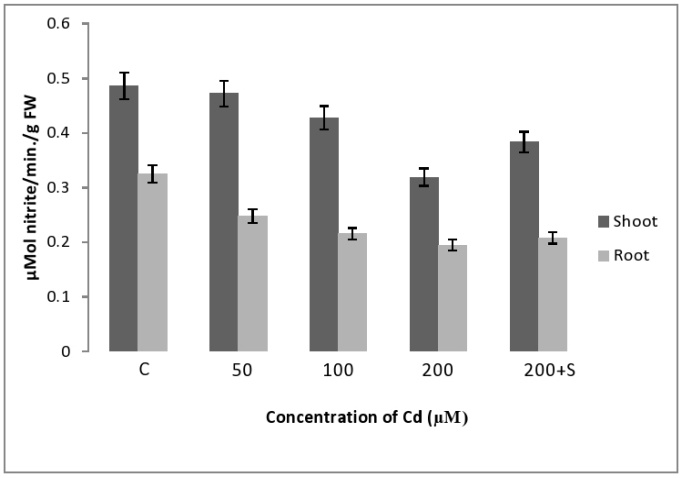

Figure 6. Activity of nitrate reductase (NR) from shoot and root of *Marsilea* plant under varying treatments (0 µM, 50 µM, 100 µM, 200 µM) of Cd concentrations and supplemented with Spermidine (200 µM Cd + 2 mM Spd) for 7 days. The data represented as mean value of observations (n=3) ±SE and put by the vertical bars in each bar. Statistical differences ($p \leq 0.05$) have been compared with student's t test.

when we did the *in vitro* assay for GS activity with NADP as an electron acceptor. Interestingly, plants showed up regulation of the enzyme activity within a threshold of Cd concentration (i.e. 50 μM in the present case) by 10% and 20% in shoots and roots, respectively over the control (Fig. 5). Thereafter, a steady decline was noted in both organs at the Cd concentrations tested. However, the maximum decline in activity was recorded at 200 μM Cd (by 58.59% and 53.06% for shoot and root, respectively as compared to control). After Spd application the enzyme activity in shoots decreased by 15.07%. In contrast, 23% retrieval of the activity was found in roots. The nitrate reductase (NR) activity was assayed using NADH as an electron donor. The NADH-NR happens to be predominant in plant system being mostly restricted in the cytosol. The activity of NR showed a declining trend in both plant organs and it was maximally curtailed under 200 μM Cd (Fig. 6). However, Spd had significantly retrieved the activity. The activity in shoots showed a decline by 34.36% but it could be recovered by 33% after the addition of Spd. Similarly, a reduction by 44.36% of NR activity in roots was also gained only by 6% in increase.

DISCUSSION

Cellular responses to environmental extremities are highly variable according to plant species. At the cellular level, the adaptability to Cd hyper-accumulation was initially adjusted by sequestering the metal mostly in the cortical zones of petioles as found in he *Marsilea* plants (Sridhar et al., 2007) Avoidance of mechanical injuries by the metal and amelioration of ROS induced oxidative damages of the tissues are the two strategies for metal hyper-accumulation (Karuppanapandian et al., 2011).Therefore, the structural deformities revealed by the SEM study in the petiole of *Marsilea* plants under Cd stress would be an outcome of oxidative damages. Spd treatment, however, might either lower the generation of ROS in its pathway or/and deterrence the tissues from ROS exposure. For the latter, Spd being a polyamine with polycationic nature can bind to negatively charged domains of membrane and thereby shield the tissues from ROS induced peroxidation (Maiti et al., 2012). Pas-mediated defense from oxidative damages has also been documented in other crops under Cd stress. In the present experiment, the primary responses to Cd sensitivity were forwarded with generation of proline in the context to water relations. Proline in general, is granted as the most common compatible solute essentially required in adjusting the osmotic balance (Handique et al., 2009). However, how proline could contribute to metal stress tolerance can be ascertained from the activities of two rate-limiting enzymes: γ-GK and γ-GPR. Thus, the synchronization of proline overproduction with its biosynthetic enzymes happens to be a noticeable feature as found in many other plants (Claussen, 2005). The osmotic imbalances in the tissues due to metal deposition become an acute need for osmoticum as also studied under Cd (Zhao, 2011).

GDH is the enzyme that donates an electron through NADH/NAD(P)H to α-ketoglutarate with incorporation of an ammonia molecule in a reversible way. This is most important for maintaining a steady-state homeostasis for keto acids

and amino acids in plants and very often remains shifted more towards the keto acid acquisition. Under abiotic stress, the amino acid biosynthesis could be subdued and featured by down regulation of GDH activity as documented in many crop species. Some authors suggest a role of GDH activity in reductive amination of keto acids and up regulation under abiotic stresses (Tripathi et al., 2013). GS is required for the reaction of incorporation of NH_4^+ into keto acids in the plants. Cd is a metal which often depletes the soluble nitrogen in the soil to be absorbed by the plants (Benavides et al., 2004). In the present study, the fall in activity of NAD(P)H-GDH both in shoot and root may meet the unavailability of NH_4^+ in the plants. More so, the supplementation of Spd is also discriminatory for maintenance of GDH activity in these organs under the same conditions. On the other hand, the enzyme activity remained almost insensitive in shoot, but a significant up regulation was found in roots under Spd treatments. The depletion of GDH activity under metal stress in shoots may be suggestive for an alternative pathway of NH_4^+ assimilation in plants (Misclaux et al., 2006). A minor rise in NADH-GDH activity observed in the present experiment (only in roots) could indicate the access of NH_4^+ which may stimulate GDH functioning. For the NADP(H)-GDH activity, plants showed a declining trend in both shoots and roots. The roots could recover the activity by several times, whereas in shoots it was hardly effective after Spd application. This is for replenishing glutamate from the keto acid pool in the roots since it is more prone to nitrogen imbalance in soil (Zhang et al., 2013). A significant amount of NH_4^+ was possibly associated with more glutamine synthase activity in the roots. Therefore, roots should have more saturation with glutamic acid as a supportive amino nitrogen balance that ought to be perturbed under metal stress.

High level of accumulation of NH_4^+ is fairly toxic in many plant species (Britlo et al., 2002). According to species specificity and cellular demands NH_4^+ availability includes photorespiratory reactions, NO_3^- reduction, salinization of organic nitrogen, degradation of storage compounds, etc. (Chaffei et al., 2004). NH_4^+ is rapidly incorporated into keto acids through a conjugated enzymatic system employed with glutamine synthase-glutamate synthase conjugate system (Masclaux et al., 2006). Glutamate synthase (GS) is required by plants to increase the pool of keto acids by de-amination reaction. Cd-induced over-accumulation of NH_4^+ might be a direct or indirect resultant of altered activity of GS as also pointed out by other workers (Haouari et al., 2011). Therefore, in the present experiment, Cd might intoxicate the plants with increased NH_4^+ concentration in tissues as reported earlier (Haouari et al., 2012). This is also corroborated with the significant decrease in GS activity as well as the increased activity of GDH under conditions of excess Cd. In addition, NADH-GDH activity may also complement the partial assimilation of Cd involving NH_4^+ which is in agreement with results on *Marsilea* (Debouba et al., 2007). NR activity under Cd stress showed a significant down regulation. A linear fall in the activity was observed in both shoots and roots, however, it was less upon Spd application. The sensitivity to Cd in shoots and roots might be explained by tissue specificity of the studied enzyme synthesis and its

modulation by Spd as well (Sharma and Dietz, 2006). The rapid fall of NR activity in the studied plant organs might be also related to the status of NADH/NAD(P)H which often undergoes changes in response to metal toxicity (Dinakar et al., 2009). This is based on the fact that under metal or any abiotic stresses photosynthetic flux to generate NADH/NAD(P)H is more limited at the initial stages of stress. Therefore, the inadequacy or depletion of NADH/NAD(P)H might cause the limitation for NR activity. PAs are reported as most useful to bind to the cellular membrane with its positive charges. Under this condition, it could also donate protons (H^+) to the cellular environment to reinforce the adequate reducing state (may be in NADH/NADPH form). Similar interpretation has also been described under conditions of salinity and metal stress and its amelioration by PAs in rice (Roychoudhury et al., 2012). Thus, in the present study, Spd might have operated in a similar way as could also be predicted.

CONCLUSION

Despite the unquestionably ill effects of Cd, *Marsilea* plants could defold their adaptability by the physiological potential with response to Spd. More so, the hyper accumulative attitude of this pteridophytic species to Cd stress might be implicated to the phytoremediation facet. A significant amount of accumulated Cd is transported into the cell wall of cortical tissues in roots, thereby reducing the possibility of transport upwards through the petiole. This shows that *Marsilea* plants can bestow their ability to tolerate Cd toxicity without changing plant's vigor. Still, the adaptability of *Marsilea* as elucidated through this experiment could help to provide some insights into metal tolerance in fern species. Moreover, the physiological traits related to the absorption and tolerance to elevated Cd concentrations may support possible biological indications for this metal under aquatic environment.

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