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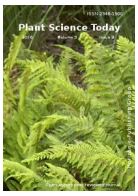

Research Article

Physiological alterations of *Salvinia natans* L. exposed to aluminium stress and its interaction with polyamine

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Abstract

In the present Investigation *Salvinia natans* L exposed to increasing concentration of Al stress and its interaction with polyamine was discussed. Among the physiological attributes Glutathione-S-transferase, glutathione peroxidase activities were up regulated against control. At transcript level glutathione peroxidase was also shown by over-expressed manner to support the lyses of peroxide. However, the application of Put had minimized these activities with same way to establish the role of polyamine under metal stress. In secondary metabolites synthetic pathway phenyl alanine ammonia lyase recorded a steady increase although the concentration of Al. Not only a single fraction of polyamine was responsible under Al stress but also pool of conjugated polyamine was up regulated. In oxidation of polyamine the activity of diamine oxidase (DAO) was more under metal stress to induce accumulation H_2O_2. In compensation for cellular depletion of reduced glutathione, dihydro ascorbate reductase activity was up regulated in plant under stress. At cellular level plants were distinctly marked with variations in heat shock proteins and established as a possible biomarker for Al toxicity. The study possibly established the affectivity in bio-monitoring of Al in field condition with exercise cellular responses of *Salvinia* plants.

Keywords
Al toxicity; western blotting; gene expression; HPLC; polyamine; *Salvinia natans* L

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Introduction

In addition to heavy metal toxicity as common in plants, the metals of less molecular masses are also offered equally detrimental. Likewise, aluminium (Al) a lighter metal is proved to be phytotoxic and becomes more detrimental under the conditions of acidic environment where pH drops below 5 (pH ≤5) (Ribeiro *et al.*, 2012). Al ranks third most abundant element covering around 8 % of the total earth's crust and abundance of it has reached almost 40 % of arable land (Kochian *et al.*, 2004). The phytotoxic forms of Al in soil mostly comprise with aluminosilicate (Al_2SiO_5, Al_2O_3 SiO_2) those are soluble after being acid hydrolysis. A substantial quantity may directly be incorporated into roots of plants in different degrees according to species variation. Owing to higher solubility of Al oxide (Al_2O_3), the water bodies and allied ecosystem adjacent to contaminated soil poses a great source of Al phytotoxicity. This is particularly, amenable for aquatic plants where

mostly in hydrated oxide of the metal. The most noticeable phytotoxicity is focused on root growth with the partial or complete inhibition in structure (Arroyave *et al*., 2011). The most sensitive zones for toxicity in roots are reported on meristematic zone in combination with other allied tissues inhibiting cell division and elongation.

The plant species which have the intrinsic potential to cope up with the effect of heavy/toxic metals are regarded as metal tolerant (Yang *et al*., 2011; Xie *et al*., 2009). A number of plant species are found to be adapted in varying degrees to tolerate the metals in adjustment of morphological, anatomical, physiological, biochemical features as well as molecular features (Ribeiro *et al*., 2012). It has already been reported some non-angiospermic species mostly the pteridophytes as like *Pteris*, *Marsilea, Azolla, Salvinia* etc. are evident as reliable bioaccumulator with displaying alteration of cellular physiologies couple with up/down regulation of genes. The plants adapting to metal tolerant are mostly focused with osmotic adjustment followed by modification of anti-oxidation system. The later could be of those that are manifested with up/down regulation of many genes directly or indirectly support the antioxidation cascade (Dhir *et al*., 2012). Pteridophytic species mostly the ferns have been a successful in different modes and have evident as potential bioremidiator for heavy and toxic metals. Like other ferns species *Salvinia natans* (L) has also been reported to be a potent quencher of heavy metals by hyperaccumulation and subsequently with the way of altering physiological activities (Dhir *et al*., 2011). The hyperaccumulation of Al in some cases is found to be modulated by application of some exogenous chemicals in plants. Polyamine (PA), an aliphatic amine has been most promising in mitigation of abiotic stress including metal toxicity, however, mostly in angiospermic plants. In few cases the metabolism of PA and its derived intermediate moieties are found participatory in metal stress or induced oxidative injuries in number of ways. However in fern, even in other non angiospermic plants the application of PA to be a modulator in those cases is less scored in earlier studies. Still in few cases like aquatic fern (*Azolla piñata* L.) record that PA could induce root abscission as indexed a special trait for resistance to toxic metal (Gurung *et al*., 2011). Therefore, PA is established to be an effective moiety to regulate the metal induced damages mostly allied to oxidative stress. Most of those were dealt with frequently using antioxidation attributes. In the present experiment some other contributory parameters were discussed in light of Al phytotoxicity along with application of PA. We observed a significant change in the root metabolic activities of *Salvinia natans* (L.) in cellular responses to modulate Al induced oxidative stress by PA. Thereby those characteristics cellular phenomena could be effective in bioindication or Al enriched water bodies.

Material methods

Plant material

Salvinia natans (Linn.) is a free-floating aquatic fern, belonging to the family of *Salvinia*ceae, class of Pteridopsida and division of Pteridophyta, was chosen as the experimental material for the present experiment.

Culture of the plant

Plants were collected from wet land, washed with deionized water and transferred to 1/4 X Murashige and Skoog medium for seven days for acclimatization (Murashige and Skoog 1962). Thereafter, plants were treated with varying concentrations 0, 240, 360, 480 µM of Al salt [$KAl(SO_4)_2$,$12H_2O$] and 480 µM of Al salt with 1 mM putrescine (Put), adjusted to pH 4.5 (Giannakoula *et al*., 2008; Parker *et al*., 1995). Those were kept in a poly house for 7 days under the ambient condition: 37 ± 1^0C of temperature, 75-85 % of relative humidity and photoperiod of 14-10 hr light and dark. After completion of treatments, plants were harvested, immediately freezed in liquid nitrogen and preserved in -70^0C for further biochemical analysis.

Assay of Glutathione-S-transferase activity (GST, EC 2.5.1.18)

It was determined according to Vestena *et al*., 2011. 1 g of fresh sample (whole plant) was taken and ground in liquid nitrogen. Then the sample was homogenized with ice cold extraction buffer composed of 0.2 M Tris-HCl buffer (pH 7.8), 1 mM EDTA, 1 mM DTT, 0.1 mM PMSF and 5 % (w/v) PVPP. Homogenate was centrifuged at 12,000 x g for 15 min at 4ºC. Supernatant was collected and followed by 100 µl of supernatant was added with 3 ml of assay mixture containing phosphate buffer (pH 6.5), 0.1 mM 1 chloro 2, 4 dinitro benzene and 20 mM GSH, activity was determined by recording the increase in absorbance at 340 nm for 3 min and one unit (U) of GST was expressed as nmol min^{-1} g^{-1} FW.

Assay of glutathione peroxidase activity and in-gel expression profile (GuPX, EC 1.11.1.9)

The plant sample was ground in a motor and pestle with the help of liquid N_2, and homogenized with 0.1 M phosphate buffer (pH 7). The homogenate is centrifuged at 15,000 x g for 20 min. The 0.5 ml supernatant was mixed with reaction mixture containing 0.1 ml of 10 mM sodium aziide , 0.2 ml of 4 mM GSH, 0.1 ml of 2.5 mM H_2O_2.Then the reaction was terminated by adding 0.5 ml of 10 % TCA after incubation at 0, 30, 60, 90, 120 seconds followed by centrifuge it at 10,000 x g for 10 min. 1 ml of the supernatant was added to

1.5 ml of phosphate buffer and 0.5 ml of DTNB reagent (0.04 % DTNB in 1 % sodium citrate) The colour developed was read at 412 nm and the enzyme activity is expressed in terms of µg of glutathione utilized min^{-1} mg^{-1} protein. The in gel staining of glutathione peroxidase was done following (Hou *et al.*, 2003; Lin *et al*., 2002), the supernatant containing 50 µg of protein was loaded in each well in native PAGE and run under cold condition. After running the gel submerged in an incubation solution of 50 mM Tris-HCl buffer (pH 7.9) containing 13 mM Glutathione (reduced form i.e. GSH), 0.004 % H_2O_2 for 20 min with gentle shaking. After that 1.2 mM 3-(4,5-dimethylthiazol-2-yl)-2,5-iphenyltetrazolium bromide (MTT) and 1.6 mM Phenazonium Metho Sulphate (PMS) were added and incubated for 20 min to resolve the gel.

The Phenylalanine ammonia-lyase activity (PAL, EC 4.3.1.24)

Enzyme was extracted from control as well as treated *Salvinia* plants. 1 g of leaf samples were crushed in liquid nitrogen and homogenized in 100 mM K-phosphate buffer (pH 7) containing 2 mM phenyl methyl sulfonyl fluoride (PMSF) and then centrifuged at 15,000 x g for 20 min at 4°C. The PAL activity was assayed by using an assay modified from D'Cunha *et al*., 1996. The 0.2 ml of the enzyme extract was added to the reaction mixture contained 100 mM Tris-HCl buffer (pH 8.5), 1 mM 2-mercaptoethanol, 15 mM L-phenylalanine. Then the mixture was incubated at 30°C for 15 min and the reaction was terminated by adding 6 N HCl. The absorption was read at 290 nm. The enzyme activity was expressed as µM cinnamic acid h^{-1} g^{-1} tissue.

Dehydroascorbate reductase activity (DHAR, EC 1.8.5.1)

Activity was measured as mentioned by Chopra and Semwal, 2011. Plant sample was ground in liquid nitrogen and homogenized in 50 mM phosphate buffer (pH 7). Then aliquote was centrifuged at 12,000 x g for 15 min at 4°C. Supernatant was collected and assayed in assay mixture containing 50 mM phosphate buffer (pH 6.5), 25 mM GSH (reduced glutathione) and 0.1 mM dehydroascorbate (DHA). An increase in absorbance due to ascorbate formation was measured at 265 nm, where, each 0.1 absorbance is equivalent to 7.14 nmol ascorbate formation, which was used for calculating DHAR activity and expressed as U mg^{-1} protein. The supernatant (equal amount of soluble protein) was separated on 10 % polyacrylamide gel electrophoresis (PAGE) under nondenaturing and non-reducing conditions, and then incubated for 15 min in an assay mixture containing 2 mM dehdroascorbate (DHA), 4 mM GSH (reduced glutathione) followed by washed with distilled water. Then, again the gel was incubated for 10 min with solution composed of 0.125 M HCl, 3 mM Ferricyanide and 3.5 mM ferric chloride (Depinto *et al.*, 2000).

Polyamine determination

PA determination was done according to Zhao and Yang, 2008 with slight modification. The samples were crushed in liquid nitrogen and extracted with 10 % (w/v) cold perchloric acid ($HClO_4$) (300 mg ml^{-1}), followed by centrifugation at 15,000 x g for 20 min at 4°C. The 0.1 ml of supernatant and reference PAs (Spd, Put, Spm) were vigorously mixed with 0.2 ml of saturated carbonate buffer (pH 9) and then mixed vigorously for 1 h with 0.2 ml of dansylchloride. The mixture was incubated at 60ºC for 1 h in dark. The dansylated PA was extracted with toluene. 100 µl of each dansylated sample was loaded on an activated (80ºC for 1 h) HPTLC silica gel plate with concentration zone using a micro syringe. The plates were run with cyclohexane: ethyl acetate (3:2 v/v) as a solvent in a glass chamber with saturated atmosphere. After complete up ward running of solvent through TLC plate, the plate was dried and followed by the spots of dansylated PAs were identified with respect to those of the standards. The spots were identified under UV lamp and scraped off and eluted with acetone. The PAs were quantified from the fluorescence measured at 360 nm excitation and 560 nm emissions by a fluorescence spectrophotometer and expressed as µM g^{-1} of FW.

HPLC analysis of PAs: HPLC analysis of dansylated PAs have been done through reverse phase (ODS; Rainin 'Microsorb') 3 mM diameter 10 cm length column using Altex-Beckman model 322 liquid chromatograph employing 254 nm detection. The C_{18} reverse phase column was filled with 5 µM particles heated at 50 ±1ºC in a water jacket and 10 µl dansylated PA sample was injected, which was eluted at a flow rate of 2 ml min^{-1} using the solvent acetonitrile: water:: 70: 30 (v/v) (Walter and Geuns, 1987).

Diamine oxidase activity (DAO, EC 1.4.3.6)

1 g of fresh plant sample from each Al doses was crushed in liquid nitrogen followed by homogenization with 0.1 M potassium phosphate buffer (pH 6.5) containing 5 mM dithiothreitol (DTT). Extracts were filtered through nylon cloth and centrifuged at 12,000 x g for 30 min at 4ºC. Supernatant was collected gently and assayed by adding 1 ml of this supernatant in to assay mixture composed of 50 mM phosphate buffer, 10 mM Put, catalase (50 unit), 0.1 % *O*-amino benzaldehyde. The absorbance at time interval has been taken at 516 nm and expressed in ρ-Mol Δ-pyrroline min^{-1} g^{-1} FW. For in-gel staining activity, electrophoretic separations were performed on non denaturing PAGE using 5 % stacking gel and 10 % separated gel with Tris-glycine buffer (pH 8) at 4ºC. The amine oxidase activity was detected by performing

in reaction mixture composed of 12.5 ml of 1 M potassium phosphate buffer (pH 7), 15 mg of Put and 1.5 mg of horse radish peroxidase (HRP), 7.5 mg 4-chloro-1-napthol with 2.5 ml of cold methanol (Houen and Leonardsen, 1992; Luhova *et al*., 2003).

Western transfer of Heat shock protein (Hsp 70)

The protein sample was isolated from 1 g of fresh whole plant samples by grinding them in liquid nitrogen followed by homogenized with protein extraction buffer containing 50 mM Tris–Cl, pH 7.5; 250 mM sucrose; 25 mM KCl; 5 mM $MgCl_2$; 3 mM EDTA; 1 mM PMSF as protease inhibitor; 1 mM DTT; 0.5 % β-mercaptoethanol. The homogenate was centrifuged at 15,000 x g for 20 min at 4ºC. The supernatant was collected and purified through 80 % ammonium sulphate cut (dyalisis). Then, the supernatant was concentrated by lyophilization at 4ºC. There after protein sample was separated through SDS-PAGE and stained with ponceau stain to visualize the banding pattern. After destaining the gel by washing with distilled water, protein bands were transferred from gel to activated PVDF membrane through western transfer. The membrane was blocked with blocking solution composed of 1x TBS and 5 % bovine serum albumin (BSA) and probed with purified primary antibody generated due to Hsp 70 protein. The detection of Hsp 70 was finally done by incubating the membrane with alkaline phosphatase-conjugated goat anti rabbit IgG. The detection of Hsp 70 was done by developing with substrate solution containing NBT-BCIP in Tris buffer (pH 9.5) (Tukaj *et al*., 2011; Tukaj and Tukaj, 2010).

Expression potential of *Salvinia natans* L. for *GuPX* gene under Al exposure

The total RNA was isolated from the *Salvinia* plants, treated with Al salt (0, 240, 360, 480 µM and 480+1 mM Put). Each 5 g of sample was homogenized with 400 mM Tris-Cl buffer (pH 8.5) containing 200 mM Sucrose, 30 mM $MgCl_2$, 60 mM KCl, 0.5 % PVP (w/v), 0.5 % PVPP (w/v), 0.1 % (v/v), Triton X-100 and 60 mM β-mercaptoethanol. The homogenate was then centrifuge at 5,000 x g for 15 min at 4ºC. The supernatant was extracted with phenol: chloroform (1:1) mixture. The upper clear aqueous layer was taken in a fresh oakridge tube and to this $1/10^{th}$ volume of 10 % sodium acetate (pH 5) was added, then RNA was precipitated by addition of 2.5 volumes of chilled isopropanol. It centrifuged at 16,000 x g for 10 min at 4ºC, then pellet was collected and washed with 80 % ethanol, dried and dissolved in 3 M sodium acetate (pH 6). The tubes were vortexed frequently with storage on ice for about 1 h 30 min to completely solubilize the genomic DNA. The tubes were centrifuged at 12,000 x g for 20 min at 4ºC to and the pellet was again dissolved in 1 ml of 0.15 M sodium acetate (pH 4.8) and 0.5 % SDS (w/v). Equal volume of phenol: chloroform (1:1) was added to this and centrifuged at 13,000 x g for 10 min at 4ºC. The upper clear solution was taken and precipitated with 2.5 volume of chilled ethanol, incubated at -20ºC for overnight. The tubes were centrifuged at 15,000 x g for 20 min at 4ºC then the supernatant was discarded and pellet was washed with 70 % ethanol and dried. The dried pellet was dissolved in DEPC treated autoclaved water. The amount of RNA was calculated by taking OD280 (1 OD = 40µg ml^{-1} RNA). To check the quality of RNA, denaturing agarose gel was run, stained with ethidium bromide, and visualized under UV-Transilluminator. Then for amplification of the cDNA for GuPX, a specific sense primer 5'AAA (r/e) 5'-AAGCAATGAACAGATTGTGGAGTTT-3´ and an antisense primer 5'aaa (R/E) 5'-AGATGAAATTACATAGATAAAGGTACAGCAG-3'were synthesized and applied to RT-PCR amplification with the following PCR master mix: cDNA- 2 µl, 10X buffer- 5 µl, 25mM $MgCl_2$- 7 µl, dNTP mix- 2 µl, Taq DNA polymerase- 1 µl, forward primer- 2 µl, reverse primer- 2 µl, DEPC treated water- 29 µl. The PCR cycle parameters: once at 94°C for 2 min; 30 times at 94°C for 1 min, 58°C for 1 min and 72°C for 2 min; once at 72°C for 10 min for final extension. Amplified bands were analyzed on 1.2 % agarose gels (Sambrook and Russell, 2001).

Statistical analysis

All the observations were recorded with three replications (n = 5) and data were expressed as mean ± SE. The statistical analysis was performed by one-way ANOVA using SPSS software (SPSS Inc., version 16.0), taking $P \leq 0.05$ as significant (Gomez and Gomez, 1984).

Results

From the facts and figures of cellular responses, *Salvinia* plant under elevated concentration of Al is clearly under stood that plants were invariably exposed to oxidative stress. However, the modified cellular activities in different parameters under varying Al concentration as compared to control are the resultant of plants induced or inbuilt resistance mechanism owing to hyperaccumulation of Al. *Salvinia* plants are also shown there bioaccumulation of Al significantly according to dose dependent manner thereby it established as a good hyper accumulatory species (data not shown here). With Al accumulation in excess initially plant responded a well tuned metal sequesteration following monitoring the GST activity. A consistent significant ($P \leq 0.05$) rising of GST activity was recorded under different dosages of Al exposure and those were 1.261 fold, 1.394 fold and 1.902 fold higher at 240, 360 and 480 µM of Al exposure respectively over control (Fig. 1a). On the other hand, when 1 mM Put was supplemented with highest concentration of Al, then the down regulation of GST activity was recorded by 29.19 % as compared to highest concentration of Al treatment (480 µM) (Fig. 1a).

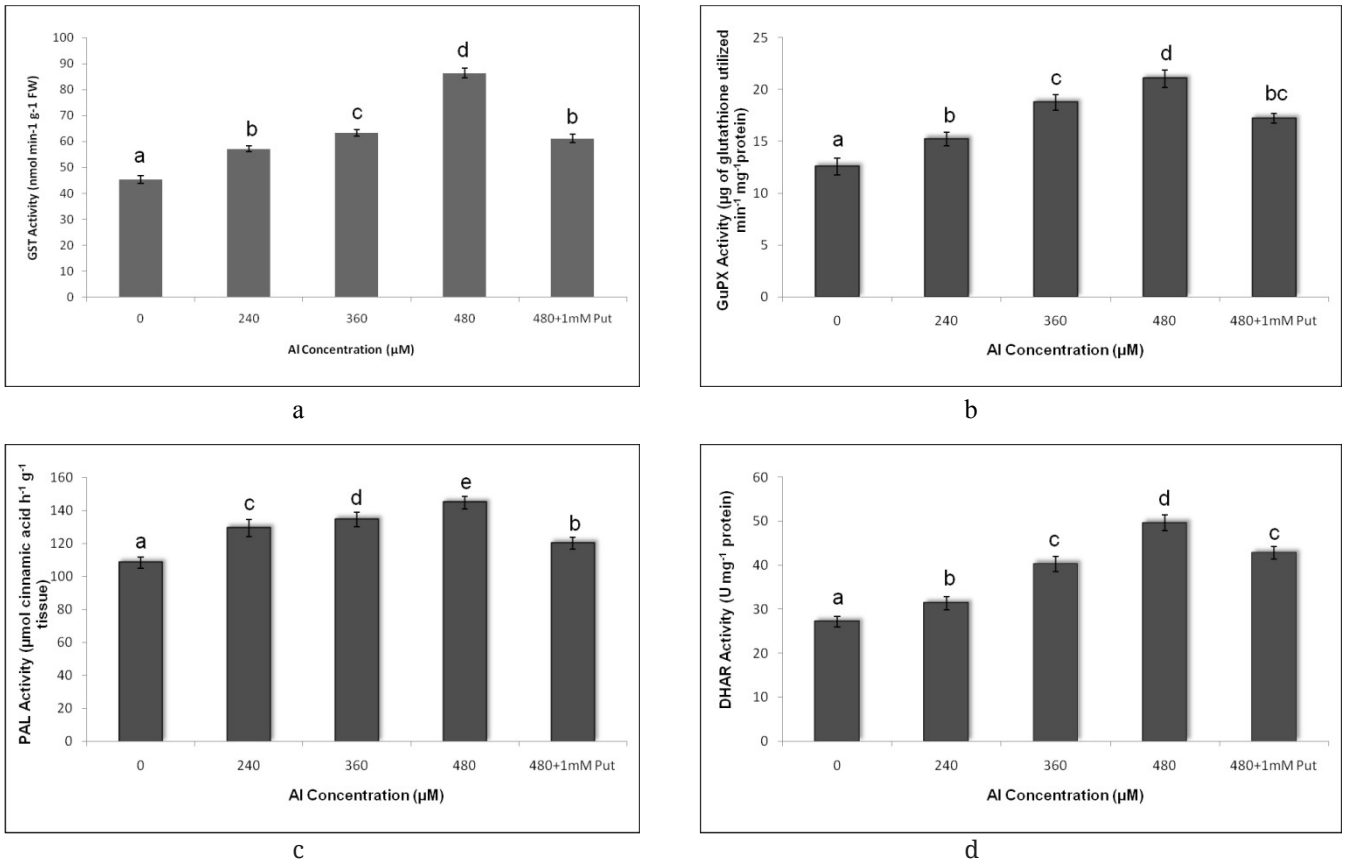


Fig. 1 GST activity (a), GuPX activity (b) PAL activity (c) and DHAR activity (d) were measured in *Salvinia* grown under varying concentration 0 (control), 240, 360, 480µM of Al and 480µM of Al supplemented with 1mM Put (480µM+1mM Put). The values are plotted from means (±SE) of replication (n=3), Bars showing different letters indicate significant differences according to Duncan's test at ($p<0.05$).

Glutathione peroxidase (GuPX) minimizes the oxidative damages by using glutathione as an electron donor. GuPX is efficient in peroxy scavenging and also can maintain the functional integration of the cell. With the increase of Al doses, the activity of GuPX increased significantly ($P \leq 0.05$) and those were recorded as 1.212 fold, 1.492 fold and 1.675 fold under 240, 360, 480 µM of Al concentration respectively as compared to control (Fig. 1b). On the other hand, while 1 mM Put was supplemented with highest concentration of Al (480 µM) and we observed that Put was reduced the activity by 18.28 % than highest concentration of Al (480 µM) (Fig. 1b). Fig. 3a showed the expression of variable polypeptides of of GuPX under different doses of Al, which has been separated on non denaturing polyacrylamide gel. In the present study, though the band numbers are same but those varied in band intensities.

In regards to phenolic derivatives and its biosynthesis which employs phenyl propanoid pathway, the rate limiting steps is attributed by PAL. It is induced in a dose dependent manner and also acts as a stress marker. Al could induce these enzymes over expression significantly ($P \leq 0.05$) by 1.194 fold, 1.241 fold and 1.336 fold higher under 240, 360 and 480 µM of Al doses respectively over control (0 µM) (Fig. 1c). With the application of Put the diminishing in activity was recorded by 16.93 % as compared to highest concentration of Al (480 µM) (Fig. 1c).

Ascorbate happens to be the most suitable antioxidant which also behaved as a substrate peroxidase. In the present experiment the status of ascorbate has also been justified with reference to Al toxicity in *Salvinia* plants and evaluated the activity of DHAR through *in-vitro* as well as in-gel studies. In an assay of DHAR we found a gradual significant ($P \leq 0.05$) increase in activity along with concentration of Al and those were recorded 1.151 fold 1.479 fold 1.82 fold at 240, 360, 480 µM of Al concentration respectively over control (Fig. 1d). Since maximum increase in activity showed with highest concentration of Al dose, therefore, 1 mM Put was supplemented with highest concentration of Al (480 µM) and we noticed that Put alleviated the activity by 13.63 % than highest concentration of Al (Fig. 1d). The enzyme sample was extracted, partially purified and run on native PAGE; different bands according to their molecular weights were resolved after the reaction with dehydroascorbate and reduced glutathione. In the present experiment, though we observed the similar number of bands for each treatment but the intensities of bands were varied significantly (Fig. 3b).

The present work is dealt to describe the oxidative stress and its concomitant effects on

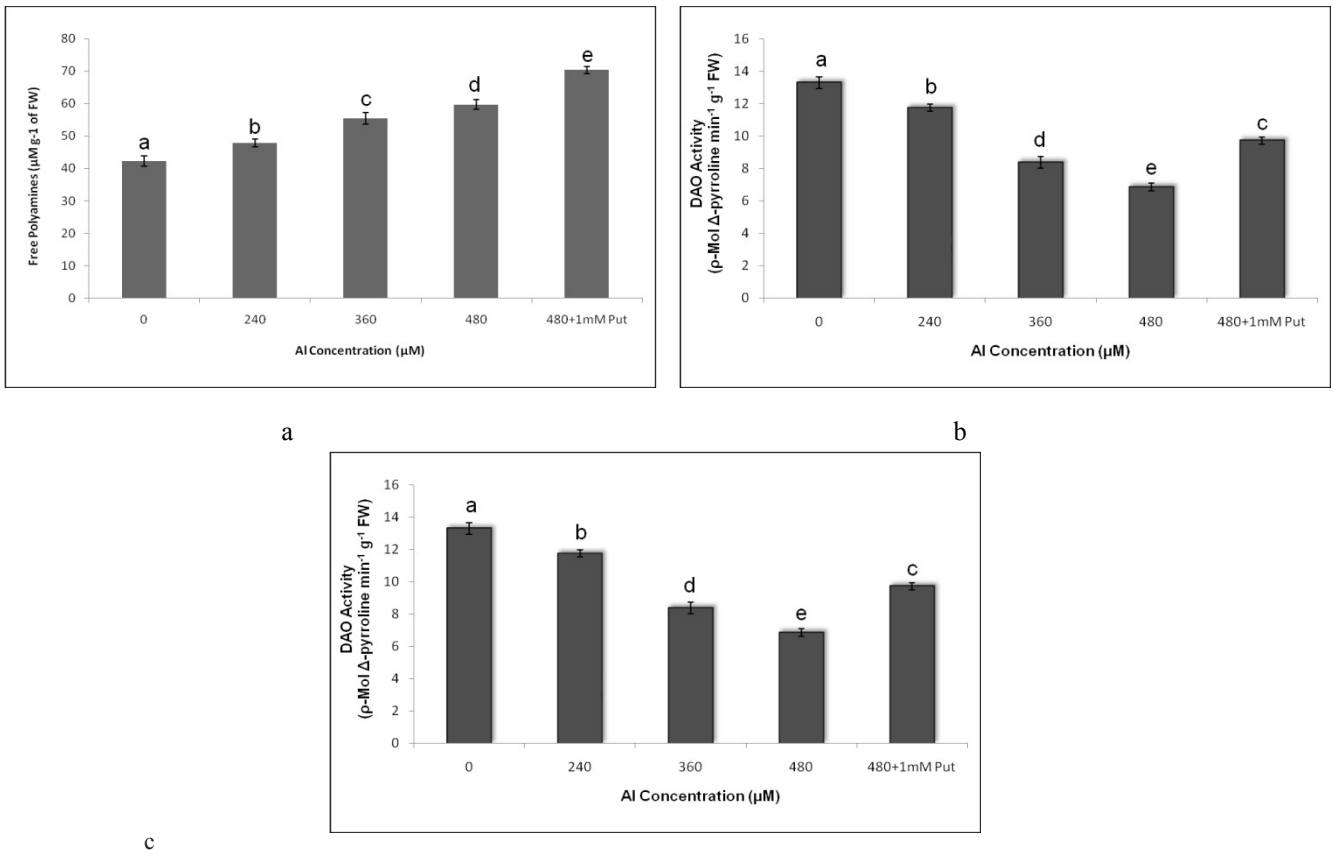


Fig. 2 Determination of free PAs (a), bound PAs (b) and assay of DAO activity (c) were measured in *Salvinia* grown under varying concentration 0 (control), 240, 360, 480µM of Al and 480µM of Al supplemented with 1mM Put (480µM+1mM Put). The values are plotted from means (±SE) of replication (n=3), Bars showing different letters indicate significant differences according to Duncan's test at ($p<0.05$).

Salvinia plant induced by Al as a toxic metal. Use of Put as PA has also been trialed to evaluate the efficiency of these elicitors to moderate the oxidative damages. In this concept the actual status of PA must be justified in plants with its all possible profile under Al contaminations and even when Put was exogenously applied. The commonly occurring PAs in plants are Put (Put), spermidine (Spd) and spermine (Spm). Those were analyzed and properly quantified with high performance liquid chromatography (HPLC). Initially, the profiles of PAs have been separated with thin layer chromatography (TLC) by running it with suitable solvent. The dansylation with PA gives a characteristic fluorescence and thus measured with reference to PA standards of Put, Spm and Spd. The TLC plate shows fluorescence spots of the PAs with distinct variation of individual spot with respect to standard PAs (Fig. 4a). Prior to this, the individual dansylated PAs were quantified through spectro-fluorimetry assay and revealed a detailed account of PAs in *Salvinia* plants. Interesting to observe that, in Put treated plant also; a significant ($P \leq 0.05$) increase of PAs was recorded. In fluorimetric assay, proper quantification was done and a linear increase of free and bound PAs content was found. However, free PAs also over expressed by 1.13 fold, 1.308 fold, 1.412 fold higher under 240, 360, 480 µM of Al doses respectively over control (Fig. 2a). Similarly, the bound PAs were over expressed by 1.227 fold, 1.404 fold, 1.617 fold higher under 240, 360, 480 µM of Al doses respectively as compared to control (Fig. 2b). As already mentioned that Put showed an inducing effect by 1.18 fold on free PA and 1.138 fold on bound PA respectively, when applied exogenously (480 µM+1 mM Put) as compared to highest concentration of Al (480 µM) (Fig. 2a, 2b). Since HPLC in general is regarded as the most sensitive way to separate and identify the individual fractions of PA, in our present experiment we have analyzed and compared the variations of PAs profile among all treatments, especially with the help of retention time, area, height, the percentage of area and height of individual peaks (Fig. 4b). This ambiguity is reflected among all PAs viz., Put, Spd and Spm. However, under exogenously applied Put treatment, more number of peaks has been observed (Fig. 4b). Therefore, we can assume that PAs could be accumulated in plant when plants were fed with Put. Rise of peaks keep parity with the obtained results by spectro-fluorimetry assay, where significant rise of free and bound PAs recorded even under exogenous Put treatment.

The biosynthesis of PA, its activity and finally its turnover depend on catabolic activities of some enzymes. The most important enzyme in this regard is amine oxidase, which oxidizes the PA into NH_3, H_2O_2 and CO_2. In the present experiment, the effect of Put was studied with reference to PA

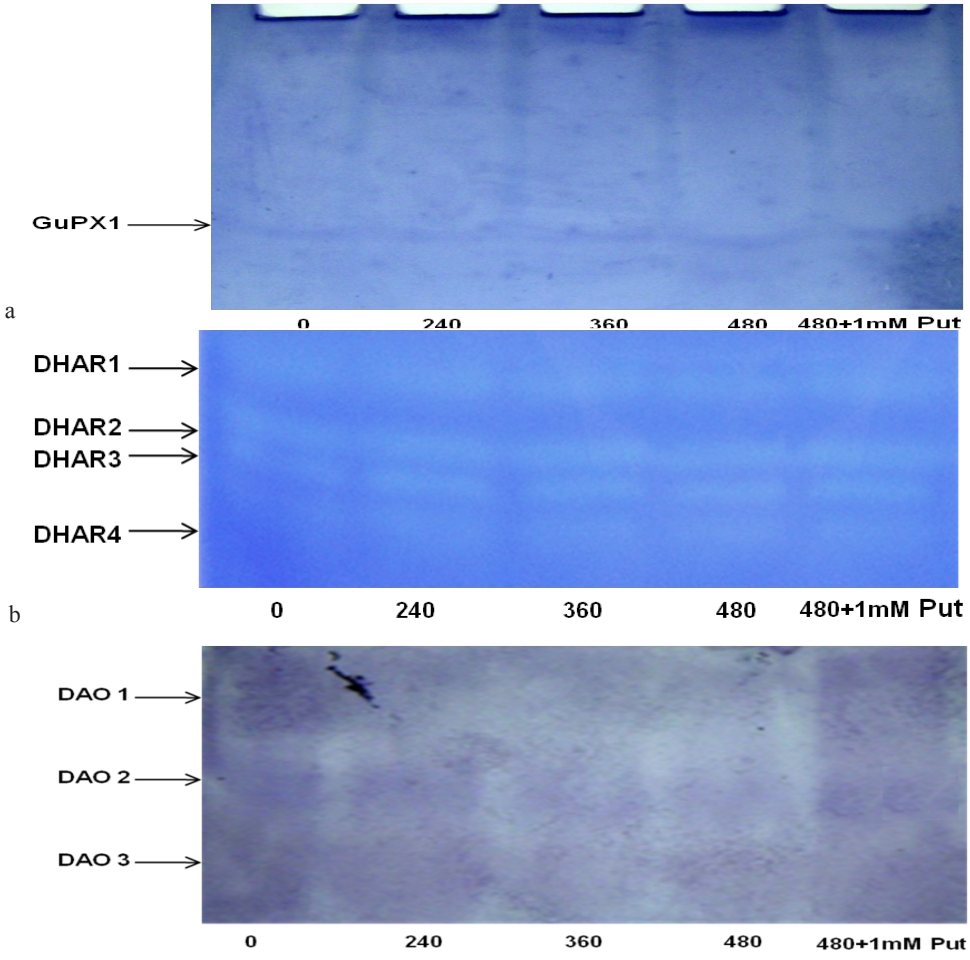
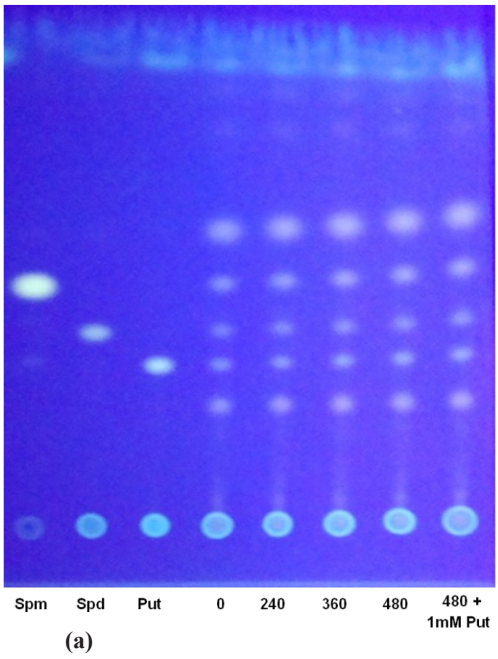


Fig. 3 Study of GuPX isoform (a) DHAR isoforms (b) and DAO isoforms (c) through activity staining from 7days of Al exposure to *Salvinia* grown under varying concentration 0 (control), 240, 360, 480µM of Al and 480µM of Al supplemented with 1mM Put (480µM+1mM Put).

catabolism by the enzyme di-amine oxidase (DAO). Both *in-vitro* and *in-vivo* assay of DAO showed significant ($P \leq 0.05$) variation among the treatments. The DAO activity was decreased by 11.59 %, 36.89 % and 48.34 % under 240, 360, 480 µM of Al exposure respectively than control (Fig. 2c). When plants were fed with Put, the activity with *in-vitro* assay was moderated and it was retrieved by 1.416 fold when calculated against 480 µM of Al (Fig. 2c). The activity and its detection *in-vivo* were done by isolation and partial purification of protein followed by separation in non-denaturing polyacrylamide gel. The isozymic proteins resolved as bands of DAO with violet colour in appearance. Interesting to note that no significant variation in band number was detected but intensities of bands were variable according to concentration gradients of Al (Fig. 3c).

Metal toxicity in plants is offered as ionic imbalances and thus the normal structural integrity of sub cellular components are deviated. Hsp are included in such a group of protein those are very conserved in nature and shows performances in a number of cellular function those are chaperon in nature. The *Salvinia* plants already have encountered some mechanical injuries out of Al accumulation as already revealed in earlier section. We have noticed the potential of plants to induce some sort of proteins which is heat shock protein in nature. The plant samples were analyzed through isolation, separation and denaturing PAGE followed by blotting on PVDF membrane. Detection of protein was done by immuno techniques using IgG-Hsp70 antibody raised against Hsp70 protein. From the analysis of the western blot there showed clear distinction between the treatments with 70 kd bands in their intensities (Fig. 5). That was detected in secondary antibody conjugated alkaline phosphatase assay system. On the other hand, application of Put had reduced the band intensities (Fig. 5). Therefore, it could be concluded that PA had been successful to moderate the expression of Hsp 70 concerned.

Already we have noticed that under condition of elevated Al doses, *Salvinia* plants undergoes oxidative stress with concomitant induction of antioxidative pathways. Among the enzymatic pathways, the peroxidase activity was found to be more induced under the same condition. Since oxidative stress is chemically characterized with an over accumulation of different free radicals mostly peroxides in nature. Therefore, lysis of those peroxides like ROS is facilitated by number of peroxidases, varying in sub cellular fractions. As already noted that peroxidase is also variable in its requirement of



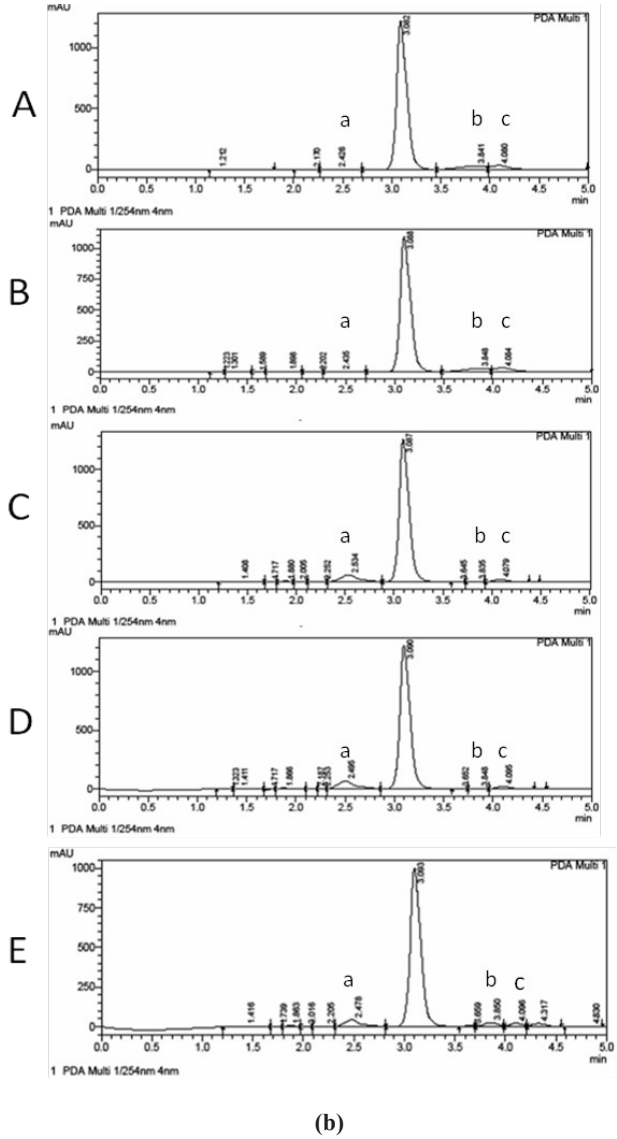


Fig. 4 Separation and identification of various fractions of PAs on thin layer chromatography (TLC) plate (a) and HPLC graph showing some differences in peak hight, area and retaion time of dansylated PAs (where a represents Put, b represents Spd and c represents Spm) (b) from 7days of Al exposure to *Salvinia* plant grown under varying concentration 0 (control), 240, 360, 480µM of Al and 480µM of Al supplemented with 1mM Put (480µM+1mM Put) (where A= 0µM (control), B= 240µM, C= 360µM, D= 480µM and E= 480µM of Al +1mM Put).

different electron donors with phenolics substances. Glutathione is another compound which is used as a source of electron, some special peroxidases and glutathione peroxidases (GuPX) is among them. As we already recorded that GuPX activity is induced under Al stress and the activity modulated by application of Put, simultaneously, we noticed some variations in band intensities through in-gel staining study. Hence for further in details study of GuPX under Al induced stress, we have tried to observed the expression level within our capability. With the view of gene expression and its associated effects on plants capacity maintaining the cellular redox we have evaluated the expression of GuPX at the transcript level. In a reaction where the total mRNA has been isolated, purified and allowed to reverse transcription reaction followed by polymerase chain reaction (PCR). The primer was designed from EST database of *Salvinia* plant (Accession No. EF620779). The product of 0.5 kb was recovered and run on agarose gel and compared with different concentration gradient of Al. It was found that the expression of cDNA was varied according to Al concentration as compared to control (Fig. 6). In addition, application of the Put down regulated such promotion in expression as compared to highest concentration of Al (480µM), however, not significantly (Fig. 6).

Discussion

From the facts and figures of the present experiment the responses of *Salvinia* plants under gradual increase in Al concentrations along with Put, it revealed that plants were essentially under oxidative stress. Initially the linear increase in activities of anti-oxidizing related enzymes like GST, GuPX and DHAR suggest that *Salvinia* plants were induced in enzymatic lysis of ROS in a co-ordinated manner following Halli-Well Asada pathway (Sharma *et al*., 2012). As such the activity of GuPX and DHAR are the efficient evidences in support of Al hyperaccumulation and plant's tolerance to oxidative stress. In fact, considering the DHAR activity for a continued supplementation of ascorbic acid from its reduced state, it is required for peroxidase reaction taking ascorbate as co-factors. A continued depletion of ascorbate in lysis of H_2O_2 by different class of

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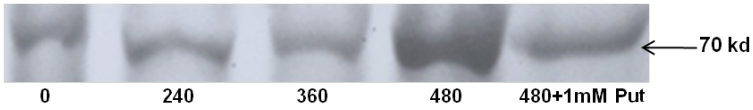

Fig. 5 Detection of the expression of induced Hsp70 (70kd) after 7 days of incubation under Al concentration in *Salvinia* [0 (control), 240, 360, 480µM of Al and 480µM of Al supplemented with 1mM Put (480µM+1mM Put)]

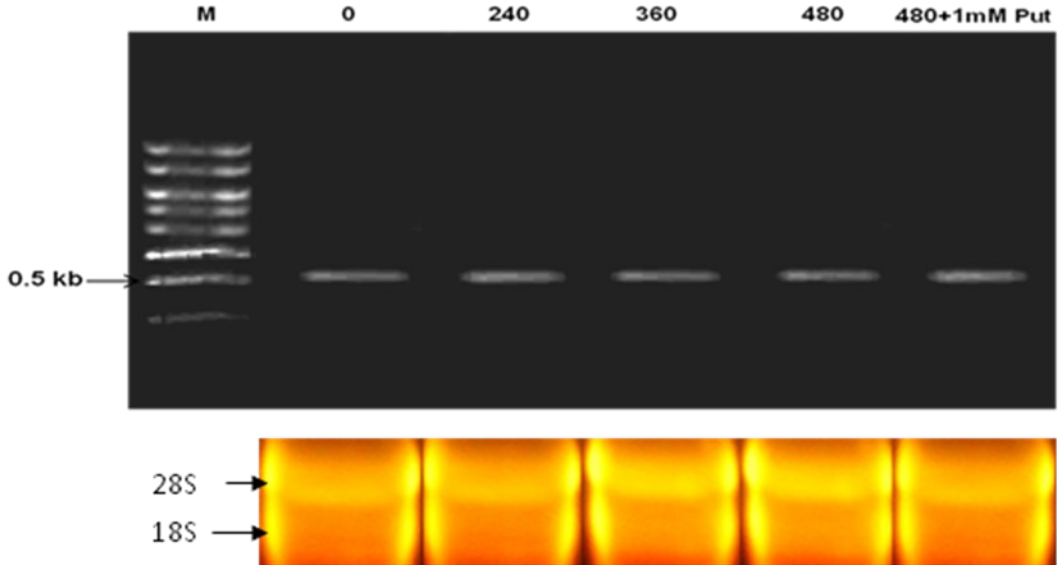

Fig. 6 Expression of *GuPX* gene by RT-PCR technique and its variation with control RNA under Al induction. A band of 0.5kb of the insert was resolved when run on 1% agarose gel along with DNA ladder (λ-DNA digested with *Hind*III) [M= Marker (λ-DNA); *Salvinia* plants grown under varying concentration 0 (control), 240, 360, 480µM of Al and 480µM of Al supplemented with 1mM Put (480µM+1mM Put) for 7days.

peroxidases has been reported in crop plants. Likewise, in *Salvinia* the enhanced activity for constant replenishment of ascorbate may also be suggestive as in compatible manner of defense mechanism in non angiospermic plant species. Moreover, the significant reduction in DHAR activity by Put may otherwise supports its antioxidant nature to balance the ROS concentration in the cell. Similar interpretation could be thought for GST, GuPX also. GST activity in metal pollution may be indexed as a common biomarker since its efficiency to form chemical legand with cations and its subsequent sequestration into cellular compartments (Vestena *et al*., 2011). It is mostly offered for heavy metals as well as some alkyl halides as osmotic perturbing agent and thereby curtails the plant water relation. For Al, particularly, in non flowering plants, the activities of GST in documentation are less observed and might be first cited in our experiment. From that point of view *Salvinia* could be granted as a reliable hyperaccumulator as well as to tolerate of metals with compartmentalization in appoplastic or non cellular spaces. Down regulation of the GST activity by Put may be meeting with two possibilities: either minimizes the Al absorption by PA or PA itself is acted as a chelating agent for the metal. Regardless of the cases, *Salvinia* are prone to regulate the GST activity, at least show evident from the present experiment. Application of exogenous PA and its concomitant mitigation of metal ion absorption were recorded earlier (Mandal *et al*., 2013). In other cases direct enzymatic lysis of ROS by classes of peroxidase enzyme which specifically use the glutathione (reduced form: GSH) as a reliable index for antioxidation pathway. GuPX is the enzyme which employs the GSH to supplement the peroxidase reaction in a variable way when other phenolics residues (as electron donors) are limiting (Hou *et al*., 2003). It is noteworthy that a single polypeptide expression might characterize the GuPX isoforms under Al toxicity in the present experiment. This was more elucidated in the expressivity of GuPX gene under Al concentrations where a significant increase was observed with RT-PCR reactions in *Salvinia*. A polypeptide and its variable isoforms when expressed in different molecular masses accomplishing the similar reaction are regarded as isozymic expression. The variable activity of DHAR in replenishing the ascorbate concentration in the tissues under elevated redox is direct evidence for antioxidation activities. In a similar way *Salvinia* plant had resolved four possible isoforms of DHAR, varying in molecular masses as a function of Al concentration in the present study. A number of possible isoforms for an antioxidizing protein may broaden the total pool of antioxidation cascade in plants under depleted redox in tissues ((Depinto *et al*., 2000). Thereby *Salvinia* plants had recorded their improved antioxidation pathways to maintain the ascorbate pool. The later is the most

predominant and indexed biomarker in plants under metal induced oxidative stress.

Plant responded well in the accumulated PA both in free and bound forms in response to Al concentrations. The activity of PA in plants under metal stress has been reported in varying manners in relation to both direct metal acquisition as well as metal induced changes of water deficit or osmotic stress in tissues. The synthesis of free PA and its simultaneous conjugation with biomolecules in the plant's cellular protection for metal induced injuries are the efficient role played by PA under oxidative stress (Fariduddin *et al*., 2013). A predominant proportion of bound form is attributed to legand or conjugant as a metal-PA complex or PA conjugant with organic moieties. These together constitute the mechanism for avoidance of metal toxicity at the cellular level attributed by PA (Alcazar *et al.,* 2010). However, plants show over accumulation of total PA pool and more shifted towards bound form under abiotic stress. *Salvinia* plant in the present experiment had not recorded much variation in bound or free forms and thereby indicated an increase of total PA along with Al concentrations. Still, possibility might be arisen that under prolong duration of Al concentration plants could allocate more bound form of PA to engage in metal sequestering. Moreover, on account of PA catabolism, the oxidative degradation is equally important with context of metal induced responses in plants. However, it recorded the plants deterioration in many cellular events when PA concentration is attended in excess in tissues. This is due to activities of some PA catabolizing enzyme to release decarboxilated PA product along with H_2O_2 as byproduct (Alcazar *et al*., 2010). The H_2O_2, however, is not a free radical but behaves as a typical ROS, often accelerates the oxidative damages induced by abiotic factors. This interpretation is much strengthens in crop plant where found a significant loss of protein and lipids moieties with over expression of diamine oxidase (DAO) and polyamine oxidase (PAO) under salinity (Lin and Kao, 2001). Our studies with *Salvinia* are also in agreement with the earlier findings where DAO activity becomes crucial for PA catabolism. Thus, the down regulation of DAO activity in *Salvinia* plants might be due to loss of enzyme function with metals like Al exposure. The fall in DAO activity with Put application could be hypothesized a significant in the plants as it reduces excess H_2O_2 accumulation out of PA catabolism. It is quite obvious that expression of gene and its intensities are dependent on duration, quantity and quality of stressors in plants. Thus, another marked gene expression signifying the establishment of stress at cellular level is the elucidation of heat shock protein (Hsp). In earlier literature different classes of Hsp are marked as cellular indices to perceive the metal stress in crop plants (Rodziewicz *et al*., 2014). Interestingly, *Salvinia* being an aquatic pteridophyte is a no exception when it recorded a distinct variation of heat shock protein after 7 days of incubation under gradient of Al concentrations. Metal induced injuries to the sub cellular fraction and its possible shielding are accomplished by appearance of Hsp in a number of variants. Maintaining cellular integrity, proper functioning of other protein with proper folding, addition / deletion of specific functional group are all belonged to possible resultants of Hsp expression (Tukaj *et al*., 2011). The gradual increase in Hsp under varying concentrations of Al and its down regulation with Put may established the bio-indication of Al toxicity in *Salvinia* plant. Moreover, the down regulation of expression of Hsp with Put treatment may also be suggestive for PA mediated cellular protection as alternative so far the plants had experienced.

Therefore, from the results and its critical analysis of the present investigation it finds that the *Salvinia* plant is prone to metal (Al) induced oxidative stress. The cellular responses those mostly attributed antioxidation pathways were well in responses and co-ordinated with Put application also. The aquatic plants mostly the macrophytes have long been exercised as an efficient bioindicators for evaluation of environmental pollution conjugated with genotoxicity (Wolff *et al*., 2012; Li *et al*., 2014). The changes of cellular responses with gradient of Al concentration undoubtedly established the reflection of plants ability to response metal stress so far the *Salvinia* in concern. More so, the expression of the genes mostly those attributed in maintaining of cellular redox are good support in their physiological alteration to such condition. Therefore, under Al toxicity *Salvinia* plant has evident as a successful quencher as well as a possible bioindicator with a number of cellular responses. Still, more fundamental studies are required particularly, at the molecular level to trace the regulatory mechanism for Al tolerance of *Salvinia* plants. However, this study may warrant the preliminary bio-indication of Al toxicity for waste water monitoring to exercise the species of *Salvinia*.

Conclusion

From the present study it is clearly revealed the *Salvinia* plants are well respondent in antioxidative stress exposure induced by Al. The most prevalent achievements as derived from the experiment is the degree of accumulation of ROS following its enzymatic lyses had opted special pattern in this fern species following its enzymatic cascade. The putrescine could down regulate the enzyme activity possibly having genotypic plasticity. On the other hand it is also well established the plants were turned with chemical signaling from polyamine biosynthetic pools inbuilt as well as induced exogenously to corroborate antioxidation under Al as metal stress.

Competing Interests

The authors declare that they have no competing interests.

Author' Contribution

Chiranjib Mandal and Subhankar Bera: Performed the experiments. Malay Kumar Adak: Designed experiment.

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