Physiol Mol Biol Plants (January–March 2013) 19(1):91–103
DOI 10.1007/s12298-012-0144-4

RESEARCH ARTICLE

Antioxidative responses of Salvinia (*Salvinia natans* Linn.) to aluminium stress and it's modulation by polyamine

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Published online: 1 November 2012

Abstract Antioxidative stress response of free-floating aquatic fern (*Salvinia natans* Linn.) was studied under increasing toxic amount of aluminium (Al) and its modulation by exogenous application of polymaine. Increased levels of superoxide (O_2^-) and hydrogen peroxide (H_2O_2) species from affected tissues suggested that plants were undergoing oxidative stress and it was concominant with increased accumulation of Al in a dose dependent manner. Application of polyamine like putrescine (Put) led to a decrease in oxidative stress as revealed by reduced level of O_2^- and H_2O_2. Al toxicity resulted into decreased biomass that was ameliorated by the application of Put. The changes observed in lipid peroxidation (MDA) and protein oxidation also indicated that plats are undergoing Al induced oxidative stress. In order to circumvent the oxidative stress resulting from Al toxicity, plants enzymatic and nonenzymatic antioxidant pathways were active. The ratio of both oxidized and reduced cellular glutathione exhibited significant variation in response to Al stress and was improved upon Put treatment. Peroxidase and glutathione were upregulated whereas catalse was downregulated under varying doses of Al. Isozyme profile of above enzymes also showed a trend with increasing amount of Al. The nuclear disintegration study using comet assay was indicative of Al induced oxidative stress. In the present study, we have explored the antioxidative response of aquatic fern *Salvinia natans* Linn in response to Al toxicity. The application of polyamine Put improved the overall antioxidative response and thus would make it a better candidate to be used as hyper accumulator of Al and other toxic metals.

Keywords Aluminium toxicity · Reactive oxygen species · Polyamine · Antioxidative enzymes · Comet assay · *Salvinia* sp.

Abbreviations

H_2O_2	Hydrogen peroxide
Put	Putrescine
MDA	Malondealdehide
ROS	Reactive oxygen species
PCD	Programmed cell death
SOD	Superoxide dismutase
POD	Peroxidase
O_2^-	Superoxide
OH^-	Hydroxyl ion
$KAl(SO_4)_2$	12 H_2O, Potassium aluminium sulphate
IMZ	Imidazole
NaN_3	Sodium azide
TCA	Tricholoroacetic acid
KI	Potassium iodide
PVP	Polyvenyl pyroliddone
EDTA	Ethylene diamine tetra acetic acid
DTT	Dithiotreitol
NAD(P)H	Nicotinamide adenine dinucleotide phosphate (reduced form)
DNPH	Dinitrophenyl hydragene
DTNB	5, 5′-dithio-bis (2 nitrobenzoic acid)
GSH	Glutathione
GSSG	Oxidized glutathione
GPX	Guaiacol peroxidase
APX	Ascorbate peroxidase
CAT	Catalase
GR	Glutathione reductase
BSA	Bovine serum albumin

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PMSF	Phenyl methane sulfonyl fluoride
DCPIP	Dichlorophenol indophenol
MTT	3-(4,5-dimethyl -2 thiazolil)-2,5 diphenyl-H tetrazolium bromine
PBS	Phosphate saline buffer
LMP	Low melting agarose
EtBr	Ethydium broimide
PAs	Polyamines

Introduction

Aluminium (Al) is one of the metallic toxic pollutants that essentially subdued the growth and development of the vegetation predominantly in acidic soil. Al and its abundance constituents are around 8 % of the total earth crust (Achary et al. 2007). In an average, Al toxicity covers around 40 % of world's cultivated land (Hede et al. 2001). Potent trivalent cationic form of Al (i.e. Al^{+3}) acts as a phytotoxic elements in the land after being solubilized in soil solution, particularly, in acidic condition ($pH \leq 4.5$). Thus, soil acidity and Al-toxicity becomes complementary for impeded growth of plants (Zheng and Yang 2005).

In general, plants grown on lands with subsoil acidity suffer from inadequate extraction of nutrients, under this circumstance; Al toxicity becomes more aggravated with the reduced and improper realization of growth due to inhibition of cell division, cell proliferation and cell elongation. Therefore, Al toxicity in the plants, preliminary, has been evident to be ideally sensitive for severe damage in the root tissues. In addition, plants display a number of allied anomalies in root functioning under Al^{+3} toxicity, like swelling of root apex, distal transition, formation of barrel shaped cells at the root epidermis etc. (Giannakoula et al. 2010). Though a wide range of cellular processes are perturbed due to Al-toxicity, at the cellular level the key mechanism for tissue disintegration is based on changes of redox status of the tissue. Many reports revealed that a burst of several reactive oxygen species (ROS) leads to imbalance of cellular redox and plants are to suffer from oxidative stress (Roy Choudhury and Pradhan 2011).

Plants cell membrane are most vulnerable for Al induced oxidative damage and in many cases significant amount of absorbed Al are concentrated in the apoplastic parts of the cell wall and other sub cellular organelle (Tamas et al. 2004). Distribution pattern are also variable among different plant species. Regardless of these, Al has been evident in inducing the ROS that predominantly includes the oxygen at its various levels of oxidation and energy states (Liu et al. 2008). The tolerance to Al induced oxidative stress in plants is implicated by the mechanisms of either down regulation of generation of ROS and/or quenching the excess energy of it (Ali et al. 2008). Some enzymes cascade, sequential in activities for cellular pathways, accomplishes the anti oxidation processes. Predominantly these are antioxidative enzymatic classes and most importantly are super oxide dismutase (SOD) and some peroxidase (POD). The SOD can alter superoxide (O_2^-) into hydrogen peroxide (H_2O_2) and molecular oxygen (O_2). POD with their various electron donors (different phenolic compounds) are widely distributed in different isozymic forms those may contribute to scavenge the oxidation potential of H_2O_2 (Gill and Tuteja 2010). Since, Al toxicity is pronounced under low pH, dismutation of super oxide (O_2^-) becomes inevitable and reduction of Al^{+3} to Al^{+2} is favored. Al^{+2}, not as a typical transition metal, however, may be assigned to reduce H_2O_2 by one electron transfer and to generate another potent ROS, viz. hydroxyl ion (OH^-). Significantly, OH^- is more efficient in peroxidation of lipids and cellular oxidation (Munné-Bosch et al. 2001). Admittedly, the potential and degrees for detoxification of ROS is highly variable in different plant species. Thus, a tolerant species is characterized with extended amicability of anti oxidation system inbuilt and/or up regulation of antioxidation mechanism under Al or other metal toxicity. Many authors have also reported for diminishing the Al sensitivity in plants by sequestering the metals into vacuoles or avoidance of it by chelating the metals with rhizospheric exudates (Dipierro et al. 2005).

Thus, variations in expression for antioxidation under toxic metal stress (Al being one of those) are often modulated by various effectors. In plant system, polyamines (PAs) are a class of straight chain aliphatic compounds with different number of amino groups (di, tri and tetra), polycationic in nature are ubiquitously involved in growth and developmental processes (Alcazar et al. 2010). Besides this, within physiological pH, polyamines owing to its polycationic nature and alkane backbone are readily offered in binding with negatively charged domains of cellular macromolecules like membrane lipids, nucleic acid, carboxy terminus of poly peptides, de-protonated phenolics, esters and others (Liu et al. 2008). Occurrence of polyamine profile and its levels in tissue is also offered as an integral protective or adaptive mechanism under diverse types of stress like salinity, water deficit, metal contamination, flooding, high temperature, chilling, high illumination, oxidative environment etc. (Roy Choudhury and Pradhan 2011). Thus, an overall protection of the cellular environment from over oxidation is furnished in a cascade of events by membrane stability, scavenging of ROS, generation of compatible solutes, maintaining steady state ion homeostasis and expression of some genes for antioxidation mechanisms. Irrespective of these, polyamines and its functioning have been evident in coordination with signal transduction and its concomitant effects at physiological effects in plant systems under stressful conditions (Gill and Tuteja 2010).

Now, it has been a practice to utilize the hyper accumulation potential of different species of angiosperm for toxic metals, Al being one of those (Boscolo et al. 2003). This becomes more dexterous with the use of hydrophytes in the contaminated water bodies and those were proven to be efficient species for phyto-remidiation strategies. However, non-angiospermic species are less explored for these purposes. Some pteridophytic species like chinese bake fern (different species of Pteris) have been evident as successful candidates, particularly for heavy metals like arsenic, cadmium etc. (Kramer 2010). *Salvinia* (*Salvinia natans* Linn.), an aquatic free floating ferns are abundant in growth in water bodies especially in marshy waste lands often contaminated with industrial effluents (Dhir et al. 2009). Therefore, *Salvinia* could be an option as a plant material for hyper accumulation of toxic metals. In order to understand the bio-chemical basis and cellular adjustment for metal tolerance, it becomes imperative to assess the extent of anti-oxidation responses due to Al induced oxidative stress. Moreover, it would also be a logical approach to monitor the modulation of anti oxidation pathways under the induction of polyamines, specially in non-angiospermic plants (like *Salvinia* in the present case), which is less explored. Thus, we summarize to propose an investigation with *Salvinia natans* (Linn.) to elucidate the cellular response of Al hyper-accumulation and to clarify the possible role of polyamine in modulation of antioxidation pathways.

Materials and methods

Plant material

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

Culture of the plant Plants were collected from marshy land, washed with running water to remove adhering salts/debris and kept in de-ionized water to leach out absorbed salts, if any. After completion, plants were transferred into culture medium (1/4 × Murashige and Skoog medium) for 7 days for acclimatization (Murashige and Skoog 1962). Thereafter, plants were treated with Al as potassium aluminium sulphate [$KAl(SO_4)_2$, $12H_2O$] 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 Put adjusted to pH 4.5 (as mentioned by Giannakoula et al. 2010; Parker et al. 1995). All the sets were kept in a glass house under the ambient condition: 37±1 °C of temperature, 70–80 % of relative humidity and photoperiod of 14–10 h light and dark for 7 days. The experiment was replicated thrice for each concentration of Al treatment. On completion of treatments, plants were harvested, frozen in liquid nitrogen and immediately stored in -70 °C for further biochemical analysis. For the analysis of growth, the intact plants were recovered from Al doses, washed thoroughly with deionized water and made completely dry under 80 °C for 5 days for constant weights. On completion, the dried plants were recorded weights and computed relative growth rate (RGR) following Jampeetong and Brix (2009).

Determination of Al content Samples were dried completely into ash at 550 °C and treated with tri acid mixture (HCl: HNO_3 :H_2SO_4=1:1:1) followed by filtration into a clear solution. The Al content was determined according to Giannakoula et al. (2010) using an atomic absorption spectrometer (ICS-AES).

Determination of superoxide (O_2^-), Hydrogen peroxide (H_2O_2) content and inhibitor study From the control and treated plants, the extra cellular generation of $O_2^-$and H_2O_2 content were assayed. For the former, 1 g of tissue was thoroughly homogenized in liquid nitrogen followed by addition of 100 mM Tris-Cl buffer pH 6.5. Homogenate was centrifuged at 6,000 x g for 30 min under cold condition (4 °C). The supernatant was added in a 3 ml of reaction mixture containing 50 mM Tris-Cl (pH 6.5), 0.2 mM nitro blue tetrazolium (NBT, Hi Media), 0.1 mM NADH and 250 mM sucrose and incubated at room temperature for 24 h in dark. The absorbance was read at 530 nm. The concentration of O_2^- was derived using the extinction coefficient of 12.8 $mM^{-1}cm^{-1}$ (Achary et al. 2007). To assess the inhibition of O_2^- by NAD(P)H oxidase, plants were treated with 1 mM imidazole (IMZ) and 1 mM sodium azide (NaN_3) in the highest concentration of Al (i.e., 480 μM). Then assay of O_2^- was done as described by Achary et al. (2007).

For detection of H_2O_2, concentration of the tissue was done according to Ghosh et al. (2011). Fresh leaf tissue of 0.5 g was thoroughly homogenized in 3 ml of 1 % (w/v) trichloro acetic acid (TCA). The homogenate was centrifuged at 10,000× g, at 4 °C for 15 min. In a reaction buffer of 0.5 ml containing 10 mM potassium phosphate buffer (pH 7) and 1.0 M potassium iodide (KI), the supernatant was added in appropriate volume followed by incubation in dark for 15 min. The absorbance was read at 390 nm. The content of H_2O_2 was determined using standard solution. H_2O_2 concentration was expressed as μM g^{-1} of fresh weight. Similar inhibition studies were used for H_2O_2 using 1 mM imidazole (IMZ) and 1 mM sodium azide (NaN_3)

In vitro assay of NAD(P)H-oxidase 1.0 g of samples were grounded into powder by liquid nitrogen and homogenized

with 2.0 ml of potassium phosphate buffer (50 mM, pH 7) containing 0.2 mM EDTA, 1.0 % (w/v) polyvinyl pyrrolidone (PVP), 0.1 % Triton, 0.1 % DTT, 1 % Leupeptine, 0.1 % BSA (bovine serum albumin) and 5 mM Ascorbate. The homogenate was centrifuged at 10,000 x g at 4 °C for 20 min. The supernatant was concentrated in lyophilizer (Heto FD 1.0) and used as enzyme source. NAD(P)H-oxidase (E.C 1.11.1.1) activity was determined as suggested by Ishida et al. (1987). The reaction was carried out in an assay mixture containing 0.05 mM NAD(P)H dissolved in 30 mM sodium-acetate buffer (pH 6.5), 5 mM manganese chloride, 0.1 mM DTT, 0.5 mM p-coumaric acid. In addition, aliquot of 50 μg of enzyme protein was added to start the reaction and decrease in O.D. at 340 nm was recorded. Using the extinction coefficient of NAD(P)H (6.22 $mM^{-1} cm^{-1}$). 1 unit (U) of activity was calculated as NAD(P)H oxidized per minute by gram of fresh sample.

Determination of lipid peroxidation and protein oxidation

1.0 g of samples from control and stress plant were extracted with extraction buffer containing 20 % (w/v) trichloroacetic acid (TCA) and 0.5 % thiobarbituric acid (TBA) followed by warming at 95 °C for 30 min. Reaction mixture was placed in ice or -20 °C for 30 min followed by centrifugation at 12,500 x g for 10 min. The absorbance of the supernatant was read at 532 nm and 600 nm and MDA content was derived according to Dhindsa et al. (1981).

For the protein oxidation in a reaction with dinitrophenyl hydragene (DNPH), the carbonyl content of 1.0 g plant sample was done. Protein oxidation assay was conducted by dinitrophenyl hydragene (DNPH) reagent according to Verbeke et al. (2000). 1.0 g sample was thoroughly crushed in 6 % (w/v) sodium lauryl sulphate (SDS) followed by incubation at 37 °C for 30 min. To this 10 mM DNPH in 1.5 mM TCA was added and further gently agitated at every 10 min up to an hour. The protein was precipitated by TCA (w/v) on ice and protein pellet was recovered by centrifugation at 15,000× g. Followed by re-extracted with 20 % (w/v) TCA. The pellet was suspended completely in 0.2 M sodium phosphate buffer at pH 7.0 and the absorbance was read at 360 nm. Finally, the protein-carbonyl complex content was calculated using the molar extinction coefficient (530 $M^{-1} cm^{-1}$) of DNPH and expressed in μM DNPH mg^{-1} protein. The protein content of the sample was determined with Bradford reagent (Bradford MM 1976).

Detection of DNA damage by comet assay

The plants were examined for any DNA damage under Al treatment by Comet assay according to Achary et al. (2007). The samples were chopped into very fine slices and from those nuclei suspension was collected in phosphate saline buffer (PBS, pH 7.4) under cold condition. A clear grease free slide, pre coated with 1 % normal agarose was taken. On the slide, the mixture of nuclear suspension with 0.60 % low melting agarose (LMP, Sigma, USA) was layered, followed by another layer of 0.60 % low melting agarose and put a cover slip on top. The whole set was kept on ice for 5 min and then cover slip was removed. The slide was immersed in a cold electrophoresis buffer (10 N NaOH and 200 mM EDTA, pH≥12) for 20 min to allow the DNA to unwind and run for 30 min at 25 V, 300 mA. The slides were then put in a solution to neutralize with 0.4 M Tris-EDTA (pH 8.0) followed by methanol for 15 min. The slides were then stained with 0.1 % ethidium bromide (EtBr) for 5 min and excess stain was washed with cold distilled water. The slides were then observed under fluorescence microscope with an excitation filter of BP 546/10 nm and a barrier filter of 590 nm. By subtracting the diameter of heads of comet, tail lengths were measured.

Estimation of glutathione content

The content of glutathione was assayed according to Paradiso et al. (2008). The plant sample was homogenized in 6 % (w/v) meta-phosphoric acid containing 1 mM EDTA. Supernatant was collected by centrifugation of the homogenates at 11,500× g for 15 min under cold condition. Moreover, aliquots of 0.4 ml supernatant was added with 0.5 M potassium phosphate buffer (pH 7.5). This was incubated in a assay mixture containing 10 mM BSA, 10 mM 5,5′-dithio-bis (2 nitrobenzoic acid) (DTNB), 0.5 mM NADH at 37 °C for 15 min. It was then cooled and absorbance was read at 412 nm. GSSG was assayed in another aliquote of the same assay mixture but treated with 2-vinyl pyrolidine to remove the GSH and reacted the same way as suggested by Yu et al. (2003). Synthetic reduced (GSH) and oxidized glutathione (GSSG) were used to prepare the standard.

Assay of antioxidative enzyme activity in vitro and in-gel To study the enzymatic antioxidative pathway of *Salvinia* plants under control and Al treatment, enzymes viz. guaiacol peroxidase (GPX), ascorbate peroxidase (APX), catalase (CAT), glutathione reductase (GR) and super oxide dismutase (SOD) were studied following appropriate protocol for extraction, purification and assay reaction according to Ghosh et al. (2011). For each type of enzyme 1.0 g of tissue was taken and crushed in liquid nitrogen followed by homogenization with extraction buffer (3.0 ml) containing 1.0 mM Tris–HCl (pH 7.7), 10 mM $MgCl_2$, 1.0 mM DTT, 0.1 mM PMSF, 0.1 mM EDTA, 0.1 mM leupeptin, 0.1 mM BSA and 2 % PVP under cold condition. The homogenate was centrifuged at 15,000× g, at 4 °C for 15 min. The supernatant containing the crude enzyme was precipitated

by 80 % ammonium sulphate followed by purification in dialysis bag with suitable buffer (10 mM KCl, 0.1 mM EDTA, 1 mM PMSF, 1 mM β-ME) under 4 °C for 6–8 h. The protein was concentrated under vacuum and dissolved in 0.5 ml of the dialysis buffer. The purified protein was used for assay of GPX, CAT and GR.

The activity of GPX (EC 1.11.1.7) was assayed spectrophotometrically using *O*-dianisidine as electron donor and hydrogen peroxide (H_2O_2) as substrate according to (Hu et al. 2009). In a reaction mixture containing (0.1 M phosphate buffer, pH 6.5; 1.5 mM *O*-dianisidine; 0.2 M H_2O_2; and equivalent amount of enzyme extract containing 50 μg of protein). The change of absorbance was monitored at 430 nm. The enzyme activity was determined according to Ghosh et al. (2011) using an extinction coefficient of *O*-dianisidine (26.2 $mM^{-1}cm^{-1}$). One unit (U) of enzyme is defined, as the time required for changing the absorbance by 0.1 per unit gram of protein. For in gel studies of isozymes of GPX, the protein was run in a non-denaturing 10 % polyacrylamide gel at 10 V/lane under cold condition (Ammar et al. 2008). The detection of specific band of polypeptide was resolved in an incubation mixture (50 mM Potassium phosphate buffer, 0.5 mM *O*-dianisidine and 0.5 % H_2O_2).

For isolation of APX (EC 1.11.1.11), 1.0 g of tissue was homogenized in 100 mM Tris-Cl (pH 7.8) buffer containing 10 mM $MgCl_2$, 1 mM PMSF, 100 mM EDTA, 10 mM DTT and 2 % PVP and centrifuged at 17,000× g for 25 min at 4 °C. For in vitro assay of APX, the reaction mixture containing 100 mM phosphate buffer (pH 7.5), 0.5 mM ascorbate and 0.2 mM H_2O_2, then equivalent amount of protein from enzyme source was added and absorbance was recorded at 290 nm (Davletova et al. 2005). For in gel staining of APX, 50 μg protein was loaded in a native PAGE which was pre run in a 2 mM ascorbate for 30 min at 4 °C and gel was run for 3–4 h at 4 °C. Thereafter, the gel was equilibrated with 50 mM sodium phosphate buffer (pH 7.0) containing 2 mM ascorbate and changed in a buffer of 4 mM ascorbate and 2 mM H_2O_2 for 20 min. Finally, the gel was stained in a solution containing 50 mM sodium phosphate buffer and 2.45 mM NBT. The reaction was stopped by adding 10 mM TEMED in the same solution.

For assay of CAT (E.C. 1.11.1.6), 50 μg protein was incubated in a reaction mixture containing 0.5 mM potassium phosphate buffer (pH 7) and 10 mM H_2O_2. The activity was determined by reading the decreasing absorbance at 240 nm and activity was detected using the extinction coefficient of H_2O_2 as suggested by Aebi (1983). For isoenzymic studies, 50 μg of protein was loaded in a non-denaturing 10 % polyacrylamide gel at 10 V/lane under cold condition. Then, incubated the gel in 0.05 % H_2O_2 and the bands were developed in a staining solution (1 % (w/v) potassium ferricyanide and 1 % (w/v) ferric chloride sequentially) for 5 min and fixed with 1 % hydrochloric acid.

Glutathione reductase (GR) (EC 1.6 .4 .2) was assayed in a reaction mixture containing 100 mM HEPES KOH pH 7.5, 0.5 mM DTT, 10 mM magnesium chloride ($MgCl_2$), 0.5 mM methionine, 0.2 mM NADH, 0.5 mM oxidized glutathione (GSSG). The mixture was incubated at 37 °C for 15 min and then immediately added with 50 μg of enzyme protein. The activity was recorded from oxidation of NAD(P)H with its extinction coefficient 6.22 $mM^{-1}cm^{-1}$. One unit of enzyme was regarded as μM NADPH oxidized min^{-1} μg $protein^{-1}$ under assay condition. The isoforms of GR was detected by electrophoresis of protein (50 μg $lane^{-1}$) in a non-denaturing polyacrylamide (10 %) native gel at 4 °C under 60 and 100 V in stacking and resolving gel respectively. The bands were developed on the gel in an assay mixture containing 0.5 mM NAD(P)H, 3.4 mM GSSG, 0.5 mM EDTA, 25 mM Tris-Cl (pH 7.6), dichlorophenol indophenol (DCPIP), 3-(4,5-dimethyl-2 thiazolyl 2,5 diphenyl-H tetrazolium bromine (MTT), under dark condition for 10 min with repeated washing by de-ionized water.

For the extraction of SOD (EC 1.15.1.1), fresh sample was homogenized in ice cold 50 mM phosphate buffer (pH 7.0) followed by centrifugation at 15,000 x g for 30 min at 4 °C (Nakano and Asada 1981). An aliquot of supernatant equivalent to 50 μg protein was incubated in an assay mixture containing 50 mM sodium phosphate buffer pH 7.0, 15 mM methionine, 75 μM NBT and 100 mM EDTA and kept under fluorescent light for 10 min followed by reading the absorbance at 560 nm. For in gel staining of isozymes, 100 μg of protein was loaded in a 10 % native PAGE which was incubated in two successive buffers: 50 mM sodium phosphate (pH 7.5) with 2.45 mM NBT, then transferred to 50 mM sodium phosphate (pH 7.5) buffer with 26.5 mM TEMED and 26.5 μM NBT in dark for 40 min, after that the gel was exposed to fluorescent light for the development of bands.

Statistical analysis

All the observations were recorded with three replications (n=3) and data were expressed as mean ± SE. The statistical analysis was performed by one-way ANOVA analysis, taking $P \leq 0.05$ and $P \leq 0.01$ as significant

Results

Al accumulation in plants and it interacts with Put

In the present study, the accumulation of Al by the *Salvinia* plant is primarily noticed by phenotypic appearances on their subdued growth of plants under Al treatments: morphologically plants recorded a diminished stature under different Al doses. A gradual decline in the growth rate with

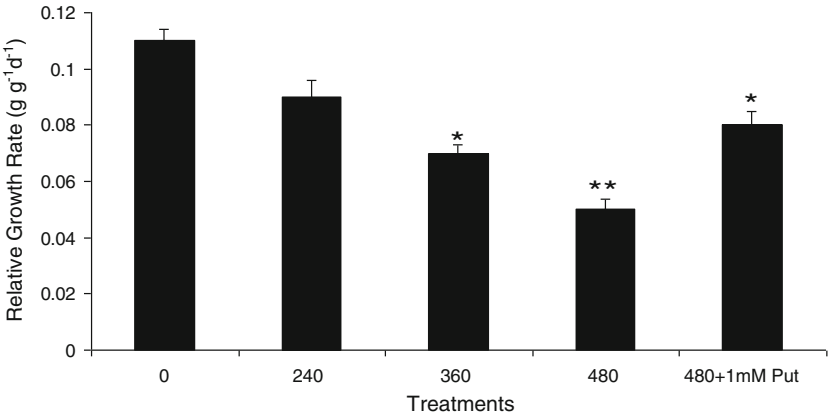

Fig. 1 Effects of Al concentration 0, 240, 360, 480 μM of Al salt and 480 μM of Al salt with 1 mM Putrescine (480 μM+1 mM Put) on growth of *Salvinia* plants. The values are plotted from means (±SE;) of replication (n=3), (*P≤0.05 and **P≤0.01)

the induction of Al exposure was recorded. The total biomass of the plants so affected were recorded in terms of Relative Growth Rates (RGR) and compared to control along with Put. A significant (P≤0.01) variation of the plants in terms of RGR under Al were recorded and it ranges from 0.11 gg-^{1}d^{-1} to 0.05 gg-^{1}d^{-1}. However, Put was evident to retrieve the growth rate by 1.6 fold when compared to that under highest concentration of Al (480 μM) (Fig. 1)

The loss of tissue weight is correlated with Al accumulation (r^2=0.85). In context to growth, there recorded a linear relationship of Al accumulation in plants grown under varying concentrations of supplied Al salt (Fig. 2). The maximum accumulation of Al was recorded in 480 μM and it was 4.56 fold over control. Whereas, Put had minimized the absorption by significant (P≤0.01 or 0.05) amount and it was 38.24 % less as compared to highest concentration of Al (480 μM) (Fig. 2).

Generation of ROS and effects of inhibitors thereon

The development of oxidative stress is a common impact in plants induced by Al toxicity. In the present study, different ROS was measured from plants under varying concentrations of Al along with Put. The extra cellular generation of O_2^- and H_2O_2 following the treatments of Al indicated a dose dependant response. A maximum rise in O_2^- and H_2O_2 content (i.e. 1.78 and 2.4 folds respectively) over control was recorded as compared to highest concentration of Al (480 μM) (Fig. 3a,b). This suggests that plants became prone to oxidative stress induced by Al in the present experiment. However, plants had minimized the generation of O_2^- and H_2O_2 when treated with Put and recorded 31.25 % and 20.18 % down-regulation respectively as compared to highest concentration of Al treatment (Fig. 3a,b). With the use of inhibitors viz. IMZ and NaN_3, there recorded a significant variation of O_2^- and H_2O_2 under Al treatments. The inhibition of O_2^- against IMZ and NaN_3 were 34.02 % and 22.91 % respectively. Similarly, the inhibition of H_2O_2 against IMZ and NaN_3 were 30.02 % and 16.51 % respectively (Fig. 3a,b).

In addition, plants possess a plasma membrane bound NAD(P)H-oxidase that mediates an electron transfer to O_2 and constitutes a significant (P≤0.01 or 0.05) accumulation of super oxide (O_2^-) and hydrogen peroxide (H_2O_2) in the apoplastic space. The maximum activity of enzyme was recorded 1.53 fold at highest concentration of Al (480 μM) and that was diminished by application of Put by 23.26 % (Fig. 4a).

Lipid peroxidation and protein oxidation under Al stress

Preliminary, plants in the present experiment had responded well for the oxidative stress by elevation of lipid peroxidation

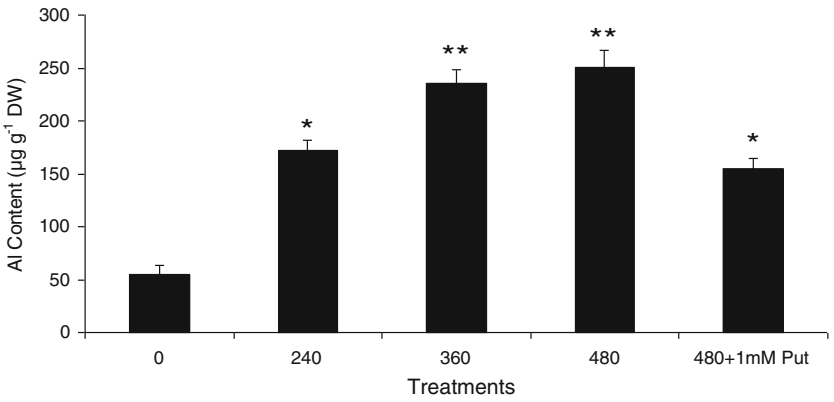

Fig. 2 Accumulation of Al in *Salvinia* grown under varying concentration 0, 240, 360, 480 μM of Al salt and 480 μM of Al salt with 1 mM Putrescine (480 μM+1 mM Put). The values are plotted from means (±SE;) of replication (n=3), (*P≤0.05 and **P≤0.01)

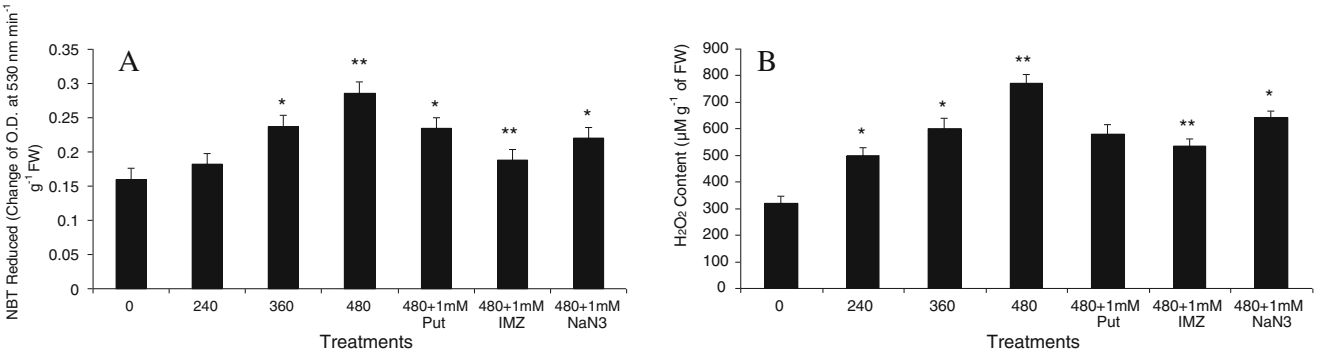

Fig. 3 Generation of O_2^- (A) and H_2O_2 (B) following treatments with varying concentration 0, 240, 360, 480 μM of Al salt and 480 μM of Al salt with 1 mM Putrescine (480 μM +1 mM Put), 1 mM imidazole (480 μM+1 mM IMZ) and 1 mM sodium azide (480 μM+1 mM NaN_3). The values are plotted from means (±SE;) of replication (n= 3), (*P≤0.05 and **P≤0.01)

in terms of MDA content under Al toxicity. Plants recorded significant (P≤0.01 or 0.05) amount of MDA accumulation by 3.44 fold at highest concentration of Al (480 μM) than control (Fig. 4b). In case of protein oxidation, it was measured with respect to the differences in carbonyl content between control and Al treated and it recorded significantly (P≤0.05) 2.72 fold over control at highest concentration of Al (480 μM) (Fig. 4c). However, Put had been recorded to down regulate the effects of Al induced oxidative stress by lowering the lipid peroxidation as well as protein oxidation by 33.94 % and 45.58 % respectively (Fig. 4b, c).

Nuclear disintegration by Al toxicity

One of the vulnerability of Al toxicity in plants is manifested by disintegration of nuclear membrane leading to DNA damage particularly, in meristematic region of roots. By the technique of alkaline disintegration comet assay a distinct variation in nuclear structure and its lysis was recorded maximally disintegrated at highest concentration of Al (480 μM) over control (Fig. 5a). It is more evident from the analysis of tail length of the comets, which were significantly (P≤0.01) variable in a concentration dependent

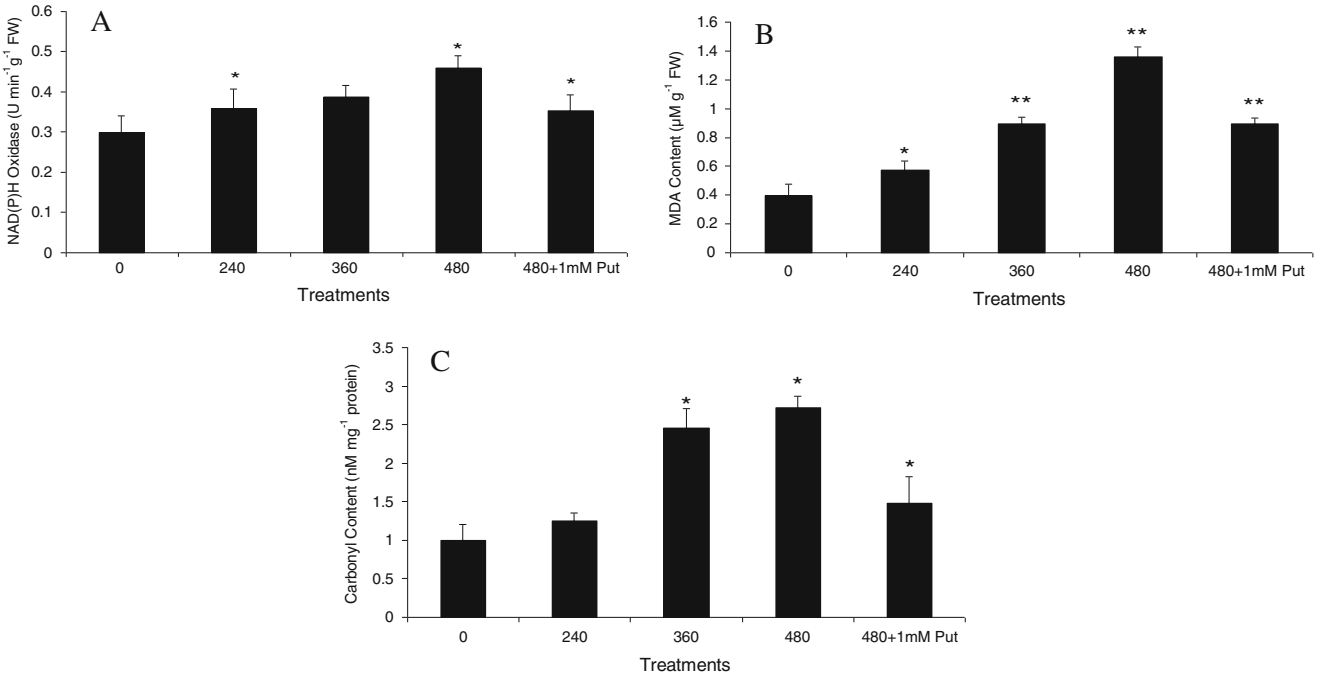

Fig. 4 In vitro assay of NAD(P)H oxidase activity (**a**), Lipid peroxidation in terms of MDA content (**b**) and Protein Oxidation in terms of carbonyl contents (**c**) and in *Salvinia* plants grown under varying concentration 0, 240, 360, 480 μM of Al salt and 480 μM of Al salt with 1 mM Putrescine (480 μM +1 mM Put). The values are plotted from means (±SE;) of replication (n=3), (*P≤0.05 and **P≤0.01)

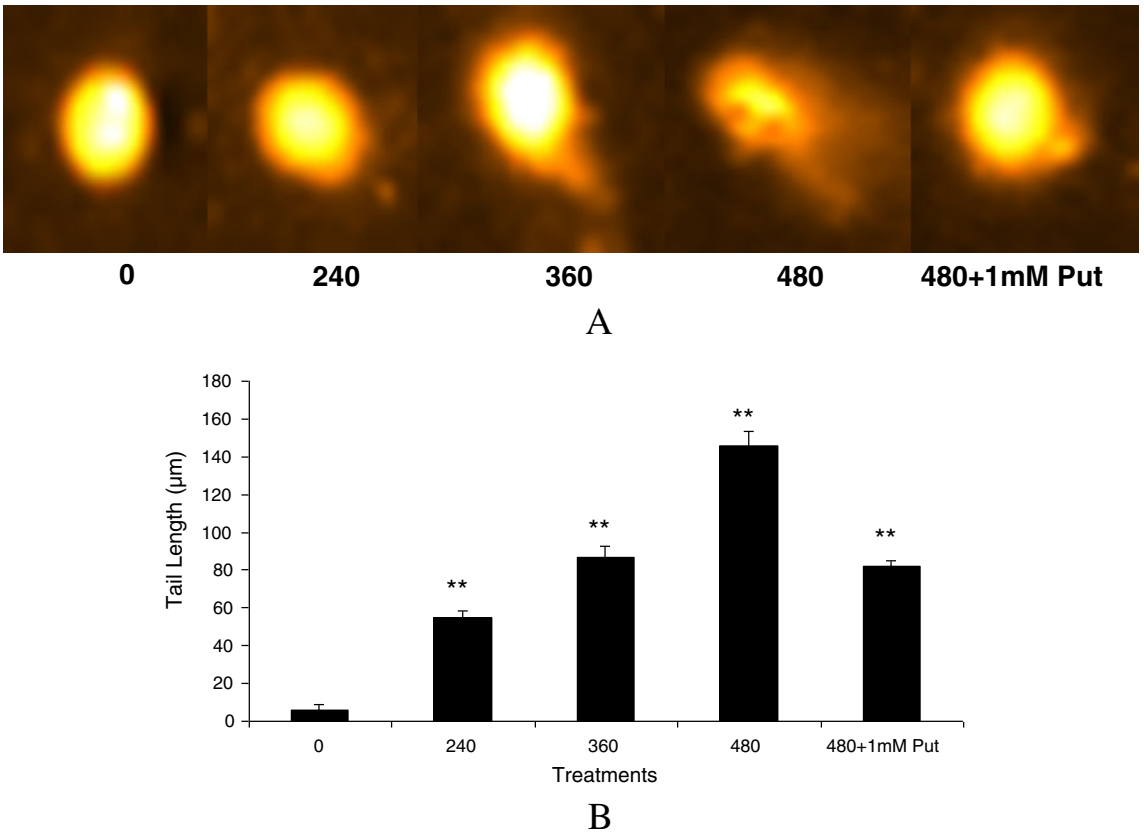

Fig. 5 Damage of the nuclei studied by Comet assay (**a**) and Comet tail length (**b**) under different concentration of Al (0, 240, 360, 480 μM) and 480 μM of Al salt suplemented with 1 mM Putrescine (480 μM +1 mM Put)

manner of Al. However, putrescine had been able to retrieve the damage of nuclei (43.83 %) from that of highest concentration of Al (480 μM) (Fig. 5b).

Variation in glutathione content under Al toxicity

In the present experiment, *Salvinia* plants recorded to accumulate some antioxidant moieties under Al toxicity. In this context, plants recorded a significant ($P \leq 0.01$ or 0.05) changes in the ratio of reduced to oxidized forms of glutathione (GSH: GSSG). Steady decline in GSH:GSSG in accordance with the Al treatments may suggest a continued recruitment of glutathione (GSH) for sustaining the reduced redox of the tissues. However, GSH:GSSG ratio maximized at 480 μM of Al and that was recorded 1.53 fold higher over control (Fig. 6a). Put, on the other hand, recorded as a reliever for Al induced oxidative stress and retrieved GSH:GSSG by 13.17 % over the highest concentration of Al (480 μM) (Fig. 6a).

Activities of antioxidative enzymes in vitro and in-gel staining

The anti-oxidative enzymes like GPX, APX, CAT, GR and SOD were studied by both in vitro assays as well as in-gel staining by running the protein on a native poly-acrylamide gel. The analysis revealed some variations in activities of GPX that was induced under Al toxicity. The significant ($P \leq 0.05$) variation was dose dependant, which recorded more activity (1.406 fold) at the 480 μM of Al over control (Fig. 6b).). However, Put application had minimized the activity of GPX by 10.51 % in respect to highest concentration of Al treatment (480 μM) (Fig. 6b). Moreover, in-gel staining for isozymes recorded three possible isoforms (GPX1, GPX2 and GPX3) (Fig. 7a).

The activity of antioxidizing enzymes like ascorbate peroxidase (APX) was studied by both in vitro assay as well as in gel studies. The activities for APX recorded significantly ($P \leq 0.01$ or 0.05) varied in a dosage dependant manner that recorded maximum (1.56 fold) at highest concentration of Al (i.e.480 μM) over control. Where as, application of Put had minimized the activity by 33.69 % for APX over highest concentration of Al (Fig. 6c). In gel staining, a band of APX (APX1) was more expressed under increasing dose of Al. However, Put had been hardly effective in modulation of those expressions as compared to control (Fig. 7b).

For other enzymes like CAT, on the contrary, there recorded a significant ($P \leq 0.05$) decline in activity with increasing concentration of Al treatments and maximally

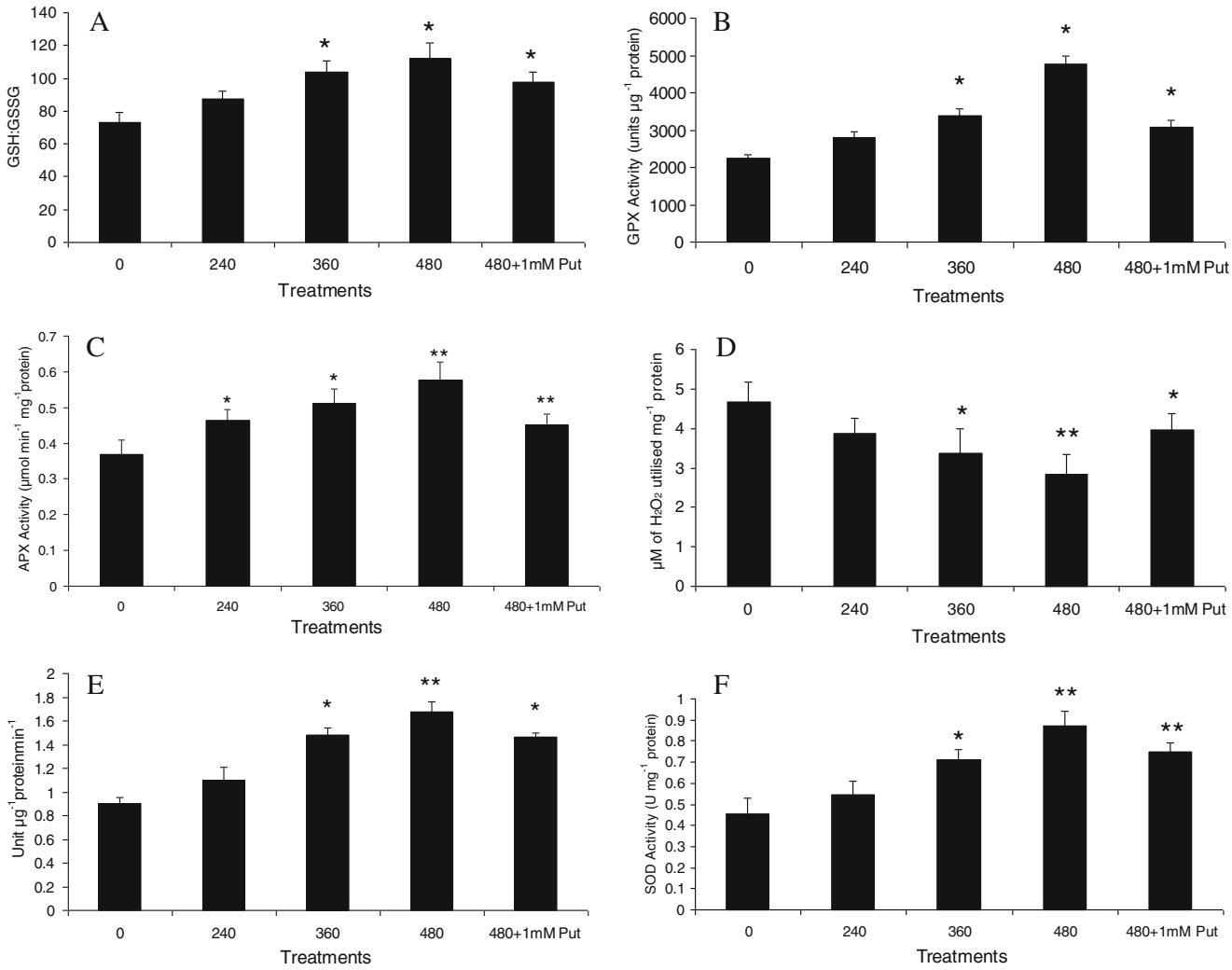

Fig. 6 Glutathione (GSH:GSSG) accumulation (**a**), Changes in activity of GPX (**b**), APX (**c**), CAT (**d**), GR (**e**) and SOD (**f**) in *Salvinia* plants grown under varying concentration (0, 240, 360, 480 µM) and 480 µM of Al salt suplemented with 1 mM Putrescine (480 µM +1 mM Put). The values are plotted from means (±SE;) of replication ($n=3$), (*$P\leq0.05$ and **$P\leq0.01$)

reduced (39.31 %) at 480 µM of Al as compared to control (Fig. 6d). The activity of catalase resume with Put treatment and it recorded 0.394 fold from highest concentration of Al treatment (480 µM) (Fig. 6d). The in-gel study of catalase showed two distinct isoforms (CAT 1 and CAT 2) (Fig. 7c) and those were variable in expression among the treatments.

Glutathione reductase (GR), the enzyme that recovers the reduced form of glutathione (GSH) in the tissue showed a significant ($P\leq0.01$ or 0.05) rise in the activity and that recorded 1.86 fold over control at highest concentration of Al (480 µM). Put, on the other hand, had effected by diminishing the activity of GR by 13.09 % under Al treatments (Fig. 6e). For the expression profile of GR, there observed more than five isoforms (GR1, GR2, GR3, GR4, GR5 and GR6) (Fig. 7d).

The activity of another antioxidative enzyme SOD assay recorded 1.92 fold increase at highest concentration of Al over control. Application of Put has a profound effect on the activity of SOD and minimized it by 27.21 % over highest concentration of Al (Fig. 6f). In case of in-gel study, three distinct isozymes bands were observed (Cu–Zn SOD, Mn SOD and Fe SOD) after resolving the gel in incubation mixture (Fig. 7e).

Discussion

Al or other metal induced oxidative stress and its impact on plants have been mostly investigated in crops. *Salvinia* being a fern species is less explored in this regard and effect of polyamine interacting with the antioxidating pathway induced by Al is a new and interesting insight for metal induced oxidative stress in plants. Al might be a potent toxic metal in terms of producing an oxidative burst in tissues with various

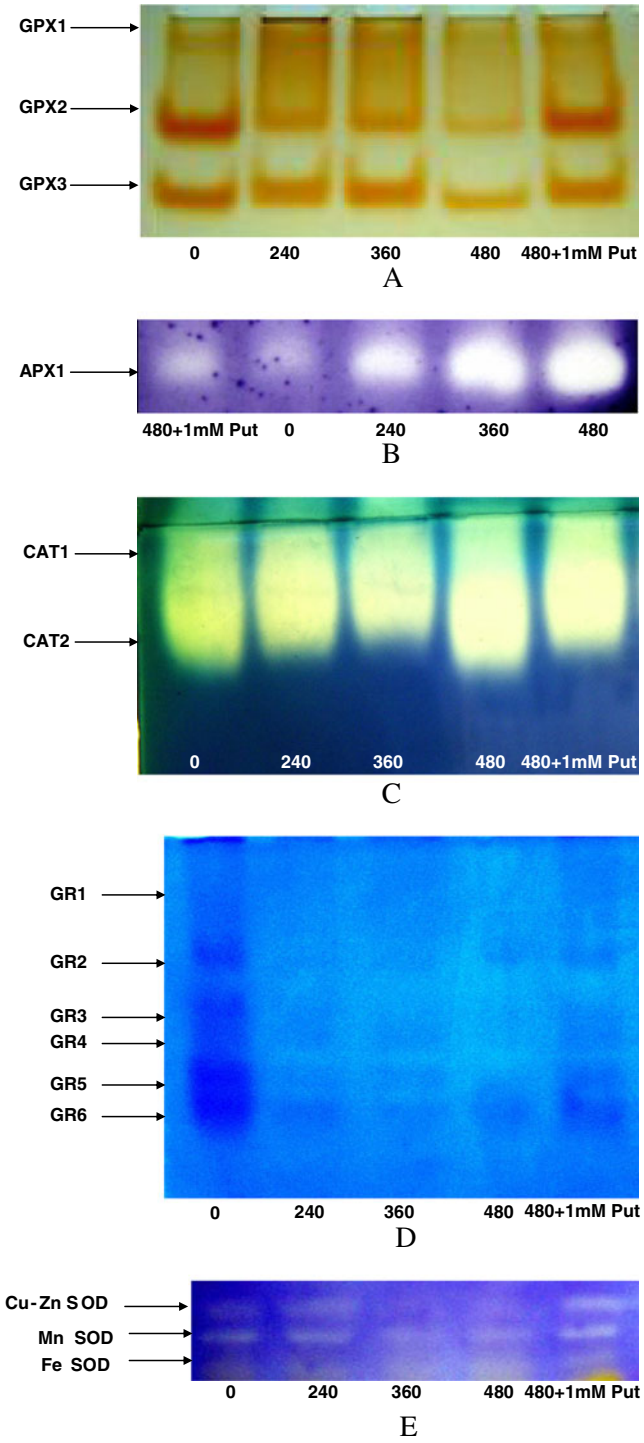

Fig. 7 Effect of varying concentrations of Al (0, 240, 360, 480 μM) and 480 μM of Al salt suplemented with 1 mM Putrescine (480 μM +1 mM Put) on separation of different isozymes of Guaiacol peroxidase [GPX1, GPX2 and GPX3] (**a**), Ascorbate peroxidase [APX1] (**b**), Catalase [CAT1, CAT2] (**c**), Glutathione reductase [GR1,GR2,GR3,GR4,GR5 and GR6] (**d**) and Superoxide dismutase [Cu-Zn, Mn and Fe-SOD] (**e**) on 10 % polyaccrylamide native gel

cellular and physiological alterations. However, some plant species can survive under highly Al contaminated area and can accumulate much amount of metals (Zeng et al. 2011). High tolerance level of Al in some plant species is accomplished by several mechanisms including exclusion, sequestering into vacuoles, sub cellular compartmentation and chelating the metal by any ligand (mostly organic acids) to inert moieties or non-reactive forms (Smiri et al. 2010). The ability of few *Salvinia* species (*S. molesta*, *S. minima* and others) for hyper-accumulation has also been illustrated for toxic or heavy metals (Sanchez-Galvan et al. 2008). Thus, we observed in the present experiment that there was a proportionate increase in Al accumulation in the tissues according to its dosages and that was under regulation of applied polyamine i.e. putrescine (Put). Interestingly, *Salvinia* being treated with Put (1 mM) responded well for retrieval of growth significantly by minimization of Al absorption. The polycationic nature of polyamine may bind to the negative domain of the cellular membranes and thereby may shield the membrane from damage by heavy metals or other toxic metals like Al and others (Stoeva et al. 2005; Chen et al. 2008). Hardly there are any heavy or toxic metals that could be able to enhance the growth and development of plants. Plants that accumulate these toxic or heavy metals are regarded as hyper-accumulators (Yang et al. 2012). So revealed from our studies polyamine (Put) did not mark any improvement for accumulation of Al in the tissues rather diminishes the metal absorption. However, Put has been evident to down regulate the ROS generation as well as enhance its anti-oxidation pathways.

Along with osmotic perturbation and specific ion effects of Al hyper accumulation, the plant becomes more prone to an oxidative burst at cellular level. Wide varieties of reactive oxygen species (ROS) with their varying oxidizing potentials are accumulated in the tissues that ensure the cellular degeneration. Lipid peroxidation and protein oxidation are those of preliminary responses to ROS induced damages in plant tissues under metal stress (Amri and Shahsavar 2010). It is interesting that Put could significantly down regulate the Al absorption. More so, application of Put has been proved to be efficient in diminishing the ROS induced oxidative damage through improvement of antioxidation pathway. Therefore undoubtedly Put becomes a faithful elicitor for reducing the oxidative stress induced by Al or by other metals. In the present study, lipid peroxidation and protein oxidation recorded a significant up regulation as a function of Al doses and that was mitigated with Put application. The protein carbamylation is an irreversible oxidation process of protein, transforms it into an inert or biologically deformed moieties and the reaction becomes more intense with the advent of ROS. The loss of protein in functional states with exposure of heavy metals is the consequences of either decline of synthetic machineries for proteins and/or an acceleration of an overall protein turn over in tissues. Functionally, oxidative stress induced protein oxidation, lipid peroxidations are almost synergistic in

operation and former is operative earlier in plants (Gill and Tuteja 2011). The trend of the present observation for lipid peroxidation and protein carbamylation are also documented in other plant species prone to Al toxicity (Pan et al. 2004; Exley 2004). Put, with its poly-cationic nature might have shielded the cellular membrane, particularly, the negatively charged domains of the unsaturated phospholipids backbones; those are more prone to oxidation by ROS. Al is more prevalent when the ROS targets the nuclear membrane overcoming the resistance offered by cell wall exudates binding the metal, sequestering in vacuole or even avoiding the enzymatic lysis in the cytosol. If these two lines of defense are not realized adequately, ROS may disintegrate the nuclear membrane with a resultant of consecutive breakage of nucleic acid (Lin et al. 2008). In the present experiment, the dose dependent induction of nuclear lyses or DNA damage is studied by alkaline disintegration comet assay. The present study focused on the DNA damage potential of Al, which was proportionately evident with the increase in comet tail length with the ascending doses. The effect of Al induced ROS for DNA breakage might be collaborated for tissue disintegration (Rodriguez et al. 2011). The protective role of Put like other polyamines on membrane stability is facilitated through the down regulation of membrane bound peroxidase induced ROS like H_2O_2 and OH^- etc. (Achary et al. 2007). At the cellular level this could be circumvented by inhibiting one of the enzymes like NAD(P)H-xidase recruited for generation of O_2. There recorded another enzyme called NAD(P)H-peroxidase that also contributes to the generation of ROS. In fact, this enzyme behaves as an oxidase that mediates the oxidation of NADPH and thus reduction of O_2 into O_2^- and H_2O_2 through a complex pathway (Zhang et al. 2008). Moreover, NAD(P)H-oxidase may induce an oxidative burst where O_2^-, H_2O_2 and OH^- are generated on the cell surface (Jung 2004). Significant amount of this is more confirmed based on the subsequent observations those recorded the diminishing of O_2^- and H_2O_2 generation when plants were treated with IMZ and NaN3 both being typical inhibitors for NAD(P)H-oxidase and NAD(P)H-peroxidase respectively. In the present experiment, a significant amount of O_2^- and H_2O_2 generation were featured in the plants under Al toxicity. Application of Put had registered significant diminishing effects on both of the super oxides.

It makes prudent that Al could be marked as an effective hazardous metal for inducing oxidative stress in plants. Essentially, plant tissues are also tuned to diminish this over oxidation by enzymatic and non-enzymatic pathways (Exley 2004). It is a sequential operation of some antioxidizing enzyme systems, those in chain of reactions resulted in cellular detoxification by the lysis of ROS. The most notably of those like superoxide dismutase, peroxidase, catalase, glutathione reductase and other enzymes are functionally expressed in plants performing the conversion of ROS into their non or less reactive forms. In the present experiment, the ability to scavenge the ROS by GPX was tested and it recorded a significant rise in activity under Al as compared to control condition. GPX, in general, constitutes a pool of all peroxidases, those catalyses the H_2O_2 with some of phenolic compounds as electron donors. However, stress induced GPX and its activities are variable according to tissue specificity. Since, root is the most vulnerable tissue for Al toxicity; expression and activity of GPX are predominant in that zone and there recorded an up regulation as evident in many studies (Sharma and Dubey 2007). However, some discrepancies are also cited in the activities beyond a threshold concentration of Al in the tissues. In those reports organelle specific GPX were more sensitive within a limit of Al concentration and showed a significant variation among those (Sharma and Dubey 2007). In the present study, the in-gel staining of GPX had hardly recorded any significant variation in band numbers as a function of Al exposure. Still, the intensities of those bands, particularly, those are of low molecular weights (anionic types) are variable in comparison to those under control. The variation in isoenzymic patterns and its distribution in tissues constitute the plant's potential for diversification of a gene (e.g. GPX, as in the present case) and its expression potential under extreme conditions (Asthir et al. 2009). A number of reports have revealed the variation in activities of other antioxidative enzymes in response to Al toxicity in tolerant and susceptible cultivars. In fact, any changes in the antioxidative enzymes may deviate the sustenance of cellular redox under oxidative stress. Among the enzymatic antioxidative pathways, APX comprises a family of isoenzymes where ascorbate is the electron donor and thus show distinct variations in structure and mode of function (Gill and Tuteja 2010). With regard to metal stress, evidences have enriched from different sources that both the amount of enzymes and its activity are involved in scavenging of ROS. APX mainly acts for conversion of dehydro ascorbate to ascorbate with another protein dehydro ascorbate reductase (DHAR) under the condition of oxidative stress (Davletova et al. 2005). Likewise, CAT, another antioxidizing enzyme not requiring any electron donors can also lyse H_2O_2. In the present case, CAT activities recorded a significant down regulation under Al and that was retrieved by Put. The ionic imbalance might deviate the affinity of enzyme from its substrate or even also denature the enzyme protein. As a whole, deflection from the optimum activity is resulted. Al was also reported to bind with phospholipids of the cell membrane that influences the ion channel proteins that inhibits specific ions flux. Ammonium (NH_4), of those is a positive modulator for CAT activity and thus it's scarcity under Al contamination particularly in acid soil might set a limitation for optimum activity (Rout et al. 2001). It is also

noted that the isozymic profiles of CAT had significant alteration in plants of the present experiment when treated with Put under Al stress as compared to control. The polypeptide for CAT1 showed no significance variation among the treatments, somehow the gene concerned is insensitive to any changes to metal exposure. Now, among non-enzymatic antioxidant moieties, glutathione (GSH), a tripeptide, is the key component for maintaining the redox of the tissues that very often attains an over oxidized state, more under the heavy metal contamination. Maintaining a high ratio of reduced (GSH) to oxidized (GSSG) is an essential phenomenon and this conversion is mediated by glutathione reductase (GR) (Foyer and Noctor 2005). Likewise, plants in the present experiment responded well in maintaining the ratio of GSH to GSSG, which happens to be an index for assessing the oxidation in the tissues under Al stress. Notably, in the present case, an increase in GSH to GSSG ratio under Al contamination and it was sustained with Put application. This has been imperative to consider the glutathione as a chief cellular antioxidant and that is precisely required in balancing the cellular oxidation by generating the ascorbic acid in glutathione-ascorbate pathway (Hamdani and Sirna 2008). Moreover, the consideration of the glutathione for sustaining a steady pool of reduced redox in the plants of the present experiment is more acceptable with the observation of GR activity. In addition, the alteration of GR activity with Put treatment is more indicative of the fact of the efficacy of Put in recycling of reduced glutathione (GSH) under Al exposure. The isoforms of GR resolved as distinct bands when run on denaturing gel and varied in their band intensities. It has been reported that activity of GR, in actual, is a composite effect of individual isoenzymic proteins and some of those are differentially expressed in plants (Hall 2002).

SOD happens to be the first line of defense for enzymatic conversion of ROS in Halliwell-asada pathway. Dismutation of superoxide (O_2^-) to H_2O_2 is attributed by SOD for combating the ROS induced damages in the tissues. In plant system the major sites of ROS generation are positioned in the chloroplast and mitochondria and therefore the most expressed isoforms are required to lysis the ROS in those organelle. SOD comprising of a family of metalloenzymes occurs in different isoforms as Cu–Zn SOD, Mn SOD and Fe SOD, catalyze the dismutation reaction of O_2^- to H_2O_2 and O_2. The variation in expression of SOD with different isozymic forms as a function of Al concentration is consistant with the findings of others where metal stress had effectively being proved as an inducer for the SOD gene (Roy choudhury et al. 2012).

In conclusion, *Salvinia* plants had been evident to be responding against Al in abundance with the changes of some cellular processes including oxidation of lipid and protein, damage of DNA, perturbance of osmotic status, imbalance of cellular redox and generation of various ROS in a system by up or down regulating the enzymatic pathways. Thus, *Salvinia* proves itself to be very efficient plant material for hyperaccumuation of metals.

In addition, although Put did not improve the absorption capacity of Al from the solution and thus Put become ineffective in stimulation of hyperaccumulation of metal. But, Put has been evident as an effective modulator to alter the cellular responses against the oxidative stress in a coordinated way either by lesser absorption of the metal or by down regulating the generation of ROS and/or by its lysis through antioxidation pathways. Moreover polyamine, like Put appears to be identified as an successful alleviator of oxidative damage induced by toxic metal like Al, still there is scope for deciphering the role of Put at the molecular level for other ferns species.

Acknowledgement Prof. Anita Mukherjee, Professor in Botany University of Calcutta, is sincerely acknowledged for her assistance in comet assay study.

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