5.1 Prelude to Discussion

The responses of plants to different abiotic stress reveal its inbuilt resistance mechanism and as a consequence, allied genes are expressed by up/down regulation through some biochemical pathways. Admitted well, among all minerals present in soil, some had harsh impact on plants growth and development. A number of ligands have induced the metal toxicity and its effect on plants through physiological and cellular changes. Likewise, aluminium (Al), a lighter metal and third most abundant of all elements is widely available in various molecular forms, however, trivalent type (i.e. \( \text{Al}^{3+} \)) is the predominantly phytotoxic (Panda et al. 2009). Since this form is most available and abundant in plant tissues, therefore, it imparts its effect on physiological activities. Al when absorbed in excess exerts specific thrust on plant cellular status: Specific ion effects and Al induced oxidative damages. The specific ion effect is responsible for metal induced water deficit stress as usual and the Al induced oxidative damages is more detrimental for induction of reactive oxygen species. In most of the cases the major effect of Al toxicity in the root zone is circumvented by ROS induced oxidative damages. This becomes more acute when acquisitions of redox active transition metal (\( \text{Cu}^{2+} \), \( \text{Fe}^{3+} \) etc.) takes place which is abundant with Al toxicity (Silva 2012). Under acidic pH, those ions becomes more activated and produce reactive oxygen species. Still it is some sort of changing redox potential of tissues as a function of ionic imbalances. This prevails also at the sub cellular fractions like chloroplast, mitochondria, peroxisome and other typically sets for the redox reaction on harboring electron transport chain. Therefore, the signaling behavior which is initiated from the root to shoots, transduce from cytosol to nucleus, and hence many cellular and physiological processes impaired in plants (Panda et al. 2009; Ribeiro et al. 2012).

In the present experiment \textit{Salvinia} plants, an aquatic fern species were taken as an experimental plant material for extent of Al accumulation and to study the impact of such
metal acquisition on plants. More interestingly, the interaction of polyamines (PAs), putrescine (Put) in the present case, with such physiological and cellular modulation highlighting the antioxidation pathway has thrown some lights on metal stress in this fern species. Initially, plant’s growth was considered as the primary determinant for assaying the impact of Al at the physiological level which was proportionately subdued with ongoing Al concentration. Since, the solution containing the potassium aluminium sulphate (the source of Al in the present case) buffer has acidic pH, it is expected that *Salvinia* plants could be absorbing the exchangeable Al rapidly being dissociated from the salt. Therefore, typical cellular and physiological parameters are required to correlate as indication with the Al sensitivity which we will be discussing in details subsequently (Mandal et al. 2012).

From the facts and figures of the present experiment, the hypothesis that was established that Al could be an effective metals as good as other heavy metals to induce oxidative stress has been proved to lot of extent (Pinto et al. 2003). Despite of increasing concentration of Al (up to 480 μM of K[Al(SO₄)₂].12 H₂O in the present case), *Salvinia* plants were able to survive at least the days of investigation. Therefore, *Salvinia* plant appears as hyperaccumulator of Al. Now, with the basis of this special trait, next to be analyzed and justified with existing references for physiological tolerance under metal toxicity. With more advanced thought for metal endurance displaying through antioxidation pathways, how a plant would be responsive for direct exposure of ROS moieties has to be determined. On this basis, *Salvinia* plants have also been evaluated in terms of some cellular and nuclear responses to direct exposure of exogenously applied H₂O₂. This has been more clarified with the interactive analysis with Put as an elicitor for amelioration of oxidative stress established by H₂O₂ application.
5.2 Effects of Al on *Salvinia* plants and interaction of PA thereon

Al or other metal induced oxidative stress and its impact on plants have been generally investigated in angiospermic plants. *Salvinia* being a fern species is less explored in this regard and interaction of PA (Put in the present case) with the antioxidation pathway induced by Al in plants is a new and interesting approach for research. Al being a potent phytotoxic metal produces oxidative stress in tissues with various cellular and physiological alterations. The main symptoms of Al toxicity are leaf chlorosis, deformation of root or root like structure developed from 3rd leaf of each whorl in *Salvinia* sp. (Gifford and Foster 1989) and stunted plant growth etc. At the tissue level the meristematic distal transition and epical elongation zones of the root tip are frequently impaired. At the cellular level those zones are affected on their cell wall and plasma membrane, sub cellular membrane and even in nucleus (Achary et al. 2008).

In the present experiment, a typical effect of Al toxicity was found in *Salvinia natans* L., in terms of relative growth rate (RGR) and net assimilation rate (NAR). With the increasing concentrations of Al, plants showed the declining trend of RGR. Similar results also found in case of NAR as foliar or leafy parts of *Salvinia* plants got affected. Since, Al curtails the proper functioning of root, therefore, absorption of water followed by translocation and proper metabolism in each part of the plants could easily be affected. The impaired water relation is manifested into plants as inadequate growth and development. In case of *Salvinia* plants, earlier records with heavy metals uptake through subdued total biomass accumulation and allied characters have also been reported (Dhir et al. 2009, Jampeetong and Brix 2009). However, application of Put showed the recovery of RGR and NAR, which may suggest the underlying mechanism of PA in relation to metal effect on growth and development as well as in allied attributes (Mandal et al. 2013b). NAR, in fact, represent the capacity of foliages to harness light energy and its subsequent conversion into photosynthates pool. Contextually, any sort of water stress may diminish the photosynthetic activities with different facets and thereby the carbohydrate reserve is deprived from its normal abundance. *Salvinia* plants, in the present experiment though have not been evaluated for photosynthetic efficiencies, still, a sharp declined in NAR showed the negative impact of Al on photosynthetic capacities.
This is more established when Put could record its potential to reverse the decline of NAR in this species, therefore, PAs mediated recovery of dry matter accumulation might be similarly effective in fern species as observed in case of higher plants under metal stress.

Al being phytotoxic in nature could interfere not only with the root but also partial or complete retardation of plant parts. Preliminary plants were analyzed for its accumulation of Al in excess and interaction with Put from the Scanning Electron Microscope (SEM) study coupled with EDX analysis (Mandal et al. 2013c). It showed excess of Al deposition dispersed over the tissues, mostly in the cortical tissues approaching vascular bundle of the modified roots or root like structure. Since trivalent Al (Al$^{3+}$) are considered to be more toxic, hence, we used potassium aluminium sulphate [KAl(SO$_4$)$_2$. 12H$_2$O] under acidic condition. Thereby in solution, it is expected to get hydroxide forms of Al. Therefore, the accumulated Al has been detected through Atomic Absorption Spectrophotometry (AAS) analysis. Trivalent Al (Al$^{3+}$) has been reported to be most effective and even toxic as referred by many distinguishing syndromes in the roots of plants (Silva 2012). A metal absorb in excess could behaved as detrimental in general in two ways: changing the established water relation in the plants and inducing some toxic moieties that very often becomes harmful (Aldesuquy et al. 2011; Liu et al. 2004; Lone et al. 2008). For the later referred to the reactive oxygen species (ROS) inducing oxidative stress (Yang et al. 2012). Al being a metal is very potent for ROS generation and its consequent effects on plant growth and development is extensively described in many literatures (Kochian 2002; Satapathy et al. 2012; Achary et al. 2012). Interesting to note that when Salvinia plants in the present experiment were treated with Put, both accumulation of Al and its resultant distribution and changes at cellular level was found to be modulated.

From the study of analytical survey of possible Al contaminated sites (Odisha, Bangalore and Kalyani), it is clear that plants have variably absorbed the metal. In comparison to control, the extent of Al accumulation varied approximately from 640 to 1330 ppm for the above mentioned places. This clearly showed the efficacy of plants to respond to Al
toxicated sites according to its hyper accumulation capability. Therefore, the accumulation of Al in excess with already discussed physiological responses may raise the feasibility for bio-indication of Al.

Likewise, the increase in ROS level and its distribution through the tissues may lead to the disruption of cellular and sub cellular fraction that eventually results in lysis of entire cell. At the tissue level, the cellular loss or cell death is detected by Evan’s blue staining. Undoubtedly, the more intense colorization of staining reveals the more proneness to oxidative damages of the tissues, especially in meristematic zone of root like structure developed from modification of third leaf of each whorl (Mandal et al. 2013b). Therefore, in the present experiment with Salvinia, Al toxicity like salinity becomes more compounded with strong depolarization of membrane protein oxidation following intense K$^+$ efflux and finally resulting in collapse of cells (Wojtyla et al. 2013). This has been mostly referred in vegetative tissues particularly root meristem in angiospermic plant species like rice, maize etc (Panda et al. 2009). In the present experiment, a linear increase in Evan’s blue uptake with the Al doses has been recorded through spectrophotometric as well as microscopic analysis. Followed by significant fall with PA may supposedly hypothesize the lesser tissue disintegration through lipid peroxidation or protein oxidation in the plants. Speculation for shielding activity of Put on cellular membranes with its polycationic nature may also support the reducing sensitivity to Al induced oxidative activity; which is significantly observed in our experiment (Mandal et al. 2012).

Attainment of subdued NAR as well as its mitigation by PA is more justified when chlorophyll content was measured in Salvinia plants. Chlorophyll, the predominant photosynthetic pigments showed a decline with one of its fractions under Al treatments in Salvinia natans L. in the present experiment. At the preliminary level, metal toxicity in relation to chlorophyll content has been attended by denaturing of the chloroplast membrane (Dhir et al. 2008). Thus, it is aggravated when metal induced ROS production and its peroxidative activity act on membrane lipid, as a result depletion of chlorophyll content and loss of its function results (Nichols et al. 2000). Al being a non redox metal
act as prooxidant and thus changes the osmotic relation and effects the green foliages with number of physiological process, photosynthetic carbon assimilation being the most predominant. The probable sources of chlorophyll disintegration are rational and those include: degradation of chlorophyll by chlorophylase, biosynthetic inhibition at few steps and loss of chlorophyll binding proteins with oxidative carbamylation. As a common consequences of the metal toxicity the inhibition of enzyme Δ amino levulinic acid dehydratase, proto chlorophyllide reductase associated with chlorophyll biosynthesis (Hukmani and Tripathy 1994) replacement of magnesium (Mg\(^{2+}\)) and ferrous (Fe\(^{2+}\)) for chlorophyll biosynthesis are most important to consider (Shekhawat and Verma 2010). Moreover, pigment system malfunctioning is attributed to metal induced solubilization of pigment protein complex from the chloroplast membrane or even with induced chlorophyllase activity (Vacha 2007). The disintegration of chlorophyll and changes it fluorescent properties is the most preliminary syndrome in a metal toxicity. *Salvinia* like other aquatic plant, the loss of chlorophyll is less affected out of water deficit but more with metal induced oxidative damages (Dhir et al. 2008). Whatever the possible causes, Put helps to regain the chloroplast membrane structure and thereby other photosynthetic activities like quantum yield, chlorophyll fluorescence, carboxylation etc. (Dhir et al. 2008; Fiedor et al. 2008). More so, *Salvinia* plants, recorded a significant down regulation of chlorophyll content. Thus, decline of NAR in *Salvinia* plants under Al concentration and resumption with Put could also be in accordance with the earlier findings.

Proline, the most water soluble amino acid, exists mainly in zwitter ionic state having both weak negative and positive charges at the carboxylic acid and nitrogen groups, respectively. Proline shares this property with other compounds collectively referred to as compatible solutes that renders support to the plants at least primarily to lower the osmotic potential (Pavlikova et al. 2008). Abiotic stress due to metal toxicity is always inductive to dehydration stress in plants followed by other secondary effects like oxidative damages. But surprisingly in *Salvinia* plants, in the present experiment, reduced the proline content as similar report established by Dhir et al. (2012) where *Salvinia* plants were exposed to heavy metal stress and showed decline trend of proline. This is in
accordance with earlier reports where hydrophytes exhibited the reduction of proline level under Cd stress (Dhir et al. 2004). However, proline biosynthesis is a multi step pathways facilitated by a number of enzymes (Celik and Atak 2012). The two distinct rate limiting enzymes: y-glutamyl kinase (y-GK) and y-glutamyl phosphate reductase (y-GPR) are appeared to be sensitive under different abiotic stress. Unexpectedly the decline of proline content under Al doses is indicative of the fact of down regulation of its biosynthesis where Pyrroline-5-carboxylate synthetase (P5CS) is regarded as a bi-functional enzyme complex that exhibits γ-GK and γ-GPR activities (Jaleel and Azooz 2009). Under metal toxicity, changes in redox state towards oxidative in nature had somehow curtailed the activity of those enzymes and thereby bio synthesis of proline was diminished (Thippeswamy et al. 2010). The effects of metal stress on these enzyme activities have been studied and revealed the regulatory property of proline in homeostasis of metal ions (Wang et al. 2010). As already reported proline biosynthesis is related to environmental fluctuations, particularly under conditions of soil salinity, metal contamination and water content. In the present study, Put could serve as osmoticum for relieving the γ-GK and γ-GPR activities and therefore positive modulation noticed in biosynthesis of proline under Al mediated osmotic stress.

In concert to osmoticum supported recovery of dehydration in tissues, another moiety, the glycine betain is also equally important. In our present experiment, Glycine betain has increased with Al stress and modulated by the application of Put. Similar increasing trend in terms of glycine betain accumulation has been reported by Dhir et al. (2012) under Cd and Cu toxicity. Glycine betain is a quaternary ammonium compound, that serves as an osmolytes by lowering the osmotic potential of the cells and thus it prevents the movement of water from the cell against the water potential gradient (Hossain et al. 2010). Therefore, its accumulation in Salvinia plant might have improved the protection of RUBISCO, maintenance of osmotic potential and membrane integrity. Undoubtedly metal induced damaging of membranes even at the sub cellular sites could be recovered from the decline in osmotic turgidity by application of glycine betain exogenously (Ashraf and Foolad 2007). So it will be quite expected that Salvinia plant over expressed with glycine betain may exercise the maintenance of tissue hydration under Al toxicity.
Plants could perceive the shock of ROS at cellular and sub cellular membrane and the disintegration of membrane could start to transduce the signal into the cell. Metal stress could render its effect by perturbation of osmotic balances in the tissues along with specific ion effects (Russak et al. 2008). Al would be of no exception and its profuseness in tissues can alter the water relation by modifying cellular membrane properties. The membrane bound proton ATPase (H⁺/ATPase) activity happens to be one of the prime concern in plants for assessment of cellular ion status, particularly for potassium. Thus a significant variation observed under various Al doses as well as Put supplementation at different duration in the *Salvinia* plant in our present experiment. A non redox metal like Al can also induce the potentiality of the root membrane and thereby a fall in ATP might pose a serious bottleneck for the cellular functions. Those H⁺/ATPase might play the important role where availability of ATP is the most crucial as substrate requirement (Kabala and Russak 2011). At cellular level, regardless of toxicity, metals might be conjugated with H⁺/ATPase by its auto phosphorylating domain. It could be logistic that the affinity of metal for binding to the domain of the H⁺/ATPase is down regulated and thereby the activity is restored from the metal toxicity (Liu et al. 2006). However, subdued effect of H⁺/ATPase activity is successfully achieved by Put in a proportional manner, so it may also be hypothesized to sustain the native structure of the membrane for proper location of the proteins (like H⁺/ATPase) and thus sustains the functioning in a proper way (Koizumi et al. 2011). H⁺/ATPase activity had been more justified when KCl and V₂O₅ applied as promoter and inhibitor respectively.

The efficacy of other PAs like spermidine and spermine had been implied with the use of PA biosynthetic inhibitor as documented in barley root under salinity (Liu et al. 2006). ROS induced H⁺/ATPase activity on membrane could be retrieved with the help of elicitors like Put. The generation of ROS, particularly at the apoplastic and intercellular spaces with some specific NAD(P)H oxidoreductase has been evident in plants (Ishida et al. 1987). Put as a PA had effected on this enzyme for moderation in activities and thereby resulting in a concomitant relieve for membrane protein oxidation. Contextually, ROS generation by the activity of NAD(P)H oxidase is another concern for metal
induced oxidative stress in plants. In the present experiment the activity of NAD(P)H oxidase revealed an up regulated trend when the plants were treated with Al salt. In an analysis of ROS and its sub cellular generation a number of cell wall and plasma membrane embedded peroxidases with varying electron donors are found in plants (Achary et al. 2008). In association with phenolic derivatives, some oxidase catalyzes $O_2$ into superoxide ($O_2^-$) and hydrogen peroxide ($H_2O_2$). This category is typically referred as apoplastic oxidases. The use of specific inhibitor for down regulation of $O_2^-$ generation has been well clarified on level of NAD(P)H oxidase expression in Allium root. With this line of approach, the use of Put has curtailed the accumulation of $O_2^-$ in *Salvinia* plants under Al stress (Mandal et al. 2012). Therefore, Put could possibly interact with NADH/NAD(P)H oxidase to modulate its activity under metal stress also. The involvement of PA under oxidative stress is more in its conjugated or bound form than free state. In fact, the functional implication of PA conjugates offer more complicated pathways involving many enzymes at cellular level of oxidative stress (Ghosh et al. 2012). With the advent of over expression of NADH/NADPH oxidase activity, the consequences were expected to be increase in ROS generation. With this we noticed a significant rise of ROS complemented with super oxide ($O_2^-$) and hydrogen peroxide ($H_2O_2$) in the *Salvinia* plants. Chemically $O_2$ and $H_2O_2$ are the products of consecutive electron reduction of molecular oxygen through shifting of the electron from its normal path (Sharma et al. 2012). $O_2^-$ is very transient in nature and is the most reactive biomolecules. On the other hand, $H_2O_2$ is rather fairly stable within the threshold value of the cellular sap. $H_2O_2$ in turn becomes more reactive with the higher concentration (variable according to plant species) particularly in reaction with $Cu^+$ and $Fe^{2+}$. In later case the turnover of $H_2O_2$ into hydroxyl (OH') under the effect of any transition metal is quite frequent in mitochondria and chloroplast. The OH' is quite diffusible like $H_2O_2$ and typically reacts with biomolecules. Therefore, the rise of $O_2^-$ and $H_2O_2$ is quite consistent with relation to oxidative stress in *Salvinia* plants of the present experiment. However the regulation of $O_2^-$ and $H_2O_2$ generation by exogenous application of 1mM Put with highest concentration of Al (480µM+1mM Put) suggests the imperativeness for induced stress and the down regulation of $O_2^-$ and $H_2O_2$ generation or ROS production by Put recorded
maximum at 7 days of duration as compared to highest concentration of Al dose (480µM).

In plant tissues the oxidative damages induced by ROS is the preliminary effects on cellular membranes particularly more in lipid profiles. The estimation of malondialdehyde content is thus set as a marker for lipid peroxidation (Pan et al. 2004; Exley 2004). A linear increase of MDA content according to the Al treatments undoubtedly suggests the oxidative damages under Al toxicity. A number of unstable moieties that are the prudent non enzymatic reaction of lipid peroxidation and those include reactive carbon derivatives (MDA), hydroxyl alkenes etc. Hydroxyl alkenes are regarded as the most frequent product of linoleic acid and arachidonic acid (Maiti et al. 2012). In enzyme mediated peroxidation path way, lipoxigenase the enzymes produces lipid hydroxide, poly unsaturated fatty acid, more specifically from inner wall of the cell membrane or from sub cellular components. Peroxy radicals are the another disintegrating moieties generated through the hydrogen replacement from unsaturated fatty acid by \(O_2\) and produced more under influence of iron (Fe\(^{2+}\)) contamination. With this environment, inductive to oxidative damages by *Salvinia* plants could be prone to lipid peroxidation activity and thereby more accumulation of MDA content occurs. However, plants with their inbuilt system of machineries can minimize the lipid peroxidation with diverse occurrence of antioxidants (Basu et al. 2010). Taking references glutathione as well as ascorbate, the two predominant antioxidising molecules can donate electrons to the dose dependent ROS either directly or through other intermediate moieties. Mono hydro ascorbate, dehydroascorbate are those commonly found in plants being played to quench the excess energy of ROS targeting lipid peroxidation. Use of any elicitors, as for example Put as in present case could be playing such a role that moderates either by radical formation or by its lysis. The guarantee for PA mediated recovery of cellular membrane is based on the facts in plant system: shielding of cellular membrane from ROS attack, primarily, and the down regulation of the ROS generation secondarily (Gill and Tuteja 2010). In fact, the development of free radicals, more under transition metals (Fe\(^{3+}\)/ Cu\(^{2+}\) etc.) can initiate the auto oxidation of electron transport chain with concomitant reduction of oxygen (Apel and Heribert 2009).
This reaction is enzymatically associated with chain elongation reactions for carbonyl radicals generated from peroxidation reaction of fatty acids. PAs could be able to hinder the reaction by donating electron from ROS to their unsaturated domain and thus keep the reaction in a subdued state (Gill and Tuteja 2010). In a different way where PAs could evoke the biosynthesis of some phenolics derivatives (like hydroxyl cinnamic acids, caffeic acid, ferulic acid, ρ-coumaric acid) under salt stress. These moieties have extended their conjugation ability by invading metals into the tissues and thereby proved its efficient exclusion mechanism. For Al, a considerable research put forward the Al exclusion mechanism, rather than provide internal mechanism for Al tolerance (Kochian 2002). Therefore, from the point of apoplastic detoxification of Al sustained by PA may explore new avenues of metal interaction in plants.

Along with osmotic perturbation and specific ion effects of Al bio-accumulation, plants experience an oxidative burst at cellular level. A vast array of reactive oxygen species (ROS) with their varying oxidizing potentials are accumulated in the tissues. As a result of which protein oxidation is also left with its detrimental effects under metal stress (Amri and Shahsavar 2010; Exley 2004). From the perspective of membrane integrity, as proteins are denatured (particularly for channel and carrier proteins) the membrane loses its biological conformation and activity. The protein carbamylation is an irreversible oxidation process of protein and the loss of protein in functional states with exposure of toxic metals is the consequences of either decline of synthetic machineries for proteins and/or acceleration of protein turn over in the tissues. Functionally, ROS induced protein oxidation and lipid peroxidations are almost synergistic in operation and former is operative earlier in plants (Gill and Tuteja 2011). Salvinia plants in the present experiment showed a linear increase in protein oxidation and thereby proved its proneness to oxidative stress. More so, the implication of the Put on sustenance of the membrane structure by moderation of protein carbamylation is important to note. The interference of PA and its inhibition to protein carbamylation in plants could be in few ways: the down regulation of ROS biosynthesis which oxidizes the membrane protein and the mechanical barrier of the protein layers on the membrane by binding with polycationic ligands of PAs (Ghosh et al. 2012). Thereby, it shields the cellular
membrane, particularly, the negatively charged domains of the unsaturated phospholipids backbones. Turnover of protease by increasing the related gene expression for chaperone moieties has also been documented in this regards (Alcazar et al. 2006). Whatever might be the reason, from the result section of present study, we find that Put curtailed the protein oxidation significantly in *Salvinia* under Al toxicity.

Metal induced signal transduction could be initiated in the plants from cellular membrane, apoplastic spaces or even at cell wall, transduced through the cytosol and finally approaches to the nucleus (Roychoudhury et al. 2011). Al could be more prevalent when the nuclear membrane is targeted by ROS 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 kinds of defense are not realized satisfactorily, ROS may disintegrate the nuclear membrane with the resultant of consecutive breakage of nucleic acid (Lin et al. 2008). If the ROS be the signaling moiety, gene expression in the nucleus would be the final impact for plants under metal toxicity. In our present experiment, the dose dependent induction of nuclear lyses or DNA damages has been studied through alkaline disintegration comet assay. The degree of DNA damages has been monitored with the monitoring of comet tail length, which is increased proportionately with the ascending doses of Al salt. More so, the effect of Al induced ROS for DNA disintegration might be collaborated with the tissue disintegration (Rodriguez et al. 2011).

The protective role of PAs with its polycationic nature is expected to bind with the phosphate backbone and thereby could supposedly minimize its lyses. Another protective role of PAs on membrane stability could be facilitated through the down regulation of membrane bound peroxidase induced ROS like $\text{H}_2\text{O}_2$ and $\text{OH}^-$ (Achary et al. 2008). However, in the present study a significant reduction of comet tail length under Put application was observed. Hence, Put acted as an effective alleviator for ROS induced DNA damages as those of other PA like spermidine and spermine under heavy metal stress (Mandal et al. 2014).

The accumulation of ROS and its elimination by different ways are characteristic phenomenon of plants responding to oxidative stress. In plant system the excess energy
of ROS moiety are minimized from its detrimental effects either by direct interaction of some antioxidant moieties or lyses of the ROS in to lesser energized fraction by set of enzymatic cascade. For the first case that is exercise of antioxidants with their unsaturated molecules can easily lead to absorb the excess electrons and thereby self oxidized. In our present experiment, *Salvinia* plants have evident with their sensitivity to oxidative damages by increasing ROS (O$_2^-$, OH$, \text{H}_2\text{O}_2$) and concomitant amelioration by Put so applied (Mandal et al. 2013b). In non enzymatic defense system, *Salvinia* plant showed a significant rise in accumulation of total phenolics including flavonoids under Al stress. The total accumulation of flavonoid is important to notice as it is the chief phenolics conjugant with their diverse molecular configuration and which can absorb maximally the energy exerted by ROS. Flavonoids with their molecular configuration has added advantages to chelate the metals and thus readily offers the radical scavenging activity (Ali 2008). This is more established with the fact of Al as furnished by electrostatic interaction in cells under low pH (pH≤ 5). The possibility may arise with regards to secretion of organic acid and formations of flavonoid residues that share the charge imbalance and facilitate the ligand formation with Al$^{3+}$ (Ryan et al. 2009). The over expression of flavonoid is recorded under Al stress in our present experiment, leads *Salvinia* plant as a bioindicator under oxidative stress. In addition the total phenolics are also appeared to be important in parallel to flavonoid accumulation for abiotic stress defense pathway. Phenolics showed similar trend of up regulation under Al concentration and thereafter minimized with Put, as followed in a number of cases where the over expression of phenolic pathways have been found to be modulated by PA Roychoudhury et al. 2011). Phenolics with their projected hydroxyl group are more favoured to conjugate with sugar moieties and remain in bound form, which is apprehended more in quenching of ROS energy. Put, somehow reduces the ROS generation thereby indirectly minimize the accumulation of phenolics including flavonoids (Ghosh et al. 2012).

However plants contain some other compounds, mostly non-thiol in nature and often used in antioxidation pathway. This is accomplished by stabilizing the cellular redox with some non proteins sulphur containing compounds. Regarding the characterization of this redox couple, the synthesis, oxidation, degradation, transport of GSH and its conjugation
with the sulphydryl groups of other compounds needs to be considered. Glutathione, a tripeptide is displayed with two interconvertible forms through the changing of oxidation status by offering its sulphohydral (-SH) groups (Semane et al. 2007). The glutathione (GSH) / glutathione disulfide (GSSG) redox couple is involved in several physiologic processes in plants and it participates in the maintenance of redox homeostasis in the cells. The redox state of the GSH/GSSG couple is defined by its reducing capacity with the half-cell reduction potentiality. It could differ in the various organs, tissues, cells, and compartments also with the changes of plants growth and development (Szalai et al. 2009). The Salvinia plants responded well in maintaining the ratio of reduced (GSH) to oxidized (GSSG) glutathione under Al stress which happens to be an index for assessing the oxidation in the tissues for the present study. Notably, from our result section we found consistent increase in the ratio of GSH to GSSG under Al contamination and which is imperative to consider as glutathione, a chief cellular antioxidant. That is specifically required in balancing the cellular oxidation by generating the ascorbic acid in glutathione-ascorbate pathway (Al-Hamdani and Sirna 2008). The signal for conversion of more GSH from GSSG is based on changing of cellular mechanism and Ca\(^{2+}\) depletion that may also contribute in salt sensitivity of plants (Rentel and Knight 2004). The high ratio of GSH to GSSG results in a reducing environment in the cells which maintains the appropriate structure and activity of protein molecules by inhibiting the intermolecular disulfide bridge formation (Szalai et al. 2009). In addition, GSH may control gene expression through glutathionylation and thiol-disulfide conversion. The metal-induced depletion of GSH has been reported in different plant species (Jozefczak et al. 2012). However, it has also been postulated that PAs could act as metal chelators (Mandal et al. 2013b) and could retrieve the depletion of the cellular redox in such a way that more GSH is retained in the tissues (Groppa et al. 2007). In the present study we recorded significant reduction of GSH:GSSG ratio under application of Put. 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.

Ascorbic acid (AsA) is another non-enzymatic component of anti oxidation path to stabilize the cellular redox (Loscos et al. 2008). Conventionally, oxidation of AsA is
operated through two sequential steps under stress condition in plant tissue, where mono-dehydroascorbate (MDHA) converted into dehydroascorbate (DHA) and AsA, whereas spontaneously AsA is reduced by APX following Halliwell Asada pathway (Sharma et al. 2012). Furthermore, reductase enzyme could replenish this DHA by a using reducing equivalence of GSH. In the present study we observed that the down regulation of ascorbate amount with increase of Al doses and interestingly PA interrupted with AsA by inducing its content when supplement with Al. Therefore, we can emphasize that AsA and GSH are important non enzymatic moieties for balancing the antioxidant demand in plant tissue (Mandal et al. 2014).

It is the plants inherent property to exercise the system donating reducing equivalent with different coenzymes moieties (NADH/NAD(P)H) to those free anionic superoxide radical. A total cellular system that accommodates the different pathways maintaining such reducing potential is called reducing power of the tissues. This is more expressed under abiotic stress inducing oxidative exposure to plant (Eckardt 2006). An elevated values of reducing power and its exercise by plant is attributed by proton donation capacity of different reducing equivalent (NAD(P)H, FADH, GSH dehydroascorbate, non thiol protein etc.) is studied in many plant species under chilling tolerant, heat exposure salinity, metal toxicity (Ferreira et al. 2007). The increase in reducing power with Al concentration and its down regulation by Put application thus may be supportive of the earlier findings. Like PA, the other exogenously applied elicitors (like proline, glycine betain, sugar alcohol, salicylic acid etc) have similar demands with their efficacies to interact with total reducing power of tissues and anyways that reduce the oxidative stress (Hoque et al. 2007; Ahmad 2010, 2012; Aldesuquy 2013).

From the ongoing discussion it becomes prudent that bioaccumulation of Al could be significantly marked as an effective hazardous metal for inducing oxidative stress in Salvinia plants. For this purposes, plant tissues are also tuned to diminish this over oxidation by enzymatic and non-enzymatic pathways (Exley 2004). Therefore, a number of antioxidative enzymes in plant system have important role to overcome from the ROS effects. Enzymatic cascades with a well regulated series of proteins can react in a
sequential manner. Such enzymatic proteins are variably as well as discriminately distributed in sub-cellular fractions of the cell with differing in their electron donors as well as kinetic parameters. These enzymes are predominantly concentrated in chloroplast, mitochondria, peroxisome and other allied cell organelle harboring electron transport chain as well as apoplastic spaces, cell membrane and cell wall interphases. The most notable isoenzymes like superoxide dismutase, peroxidase, catalase, glutathione reductase etc. are functionally expressed in plants; convert ROS into their less reactive forms.

SOD happens to be the first line of defense system for enzymatic conversion of ROS in Halliwell-asada pathway. Plants initially starts with dismutation of superoxide (O$_2^-$) to H$_2$O$_2$ and O$_2$ with one electron reduction by SOD for combating the ROS induced damages in *Salvinia* plants. In plant system, chloroplast and mitochondria are the major sites of ROS generation and therefore, the isoforms are mostly expressed to lysis the ROS. SOD comprising of a family of metallo-enzymes as it required metallic cofactors, occurs in different isoforms as Cu/Zn SOD, Mn SOD and Fe SOD (Achary et al. 2008). In our experiment, the induction of SOD activity were observed with the increasing of Al concentration as well as with higher duration and its modulation with Put has distinctly demonstrated in the result section. However, as the highest activity shown in 7 days of duration in our experiment, hence, we have performed the enzymatic expression through in gel study at that duration only and observed the variation in expression of SOD with different isozymic forms as a function of Al concentration. This is consistent with the findings of others where metal stress had effectively being proved as an inducer for the SOD gene (Roychoudhury et al. 2012). However, further in detailed study is required to check the sensitivity of those isoforms with requirement of different inhibitors for their proper identification in *Salvinia* plants.

In plants, the next line of defense is furnished in relation to detoxification of ROS are performed by peroxidase, which is distributed in various cellular organelles and can deviate the sustenance of cellular redox under oxidative stress. In our present experiment we have concentrated on two most predominant isoforms: Guaiacol peroxidases (GPX)
and Ascorbate peroxidase (APX). The phenolic residue as electron donor is guaiacol as commonly used for GPX. In 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). The ability to scavenge the ROS by both the enzymes tested in case of both showed similar trend. It recorded a significant rise in activity under Al treatments, which depends on doses and duration. The GPX and APX activity recorded maximum at highest dose of Al treatments (i.e. 480 µM of Al) and highest duration of incubation (7 days) in our present experiment as compared to control condition. However, the application of PA (Put in present experiment) could mitigate the enzymes activity. Peroxidases catalyze the H$_2$O$_2$ with some of phenolic compounds as electron donors. However, the activities of those enzymes are variable according to tissue specificity. Since, root is the most vulnerable tissue for Al toxicity therefore 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). This refers to the accumulation of H$_2$O$_2$ in the cellular environment under metal toxicity and detoxification of H$_2$O$_2$ by GPX and APX are regarded as regulation for threshold values of H$_2$O$_2$ which plays a role in signaling pathways (Mandal et al. 2013b). Therefore, the induction of GPX, APX and other H$_2$O$_2$ lysing enzymes is accepted as stringent regulation of cellular concentration rather than a complete eradication. As highest activity of peroxidases shown at 7 days of treatments, hence, for isozymic profiling through in-gel study of peroxidase performed at 7 days only. The various isoforms of GPX and APX under Al treatments and differential expression pattern have been observed, though hardly there is any difference in band numbers but in band intensities in *Salvinia natans* L. A number of isozymic variations in angiosperm have been reported for differential antioxidative responses under salinity stress (Ghose et al. 2012). Moreover, the variation in isoenzymic patterns and its distribution in tissues constitute the plant’s potential for diversification of a gene and its expression potential under extreme conditions (Asthir et al. 2009). Therefore, it is another way to relieve the plants from overdoses of H$_2$O$_2$. In fact it is the amount of H$_2$O$_2$ produced as compared to enzyme concentration of that duration for stress imposition and the genotypic discrimination that actually determine the ways of H$_2$O$_2$ detoxification. Thereby plants reduce the extra load of H$_2$O$_2$ over the required concentration for cellular
signaling purposes. Contextually the purposes of PA as already documented either to reduce the ROS generation or/and induction of enzymatic pathways of these two peroxidases could also be illustrated with our results in relation to Put mediated enzymatic recovery. Put has also been implicated as a molecule to generate H$_2$O$_2$ by its own catabolic pathways with diamine oxidase (DAO). H$_2$O$_2$ so generated supposedly may be triggering other antioxidative moieties so that the over expression of GPX and APX are revealed. Whatever the case may be, PA undoubtedly has improved the overall antioxidation pathways under Al toxicity as reported in other studies (Chen et al. 2008).

Unlike GPX, APX and other peroxidases, another class of antioxidizing enzyme is catalase (CAT), which not requiring any electron donors but can also lyse H$_2$O$_2$ derived from other biochemical reactions in plants. Hence in peroxisome, glyoxisome and other sub cellular fractions, H$_2$O$_2$ derived by glycolate metabolism is targeted by CAT. In our present experiment, CAT activities recorded a significant down regulation under Al concentration irrespective of duration than control. On the other hand, CAT activity was significantly retrieved by Put. The ionic imbalance might discriminate the affinity of enzyme from its substrate or even also denature the enzyme protein; 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, inhibit specific ions flux (Guo et al. 2013). Thus, the scarcity of ammonium under Al contamination particularly in acid soil might set a limitation for optimum activity (Rout et al. 2001). At optimum duration (7 days in the present case), the variation in isozymic expression in Salvinia plants with their band patterns and intensities under Al toxicity is an indication for oxidative stress mitigation. Important findings have been observed that the isozymic profiles of CAT had significant alteration in Salvinia plants of the present experiment when treated with Put under Al stress as compared to control. A marked decline in CAT activity was also documented with similar explanation in mustard seedling, pea, radish and even rice also under varying inducers (Ghosh et al. 2012; Choudhary et al. 2010).

Antioxidation system in plants is required to maintain an adequate redox of the tissue even under oxidative exposure. Now, among nonenzymatic antioxidant moieties,
glutathione (GSH), a tripeptide, is the key component for maintaining the redox state in plants often attains an over oxidized state, more under metal toxicity. According to Halliwell–Asada pathway, the interconversion between ascorbate and dehydroascorbate are accomplished with subsequent donation of electrons in reversible way (Halliwell and Gutteridge 1999). Almost in all the cases glutathione with its reduced forms (GSH) plays the role of electron donor and acts as a reductant. Therefore, maintaining a high ratio of reduced (GSH) to oxidized (GSSG) glutathione is an essential phenomenon and this conversion is mediated by glutathione reductase (GR) (Foyer and Noctor 2005; Yannarelli et al. 2007. With this concept the activity of GR and its existence in multiple forms sets a marked feature for oxidative stress tolerance in plants. GR constitutes of a multigenic family and expressed with variable isoforms in plant organs (Ding et al. 2012). The GR activity consistently induced with the increase of Al concentration and also with the duration. The optimum results obtained under highest concentration of Al treatment (i.e. 480 µM) at 7 days of duration over control. In addition, the alteration of GR activity with Put treatment is more indicative of the fact for the efficacy of PA in recycling of reduced glutathione (GSH) under Al exposure. The varying number of isoforms as well as different intensities of bands at highest duration (7 days of treatment, as maximum activity noted) confidently advocates the expression potential of GR under Al toxicity. It has been reported that GR activity, in actual, is a composite effect of individual isoenzymic proteins and some of those are differentially expressed in plants (Hall 2002). In any case PA is evident in service for maintenance of cellular stability appropriately even in an indirect manner and under non denaturing gel; its intensities might vary for GR activity for depletion of cellular redox in terms of GSH.

Glutathione S transferase (GST) is the enzyme which involved with detoxification processes and catalyzes the conjugation of several xenobiotics to reduced glutathione (GSH) which has the ability to donate an electron from its sulphohydryl (-SH) group to form a ligand with free anionic superoxide radical. In the present study, GST activity could be thought as an adaptive feature for *Salvinia* for encountering oxidative stress with an over expressed activity of the enzyme. The level of tolerability to ROS particularly, the metal induced super oxide and even to other xenobiotics forms ligands with
glutathione (Nahakpam and Shah 2011) are also observed. GST attracts its notification as reliable traits for protection of sub cellular fraction particularly for membrane as it could sequester or compartmentalize the oxidative moieties into vacuoles or apoplastic spaces (Mannervik and Guthenberg 1981). In our present experiment in Salvinia plants, GST activity induced under Al concentration, as the similar trend has been reported earlier in Soybean under Al contamination (Zhena et al. 2007) and Put act as modulator by diminishing the activity. Thus, the observed increase in GST activity in the Salvina natans L. exposed to Al may be considered to be indicative of an oxidative stress induced by that metal. Concomitantly, the induction of GST happens to protect the Salvina plants from oxidative injury, functioning as the glutathione peroxidase, by using glutathione to reduce organic hydroperoxides (Vestena et al. 2011).

From the result section we have noticed that under condition of elevated Al doses, Salvina plants undergone an oxidative stress with concomitant induction of antioxidative pathways. Among the enzymatic cascades, glutathione peroxidase (GuPX) plays an important role to minimize the oxidative damages by using glutathione as an electron donor (Sani et al. 2012). GuPX has the efficacy in peroxyl scavenging mechanism also and it maintains functional integrity of the cell. GuPX has another function, to reduce lipid hydroperoxides to their corresponding alcohols and reduce free H₂O₂ to H₂O (Ragavendran et al. 2012). The induction of GuPX activity recorded in result portion with the increasing concentration of Al as well as at maximum duration. Moreover, elicitors like Put reduced the GuPX activity significantly. From the result section, another important variation has been noticed with in gel SDS PAGE staining, where intensities of bands are varied under Al concentration and also with Put treatments. The variation of band intensities might be occurring due to various expression levels of GuPX gene in Salvina plant. This has also been studied by cDNA profiling through amplification and followed by resolving in agarose gel. We have noticed some variation in intensities.

In regard to phenyl propanoid pathway, the rate limiting steps is attributed by Phenyl alanine ammonia lyase (PAL) on phenyl alanine act as a precursor. PAL is the most cultivated rate limiting enzymes that is behaved as a marker for characterization and
illustration of phenolic moieties generated in plants (Hawa et al. 2012). Thus, Al being a prooxidant and its concomitant nature to induced oxidative stress is guided by the *Salvinia* plants with PAL activity. The variation in expression with better adaptability of PAL under salinity and thereby its resultant in increase of subsequent polyphenolics makes the plant more tolerant to oxidative stress (Liu et al. 2008, Basu et al. 2010). *Salvinia* plant in the present experiment is not exception of that. The over expressed activities of PAL has moderated by the Put application lowering the accumulation of phenolics in plants and may suggest that PA could be trying to restore the adequate redox of the tissues and thus compensate the demand of phenolic biosynthesis. It could be speculative as well as supportive also that application of Put; a diamine could be a source of H$_2$O$_2$ by oxidative deamination. H$_2$O$_2$ is known to be an effective secondary messenger to evoke other antioxidation pathways, particularly, the enzymatic ones those may subside the need of polyphenolics (War et al. 2012).

As already mentioned that ascorbate is the most unique and abundant antioxidant which serve as electron donor in energetic antioxidation pathway (Hossain et al. 2010). Besides its role in direct quenching of O$_2^-$, OH$, some peroxidase, mostly chloroplastic and are characterized to lyse H$_2$O$_2$ with ascorbate as the substrate. Therefore level of endogenous ascorbate in plants should be maintained optimum to support the ascorbate glutathione (ASA-GSH) cycle particularly under conditions of oxidative stress. Glutathione reductase (GR), dehydroascorbate reductase (DHAR) and monodehydroascorbate reductase (MDHAR) are the key enzymes responsible for maintenance of redox (AsA and GSH) pool of the cell (Chopra and Semwal 2011). Two important enzymes: MDHAR and DHAR are indexed with regard to ascorbate metabolism under oxidative stress. In fact univalent oxidation of ascorbate in APX mediated reaction leads to the formation of Monodehydroascorbate (MDHA). The later has two facets according to cellular demands: direct replenishment of ascorbate by MDHAR and non-enzymatic disproportionate conversion of Dehydroascorbate (DHA) that may undergo enzymatic conversion to ascorbate before it is lost. Therefore, DHAR is now indexed as key regulatory enzyme in ascorbate metabolism (Wang et al. 2010). *Salvinia* plant recorded an inducing activity of DHAR which is indication of the strength of recovering ascorbate and could be utilized
under oxidative stress. Put had diminished the DHAR activity by up regulation of H$_2$O$_2$ generation so that more ascorbate is required for APX activity.

Peroxidases are variably distributed in different sub-cellular fractions, cell wall and even in apoplastic spaces. Peroxidase, which is typically found in cell wall and mostly required for cell wall lignifications accomplished by catalyzing the oxidative polymerization of phenolics residues called wall bound peroxidase (Larue et al. 2010). This belongs to type III peroxidase, constitutes those which required phenolic residues as electron donor and H$_2$O$_2$ might be used as a substrate to be lysed by wall bound peroxidases to generate free radicals, which are required for cross linking or polymerization of phenolics on the cell wall (Aptith et al. 2009). Lignifications on the cell wall could be indexed as an adaptive trait for metal tolerance in plants. In our experiment, induced activity of wall bound peroxidase is found in Salvinia natans L. which might be a trait for Al induced resistance. Moreover, when the enzyme was run in polyacrylamide gel different isozymic bands, varying in molecular sizes and intensities were resolved (Mandal et al. 2013c). As expressed in other study, where the over expressed bands for different isozymes of such peroxidases were characterized in plants under Al contamination (Almagro et al. 2009). Possibly that might be suggestive to the fact that environment mediated gene regulation to support lignifications. On the other hand Put somehow relieved the water stress and also down regulates the activity under Al toxicity, followed by chances of less mechanical injuries and thus activities was also down regulated.

The toxicity of any metal imbalances is preliminarily the water relation in plants. The later undoubtedly be an important factor for curtailing any sort of cellular pathway for anabolic and catabolic metabolism and thus photosynthetic carbon fixation becomes the predominant of those. Thus with the view of photosynthetic performances under Al toxicity the behaviour of Salvinia plant was analyzed in terms of few enzymatic assay. Phospho enol pyruvate carboxylase is an enzyme that catalyzes the conversion of PEP to OAA which is then subsequently decarboxylyzed and significant amount of pyruvate produced during this process. Concomitantly, CO$_2$ might be metabolized by the leucoplast for more complex bio-moieties including various carbon compounds mostly
required for carbohydrate metabolism. PEPC, however, is regarded as the key enzyme for C₄ photosynthesis, still, its non photosynthetic form as found in cytosol of C₃ plants, those are also equally important (Sangwan et al. 1992) Salvinia being a typical C₃ plant are characterized with an increased activity of PEPC which was assayed in coupled enzyme assay with malate dehydrogenase. Similar increasing trend also reported under stress condition according to Idrees et al. (2010). The over expression of PEPC activity in an increasing order as a function of Al concentration undoubtedly suggest the involvement of more carbon flux in addition to normal photosynthetic carboxylation. Salvinia being a typical aquatic plant, dissolve carbon in the form of bicarbonate are more accessible than gaseous counterpart. Therefore, induction of PEPC with a preferred substrate as bicarbonate may be indicative of carbon acquisition in non photosynthetic ways. Non photosynthetic PEPC are also important for reversible phosphorylation in various tissues of C₃ plants, especially during its role in C/N metabolism (Chastain et al. 2002). It also important to broaden the possibility of other keto acids those could be replenishing the ongoing TCA cycle. Possibility cannot be ignored that the impairment of normal TCA cycle under oxidative stress might be ameliorated with incorporation of keto acids (oxaloacetate and malate) from other sources (Arora 2002). From this over accumulation of keto acid might goes another possibility in ligand formation for binding of Al. Thereby possibility may arise to fulfill the role of PEPC in metal scavenging processes with reference to Salvinia, in non angiospermic plant in the present experiment.

NADP-ME is the most regulatory enzyme for photosynthetic pathway except the carboxylating enzyme as RUBISCO. Characteristically, NADP-ME behaves as decarboxylating protein predominantly sitting in bundle sheath of chloroplast (reference to C₄ type only) and engaged in generation of pyruvate from malate with a release of CO₂ (Wingler 1999). Additionally, with non photosynthetic NADP-ME isoforms particularly in C₃ plant makes an alternative provision for NAD(P)H⁺H⁺. Specifically, this enzyme maintains the inter cellular pH in synchronization with activation of pyruvate carboxylase (PEPC). The NAD(P)H⁺H⁺ as retrieved from the NADP-ME mediated reaction is used in biosynthesis of proline and manitol as compatible solutes (Chi et al. 2004). Under metal stress, regeneration of cell membrane lipid is partially accomplished by supplying of pyruvate and NAD(P)H through fatty acid biosynthesis (Sagi and Fluhr 2006). Therefore,
analysis of NADPME in Salvinia plants under Al interference may be imperative to justify. The results of the present study as illustrated in sensitivity of metal was tested thoroughly under exposure of light and dark. Through this treatment, a synchronization of this activity was recorded which essentially indicated the light mediated promotion of the enzyme. Undoubtedly, the Salvinia plants were undergone a photosynthetic impairment under Al toxicity as recorded in earlier studies with the fluctuation of photosynthetic flux (Gardner and Al-Hamdani 1997). Therefore the promotion of activities for NADP-ME could be attributed essentially to support the photosynthetic behavior in the present experiment also. Metal stress and its tolerance could be employed in a significant amount of fixed carbon (mostly, in the form of organic acid with different carbon residues) into the generation of compatible solutes and thereby releasing the plants deficit for organic acid. Understanding the biochemical mechanism involved in carbon metabolism by NADP-ME the inter conversion of organic acid for replenishment of the deficit mentioned could be understood in a significant beneficiary way to plants (Celik and Atak 2012). Metal toxicity and its induced development of water deficit and its concomitant stomatal behavior have thoroughly being justified in respect to NADP-ME activity. Plants induced NADP-ME activity is circumvented with more retention of hydration and thus cellular osmotic turgidity is sustained. In the present experiment the variation in said enzymatic activity amongst Al treatments may be supportive for their inbuilt or it’s over induced potential to resist the dehydration state of the tissues out of Al stress. For metal stress with its secondary effect to generate and over production of ROS could be mitigated by induction of antioxidative enzymatic cascade. The attention of NADP-ME is drawn in favour of stomatal regulation with the support of supplementation of organic acid into the guard cell which undergoes deficit under water stress (Doubnerova et al. 2009). Therefore, the up regulation of NADPME activity could lead to conclude possible impact on stomatal functioning which might be perturbed under Al exposure. The sustenance of NADPME activity of the Salvinia plant with the aid of Put is interesting to note. A number of report suggests that PA had direct effect on restoration of soluble protein mostly those constitute the membrane structure as well as the chloroplastic protein for photosynthetic reaction (Chi et al. 2004; Liu et al. 2007). Therefore,
application of Put on plants as shown in our experiment to suppress the NADP-ME activity results by less organic acid flux to the chloroplast (Hyskova et al. 2014).

From the result section in our present study, the responses of *Salvinia* plants under Al contamination had showed significant physiological variation to metal toxicity. This has been more informative when PA as Put is applied along with Al concentrations. Metal toxicity interfered by PA has been quite an interesting domain in elicitor mediated plant responses (Bergmann et al. 2001). PAs as quite elaborately studied in plant’s growth and developments are referred as aliphatic, straight chain aminated moieties in alkane backbones. It is highly protonated at cellular pH behaving as ideal polycationic in nature and show its affinity to bind with negatively backbone of cellular and sub cellular fractions in plants (Takahashi and Kakehi 2010). Those include mostly the cellular membrane, negatively charged protein residues, carboxylic acids, fatty acid chain and negatively charged domains of nucleic acid. Admitting this, we assayed PAs concentration of both free and bound fractions from the Al treated *Salvinia* plants supplemented with Put also. From the result section it is clear that the various fractions of PAs were not bearing any significant consistencies among the treatments but, the total PA recorded to be increased through the Al treatments. Whereas, when Put was fed to plants, the up regulation of PA accumulation was recorded. From the demonstration of PA in plants, it is the bound PA that is more offered to be over expressed under abiotic stresses (Ghosh et al. 2012). In the present experiment, therefore, increase in bound PAs as a function of Al concentration may be supportive of the fact that the readily biosynthesized PAs are more allocated to conjugate with some cellular moieties. Hydroxy cinnamate like p-coumaric acid, caffeic acid are more common in conjugation with di, tri and even tetra PAs in plants under stress (Quinet et al. 2010). The discriminatory nature of individual PAs and its varied supportive role are the stronger evidences for genotypic potential of a plant species to express the PA profiles according to doses of stress, duration of stress, plant ages and other factors. This is also documented that neither of individual PA could compensate directly any short fall of metabolic flux of the tissues regardless of plant species (Gill and Tuteza 2010; Liu et al. 2004). Therefore, it is confirmed that bound PAs are more required in abiotic stress tolerance mechanism. Our interest to ensure that the
PA content with its fractions in *Salvinia* plants grown under Al toxicity, could be focusing some insight of PA interaction with cellular processes. Therefore, we have adopted a fast, inexpensive, and reliable method for PA quantification. In our present experiment, Thin layer chromatography (TLC) techniques have been performed with dansylated PAs extracted from each treatment and also with dansylated synthetic standard (Put, Spd and Spm in the present case). Thereby, PAs have been easily identified by comparing with each standard against each spot marked from experimental material. This was followed by scraping of individual spot and its elution through suitable solvents following spectrofluorometric analysis. However, for purification and more authentications of PAs, the study was more supported with reverse-phase high performance liquid chromatography (HPLC) system which is suitable for direct determination of PAs in crude extracts, with good recovery within short analysis time. The resolution of the common PAs from their less common homologs is better when compared with the HPLC systems than that found with TLC techniques. More so, the preliminary identification of the unknown peaks of plant extracts could be done with the reproducible retention times obtained from the HPLC of PA standards. In addition, each peak was spiked with known amounts of a particular standard, and by measuring of the areas of specific peaks confirmed the nature of the unknowns. The correlation of PAs fraction by HPLC and TLC further supported the nature of the peaks observed in all samples studied in this investigation. The variation in peaks of PAs clearly revealed that it was significantly accumulated in *Salvinia natans* L. under Al treatments and also supplemented with Put in the present experiment.

As already mentioned that plants could maintain a steady state pool of PA under normal condition, therefore, some inbuilt cellular mechanism must be on to maintain the up/down regulation of PA, particularly under stressful conditions. A fine regulated synthesis of PAs and its turnover by committed enzymatic steps is the key factor for allocation of free and conjugated PAs (Bouchereau et al. 1999). Thus for the biosynthesis of PAs is initiated with the formation of diamine Put where ornithine decarboxylase (ODC, ) or arginine decarboxylase (ADC) are required for direct or indirect pathways respectively. For ODC, it is one step process where ornithin is converted into Put. In the
ADC catalysed reaction the arginine is converted into agmatine and N-carbamyl Put respectively by agmatine iminohydrolase (AIH) and N-carbamoyl Put amidohydrolase (CPA) respectively (Alcazar et al. 2010). Once Put is produced via ODC or ADC, it serves as a precursor of triamine (Spd) and tetramine (Spm). Spd and Spm are formed by successive additions of aminopropyl groups and catalyzed by Spd synthase (SPDS) and Spm synthases (SPMS) respectively. Decarboxylated S-adenosylmethionine (dcSAM) is the donor of aminopropyl groups, is formed by decarboxylation of SAM in a reaction catalyzed by SAM decarboxylase (SAMDC) which is the most targeted enzymes in this pathway (Slocum et al. 1984). A number of studies concerning functioning of SAMDC revealed over expression or even down regulation (Ghosh et al. 2012). In the present experiment, Salvinia plants have also monitored with expression potential of SAMDC through immuno blotting techniques. A distinct variation in protein expression through the courses of Al treatment has supported the views for the role of SAMDC in metal stress. SAMDC has another impetus to the plant metabolic profile that it happens to be the check point whether plants are preferred to shift the downstream reaction either to PA (Spd/Spm) or ethylene biosynthesis (Martin-Tanguy 2001). It is the genotype specificity as well as cellular requirement to combat the types of abiotic stresses that plants prefer a steady over expression of SAMDC activity. Undoubtedly, it leads the plant for accumulation of more PAs which has been intensively studied under abiotic stresses. The application of Put recorded an over accumulation of total PA pool in Salvinia plants under Al treatments. These supposedly favour the plants for deposition of PAs for further use as and when necessary to meet the abiotic stresses. However, it needs further extensive analysis to come to an established conclusion supporting the precise role of SAMDC alone or in combination with other enzymes favouring the overproduction of PA in a regulatory state.

Plant responses to abiotic stress and its up/down regulation of PA profile are discriminatory according to its various fractions. Hardly any of those could be precisely be an index in consistent manner for any sort of abiotic stress. The excess accumulation of free Put sometimes appears as more toxic to plants and also set a demerit to plants under abiotic stress (D’Cunha et al. 1996). This was so anticipated with the generation of
H$_2$O$_2$ over the threshold concentration with oxidation of Put. PA metabolism in plants may involve the turnover of this moiety through oxidative deamination by the action of diamine oxidase (DAO) and PA oxidases (PAO) (Fariduddin et al. 2013). DAO is Cu containing enzyme which catalyzes the oxidation of diamines (like Put) on the primary amino groups. As a result NH$_3$, H$_2$O$_2$ and 4-aminobutanal are produced which is further converted to $\gamma$-aminobutyric acid (GABA) via $\Delta$1-pyrroline (Moschou et al. 2008). The oxidative intermediate molecules are earlier thought to be evolved as an outcome of hypersensitive reaction (HR) on plant pathogen infestation or direct metal or abiotic stress influences. Later on it was established that oxidative deamination of some endogenous compound might be source of ROS development (Achary et al. 2012). PA could be more available to generate ROS by several enzymes like NADH/NAD(P)H oxidase (Ishida et al. 1987), some Cu-containing amine oxidase (Quinet et al. 2010) and PA oxidase (Martin-Tanguy 2001). The activity of these enzymes is otherwise required for a useful provision of H$_2$O$_2$, a key factor for different peroxidation reaction, particularly, on cell wall (Almagro et al. 2009). Wall bound peroxidase are most favoured to be over express in relation to cross linking of phenolics residues leading to lignifications (Gill and Tuteja 2011). Therefore, PA catabolism indirectly may circumvent the lignification pathways for tissue strength under metal stress. However, DAO activity was reduced under AI stress in the present experiment but Put could retrieve its activity. In earlier report to support the increased level of Put in metal treated leaf discs, ODC enzyme was primarily responsible. It also showed in wheat leaves, the increased Put content under metal in excess might be synergistic to an inhibition of DAO activity (Groppa et al. 2007). More over in the present case the over activity of DAO is also revealed from the few polymorphic isoforms revealed through in gel studies under AI stress. The expression potential of diamine oxidase were co-operative and concentration dependent of AI toxicity so revealed from the band intensities.

HSP happens to be the most conserved protein which is universally present among the plant genotypes, however, with considerable variations. These variations may exist with tissue specificity, degrees of expression and homology of sequences. HSP are encoded to a large number of families of highly conserved genes and at the cellular level it functions
as molecular chaperons to perform a number of functions. In consecutive mode these proteins shares with protein folding glycosylation, assembly of heteromers, degradation as well as translocation (Goswami et al. 2010) Hsp are found in almost all cellular compartments. Hsp constitutes with variable molecular ranges and amongst those 70kDa Hsp (Hsp-70) appears to be the most predominant. Characteristically, metal stress being analogous to oxidative stress has been reported to be involved in the induction of Hsp-70. Admitting these, Al granted as a proxidant, is hypothesized to induce the Hsp-70 in Salvinia plant in the present experiment. Thus, we have documented the Al toxicity with reference to Hsp-70 induction. This clearly supports our hypothesis that Salvinia plants are also prone to oxidative or heat related damages, thereby, it has imprinted the cellular impacts in Hsp-70 over expression. More importantly, the expression potential shown by Salvinia plants at the molecular level could be occupying the potential place of biomarker or bio-indicator regarding environmental quality concern (Achary et al. 2012; Tukaj and Tukaj 2010; Tukaj et al. 2011). Salvinia in the present experiment established as significant respondent showing the Al induced toxicity at the cellular level (Mandal et al. 2013b). At the molecular level the specific biochemical changes attributed by various pathways under gene regulation, can be sited with Hsp-70 expression in aquatic environment. Few plant species from freshwater have been reported as strong indicating species with co-linearity of metal accumulation (Dhir et al. 2011). Therefore, in our present study, it could not be less, at least show evident from the Hsp-70 over expression in Salvinia plant taking as an effective bio-indicating species for Al. On account of Put application it could be thought that Salvinia plants had recovered the Al toxicity in any such way that might not be demanding the cellular shielding and thus protection by Hsp-70 gene to be over expressed.

As already mentioned that glutathione is implicated with its multiple roles in plant system to impart the defense mechanism against oxidative stress. This has also been evident in many crop species as well as corroborated with our experimental material as Salvinia that GSH is behaved as sensor for cellular redox that very often undergoes towards more oxidized state (Milla et al. 2003). To accomplish the cellular defense and to replenish the reduced glutathione from the oxidized and reduced transition (GSSG-GSH), a number of
genes coordinate the cellular antioxidation property. Among those, glutathione peroxidase (GuPX) constitute a family of enzymes that targets a number of molecules for reduction of $\text{H}_2\text{O}_2$, organic hydro-peroxides, lipid peroxides, alkyl peroxides etc., taking glutathione as electron donor (Ragavendran et al. 2012). According to multigenic origin, GuPX represents several isoforms in plants with varying molecular structure, substrate specificity and sub cellular localization. In the present experiment, we have analyzed the expression pattern of GuPX in *Salvinia* plants from Al induction along with Put supplementation. By using RT-PCR technique with designed gene specific primers from published GuPX ESTs sequence (Accession No. EF620779) the expression was analyzed. In brief, we recovered cDNA, run in agarose gel and the band of 0.5 kb was retrieved. From the results the RT-PCR fragments show the signal for induction of Al accumulation in a dose dependent manner. Moreover, the expression was down regulated when Put was added. It has earlier been observed that PA in any form (di, tri and tetra amine) can reduce the oxidative exposure out of salt, metals and even extreme temperature also. It is widely accepted that lowering of ROS activity and its accumulation either by any chemical elicitors like Put could be cited of those which could bring back the adequate redox of the tissues. Therefore, regulation of GuPX mRNA level might be down regulated as shown in the present experiment by Put application, however, not significantly. A number of plant species have been reported to over express the GuPX transcripts by increasing the expression of GuPX according to metal stress (Fu 2014). To support our findings it was earlier reported by by Sugimoto and Sakamoto (1997) that expression of GuPX enhances when *Arabidopsis* seedlings were treated with NaCl. Interesting to note that salt increased transcript level of GuPX are variable according to tissues specificity and there found no direct correlation with activity of GuPX against transcript abundance (Chang et al. 2009). Still, in our experiment the pattern of abundance of transcript under Al as well as Put supplementation followed the similar patterns when enzyme activity *in vitro* was concerned. However, with regard to *Salvinia* plants, an aquatic pteridophytic species could be employed for bio indication under Al toxicity with transcript profile of GuPX.
Incorporation of nitrogen in the plant system is primarily based on availability and acquisition of nitrate, ammonium salt and di-nitrogen (N\textsubscript{2}) moieties as external nitrogen sources. Ammonium is the final form of inorganic nitrogen prior to the synthesis of organic nitrogen compounds. This could be produced via internal metabolic reactions, like photorespiration, hydrolysis of nitrogen molecules and amino acid conversion etc. (Ireland and Lea 1999). Deposition of metals in the soil over a limit may cause plant toxicity, which impedes the nitrogen metabolism. Nitrogen remobilization is essential for natural leaf senescence to support the new organ emergence and growth (Masclaux et al. 2001). Therefore, the responses of plants for nitrogen metabolism under Al contamination is required to study in reference to physiological and biochemical aspects in \textit{Salvinia} plants in the present case with reference to PA interaction. Nitrate reductase (NR), the enzyme which initiates the first step of inorganic nitrogen reduction in the plants by donation of two electrons to nitrate salt using NAD(P)H as electron donor. A number of isoforms of NR are abundant in plants according to its location in sub cellular fractions. NR activity showed a linear decline with ongoing concentration of Al which could retrieve with application of Put Sharma and Dietz 2006) The rapid fall of NR activity could preliminarily be related to alteration in NADH / NAD(P)H pool in the tissues which often undergoes a change under metal toxicity (Dinakar et al. 2009). This is based on the fact that under metal or any abiotic stresses, generation of NADH/NAD(P)H is the key shortfall for products of photosynthetic light reaction. Therefore, the inadequacy or depletion of NADH/NAD(P)H might pose the limitation for NR activity. PAs are reported as most useful to bind to the cellular membrane with its positive charges. Under this condition, it could also donate protons (H\textsuperscript{+}) to the cellular environment to reinforce the adequate redox in reducing state. Therefore, the retrieval of oxidative changes under metal stress and its recovery of NR activity by PA has also been documented earlier (Athwal and Huber 2002) and established the PAs in amelioration of nitrogen metabolism under metal toxicity (Wang et al. 2012; Roychoudhury et al. 2012). Glutamate dehydrogenase (GDH) is the enzyme catalyzing the reductive amination of 2-oxoglutarate (2OG) and the oxidative deamination of glutamate in plant tissues (Restivo 2004). With regard to nitrogen metabolism, GDH is the key enzyme required to maintain a steady conversion of keto acid to amino acid. It is very often shifts towards more keto
acid acquisition for sustenance of respiratory reactions Haouari et al. 2013), particularly, under stressful conditions. The keto acid initially behaves as a precursor for amino acid biosynthesis through transamination reaction. The biosynthesis of amino acid under abiotic stress, could be enhanced and featured by up regulation of GDH activity as documented in many crop species (Tripathi et al. 2013). In the present experiment, the increase in activity of NAD(P)H-GDH follows the catalysis of ammonium (NH$_4^+$) in the plants under Al toxicity. More so, the supplementation of Put reduced the GDH activity under same condition. In case of NADH-GDH activity, increasing trend showed in Salvinia plants could be postulated for the access of NH$_4^+$ accumulation which may stimulate the functioning of GDH. Therefore, another alternative pathway may exist in plants to supplement the NH$_4^+$ in tissues that is circumvented by NADH-GDH activity in cytosol /mitochondria (Reguera et al. 2013). NADH-GDH may play main role in ammonium assimilation and be one of the important mechanisms of plants to resist metal stress. Thus, GDH keep its special contribution in nitrogen metabolism for abiotic stress tolerance in plants. Glutamine-2-oxoglutarate aminotransferase (GOGAT) belonging to the family of oxido reductases. The level of ammonium ion (NH$_4^+$) is most important to be maintained within the threshold concentration in plant tissues. It is because of high level of accumulation of NH$_4^+$ is fairly toxic in many species. There happens to be a few alternative sources of NH$_4^+$ in plants besides direct absorption of NH$_4^+$ by roots. According to species specificity and cellular demands, NH$_4^+$ availability includes photorespiratory reactions, NO$_3^-$ reduction, degradation of storage compounds etc. GOGAT causes a major re-assessment of the way in which ammonium is assimilated in the plants related to ferredoxin- and NADH-dependent enzymes (Lea and Miflin 2003). GOGAT acts on the CHNH$_2$ group of donors with NAD$^+$ or NADP$^+$ as acceptor and it has cofactor like FAD, FMN iron, sulfur etc. It has been shown that enzymes of nitrogen metabolism are differently affected by Al stress (Syed et al. 1998). In contrast to GDH, plant registered a decline in activity of the NADH-GOGAT under Al stress in the present experiment. Interesting to note that, the application of Put retrieves the NADH-GOGAT activity significantly. In concert to GOGAT, is another enzyme in this cascade of nitrogen metabolism is Glutamine Synthetase (GS), where the glutamate is aminated in an ATP dependant reaction into glutamine. The molecular weight and genetic studies
revealed that GS happens to be the key regulatory enzyme which operates in ammonium incorporation into carbon skeletons. In conjugation enzyme system it stabilizes amino acids and its amine derivatives by GS-GOGAT cycle in plants (Gouia et al. 2000; Shora and Ali 2011). According to tissue specific location, GS exhibits distinct variation in their isozymic abundance in different plant species (Wang et al. 2008). However, the soil salinity or metal contamination has a direct impact on ammonium assimilation in subdued activities as is recorded from the present experiment with *Salvinia*. The degree of $\text{NH}_4^+$ accumulation in plants probably reflected the changes of $\text{NH}_4^+$ metabolizing enzymes in the tissues. In fact, sensitivity of $\text{NH}_4^+$ to plant species is highly variable which is reflected in photorespiratory assimilation involving the GS activity in mitochondria (Wang et al. 2008). However, in our present experiment under Al stress, *Salvinia* showed a steady decline in GS activity. Put somehow managed the subsequent reactions and appeared as retriever for GS activity. Therefore, as a whole, supplementation of Put as a treatment helps in beneficial way to balance the amino acid and keto acid proportionately and bring back the plants potentiality to continue its proper nitrogen metabolism. Our results keep in parity which postulated that the GS-GOGAT pathway inhibited by Al was the major route of ammonium assimilation, whereas the NAD/NAD(P)-GDH finds alternative pathway, which might partially assimilate Al-induced ammonium accumulation in tissues. Therefore, it could be assumed that GS as well as GDH played synergistic roles in scavenging of excessive endogenous ammonium in *Salvinia* plants under Al contamination.

Therefore, from the facts and figures of metal induced impairment of chlorophyll and concomitant photosynthetic activities; plants become deprived of adequate carbohydrate status. Assessment of carbohydrate profiles, its generation by biosynthetic pathway and allocation in other biomolecules should be the determining attributes for any plant. Photosynthesis is the major physiological process and commonly modulated in various dimension under any form of abiotic stress. Thus, Al being a metal is considered for the carbohydrate metabolism of *Salvinia* plants. Preliminarily, it was quite expected that plants could be changing in photosynthetic efficiency as already evident from its chlorophyll content, NAR and total dry matter accumulation when measured in the form
of RGR. Thus, a significant depletion of reducing sugar according to concentration of Al is major concern of this experiment. In angiospermic crops under acidic soil, significant amount of reducing sugar along with total carbohydrate was recorded under Al exposure (Halman et al. 2013). Along with reducing sugar plants shows some established hydrolytic activities with gear up solubilization of storage carbohydrates. Under stress condition the homeostasis of cellular pools for total reducing sugar is imbalanced (Rosa et al. 2009). Therefore, an induction of osmolytic activities particularly depletion of water potential also found under metal stress (Basset et al. 2010). In our studies *Salvinia* plants could be pointed out, on the contrary, with a declining trend of reducing sugar throughout the Al doses. More so, it was accompanied with declining of total carbohydrate also. Metal induced perturbation of water relation is reflected; otherwise, fall in water potential i.e. water deficit stress is ensured. Reducing sugar is very often allowed to perform directly or indirectly as osmolytes (Mousavi et al. 2009). For the later, conversion of reducing sugar into sugar alcohol and other small glycosides are fairly encountered in plants are the osmolytes (Mousavi et al. 2009). Under acidic soil the hydrolysis of Al into trivalent form as reported to be potent toxic for plants particularly inducing the anomaly of metabolism in root tissues. Under acidic cell sap the cell wall bound invertase showing optimum activity at low pH is more induced. A few references particularly in aquatic crop species are found with significant increase of invertase activity under Al contamination. Therefore, when invertase was isolated from cell wall and allowed to incubate with substrate it shows a significant over expression. So, higher invertase activity with the increasing concentration of Al may facilitate more reducing sugar to release sucrose, to involve in verification avoiding cellular dehydration as well as coagulation of membrane protein (Prado et al. 2010). A number of crop species like corn Soybean, wheat, cotton, chick pea are also shown to be adaptive with over expression of those sugar compounds for their tolerance even under metal induced osmotic stress (Basu et al. 2010). The application of Put as perceived by the *Salvinia* plants as an efficient reliever to the carbohydrate status of the plant under said condition. The suppression of the acid invertase with concomitant drop in reducing sugar could be correlated, at least as for compensating role of osmolytes by PA. PAs with its other cellular performance have also been reported as osmo-protectant either shielding the hydrophobic domains of the
biomolecules and behaving itself as osmolytes (Aldesuquy et al. 2011). Therefore, PA could be acting as osmolytes even effective in a similar way as recorded in non angiospermic plants like *Salvinia*. *Salvinia* plants are found to be grown extensively and hence the plant is expected to be highly photosynthesizing under natural condition of environment. Sucrose is the most important moiety in photosynthetic process. In fact, sucrose synthesis through proper pathway enables plants for allocation of complex carbohydrate for dry matter accumulation. Sucrose phosphate synthase is the key regulatory enzyme for photosynthetic carbon metabolism, particularly, under stressful situation. SPS activity is reported to be modulated by diverse system of abiotic stresses. The later may be linked to a situation which influences or deviates the normal system of photosynthetic generation and its allocation. Possibility arises for inhibition of SPS activity either with ligation of UDP glucose and fructose into sucrose phosphate or/and the inadequate rate of carboxylation for synthesis of hexose sugar (Huber and Huber 1996). *Salvinia* plants have been marked to be significantly influence in photosynthetic rate and allied photosynthetic characters under contaminated water bodies predominantly with metal toxicity (Mandal et al. 2013b). Al induced photosynthetic inhibition and thereby concomitant decline in sucrose synthesis as featured by SPS activity has been documented in some other crop species (Chen 2005). Therefore, the *Salvinia* plants in the present experiment are an example of deviation of increased SPS activity even under Al stress. The pit fall in photosynthetic rate characterized by other leaf character is concomitantly affected in generation of current photosynthatase that is sucrose. Physiologically sucrose synthesis and its following allocation are determined by demand of reduced carbon in the leaf tissue, more in the chloroplast. The chloroplast being a site of starch synthesis converts sucrose into starch for storage carbohydrate in a complex reaction (Stettler et al. 2009). It is the inherent characters to sustain a continued trend for transportation of the sucrose into chloroplast under optimum condition of light and carbon dioxide concentration. This depletion of sucrose from the leaf tissues tends the plant to more carbon fixation following reduction through Kelvin cycle pathway. Thus possibility may arise to keep the SPS activity optimum or even decline in photosynthetic rates. This rational could also be fit for possible reasons of reduced NAR as well as total dry matters acquisition in the plants under Al contamination. With ongoing synthesis of
sucrose playing a pivotal role in photo assimilation plants are tuned with interconversion of organic acid in downstream anabolic reaction. Those acids are quite interconvertable as an intermediate residue of TCA cycle (Musrati et al. 1998). Malate dehydrogenase (MDH) is the enzyme that is understood one of the key regulatory proteins in TCA cycle. Out of all available forms MDH happens to be more accomplishing the dehydrogenation reaction in chloroplast and mitochondria from malic acid in a reversible way with NADP and NAD as cofactors (Kumar et al. 2000). Besides this, the most abundant isoforms of MDH is cytosolic and required for conversion with NAD which is also accounted in the present case with *Salvinia*. Under increasing concentration of Al, the conversion of malic acid into OAA is hypothesized as playing dual roles; the rapid supplementation of OAA as substrate to different amino acid biosynthesis through amination reaction and the utilization of malic acid as a chelating substance with metals (Hamel 1999; Ma 2000). With regard to the later, specificity for Al toxicity by a tolerant species is characterized with production of significant amount of malic acid to form ligand with cations (Yang et al. 2011). Therefore, the active concentration of Al could possibly be subdued upto the extent that appears less toxic in the cytosol. *Salvinia* plant an aquatic C3 species has shown an extensive growth in some case when absorb a higher concentration of metallic pollutants.. This possibly is indicative of the fact of enhanced activity of MDH that might be linked to energy yielding pathways (like TCA cycle). Al with its interference in carboxylic acid metabolism that enables one step towards siderophore like activities is interesting to note (Dakora and Phillips 2002; Huang et al. 2013). Later compound is more amenable for metal exclusion mechanism mostly offered in aquatic pollutants resistance. With reference to *Salvinia* in the present experiment may be few of those aquatic macrophytes which over express the malic dehydrogenase activity as a signal for bioindication to Al toxicity.
5.3 Effects of H$_2$O$_2$ on *Salvinia* plants and interaction of PA thereon

In the previous chapter it has been clearly implicated that Al tolerance is attended by the following consecutive major physiological domains by plants:

Maintenance of a favorable plant water states during the stress and by the recovery of Al induced oxidative stress directly or indirectly imposed to plants, therefore, possible amelioration was discussed in each domain, in terms of the imposed problems and its possible solutions. In this aspect the existing anti-oxidizing system in plants were found to be modulated with PA and it was satisfactorily justified by the elicitors in successful plant protection. H$_2$O$_2$, the most stable and even a safe ROS found irrespective of tissues as a byproduct of some metabolic reactions. For the later case, photosynthesis and photo respiration would be most suitable for reference (Sharma et al. 2012; Slesak et al. 2007). Therefore, if the plants are directly exposed to H$_2$O$_2$ in varying concentrations, expectedly the physiological reactions would be proportionate. Moreover, the methodologies and protocols become similar in nature that could be extending on the nature of variation between Al and direct H$_2$O$_2$ induced oxidative stress. The selective traits if becomes identical or even similar then H$_2$O$_2$ could be responsible as a factor for oxidative damages generated indirectly by Al induction. Admitted well the intricacies may appear for the confusion whether the applied concentration of H$_2$O$_2$ could be unsafe when compared to its threshold concentration between existing H$_2$O$_2$ pool in the tissues. With this view we have set a simple experiment how a direct exposure of H$_2$O$_2$ could effect the plants metabolic status in a similar way or in different form as that under Al to re-establish the oxidative nature of this specific ROS. It is more speculating that H$_2$O$_2$ could alternately be generated with catabolism of PA, particularly, with diamine oxidase activity where Put is a substrate. Thereby, the catabolic pathways of Put enriching the plants with this specific ROS (H$_2$O$_2$) may also throw some insights regarding PA sensitivity of *Salvinia* species. So, the following analysis from the results and its possible interpretations may be helpful to gather information on *Salvinia* plants with its possible modulation by Put to H$_2$O$_2$ induced oxidative damages. Initially the accumulation of H$_2$O$_2$ was detected by both *in-vitro* and *in-vivo*. From the linearity of H$_2$O$_2$ accumulation with respect to exogenously applied H$_2$O$_2$ concentration shows that plants were evoked to
more oxidative stress. The H$_2$O$_2$ may appear in the tissue directly from two sources i) metabolism of glycolate through its subsequent reactions occurring in the peroxisome or glyoxisome and ii) a single electron transfer with the super oxide (O$_2^-$) in generation of ROS from electron transport chain (Sharma et al. 2012). For the first the concentration of H$_2$O$_2$ may not necessarily supersede the tissue level and thereby may be involved in some inductive process rather than disintegration of tissues. An oxidative over expression particularly in the biotic infestation may initiate many genes to up regulate the antioxidative pathways. In the present experiment, in-vivo detection of H$_2$O$_2$ has also been carried out with specific staining. Therefore, a concomitant increase in DAB activity has shown over folded intensity of staining as well as for Put application is a strong evidence for tissue sensitivity in *Salvinia*. ROS, with special reference to H$_2$O$_2$ have also been referred to interact with PA.

The cellular functioning on which the H$_2$O$_2$ interaction with Put takes place is the conversion of O$_2^-$ to H$_2$O$_2$, which is an outcome of spillage of electron from excited chloroplast or / and mitochondria. Therefore, at source level H$_2$O$_2$ is down regulated by the Put in plant tissues (Mandal et al. 2014). Possibility cannot be ignored that tissue permeability may be a factor for H$_2$O$_2$ to overcome plasma membrane and thereby accumulation results. Conventionally Put being a PA could be able to interact with its positively charged domains to the negatively charged backbones of the cell membrane. However, non specific transporter of H$_2$O$_2$ is reported so far on plant membrane. The attention of shielding nature of PA over the cell membrane is still questionable, at least, for H$_2$O$_2$ (Alcazar et al. 2010). Therefore, from the understanding of nature of H$_2$O$_2$ it becomes prudent to justify the impact of its effect on cellular metabolism. H$_2$O$_2$ though appeared as safer ROS within the tissue concentration; still, it can induce the detrimental activities on almost all cellular events (del-Rio et al. 1992; Upadhyaya et al 2007). Starting from membrane destabilization with lipid and protein oxidation to nuclear destabilization of chromatin material is induced within its wider range. Initially the loss of membrane permeability with dissolution of cell membrane results in a significant accumulation of malondealdehyde and carbamyalted derivatives of proteins. In the present experiment it’s of no exception where a significant accumulation of those
chemicals were detected in a dose dependent manner of H$_2$O$_2$ applied exogenously. The potent cytotoxicity of H$_2$O$_2$ has initially recorded with in-vivo induced chlorophyll degradation in a similar way done under water deficit and metal stress (Sairam and Srivastava 2000). The increasing concentration of H$_2$O$_2$ induced lipid peroxidation and its concomitant increase in membrane permeability indirectly supports the destabilization of the membrane following accumulation of peroxide along with degraded chlorophyll (Mandal et al. 2013a). It was also in agreement that Put could ameliorate the oxidative damage caused by H$_2$O$_2$ as compared to other abiotic stresses particularly for metal toxicity. Put may either be acting on generation of H$_2$O$_2$ by blocking the spillage of electrons from photo systems or can act in a protective manner shielding the susceptible bio molecules with its poly cationic attachment (Fariduddin et al. 2013; Sawhney et al. 2003). In more details the recognition of Put as a stress reliever is evident from its down regulating influences on some oxidases required to generate H$_2$O$_2$. This essentially links the ROS metabolism with the aid of NADH/NAD(P)H from energy yielding pathways. Chloroplast and mitochondria are the major sources of misfiring of electron more specially in presence of transition metals (Cu$^{2+}$ and Fe$^{2+/3+}$). A wider variety of peroxidases like limited substrate oxidases type III peroxidases and NAD(P) oxidase are activated under such conditions. Of those some produces directly H$_2$O$_2$ and others through some reactive intermediates like O$_2^-$ and $\frac{1}{2}$ O$_2$. PA has been found to interact those enzymes particularly NADH/NAD(P)H oxidase. One of the possibilities, that H$_2$O$_2$ is widely circulating owing to its high rate of diffusibility. The cellular concentration of H$_2$O$_2$ when crosses its threshold values may induce some sort of dehydration state (Cheeseman 2006). This phenomenon is clearly indicated in the present experiment as it shows a steady decline of the proline content. In usual form the proline acts as an indicator of osmotic stress where it behaves as a compatible solute to lower the osmotic potential. Unexpectedly, the decrease of proline content under H$_2$O$_2$ treatment is indicative of the fact of the down regulation of its biosynthesis. In a multistep pathway for proline biosynthesis the distinct rate limiting enzymes $\gamma$-glutamyl kinase ($\gamma$-GK) and $\gamma$-glutamyl phosphate reductase ($\gamma$-GPR) appears to be sensitive under different abiotic stresses. The changes in redox in metal contamination more towards oxidative in nature had somehow subdued the activity of those enzymes and thereby biosynthesis of proline
was curtailed (Thippeswamy et al. 2010). The effects of heavy metal stress on these enzyme activities have been studied and revealed the regulatory property of proline in homeostasis of metal ions. Put as found in the present study with a positive modulator in biosynthesis of proline under H$_2$O$_2$ mediated osmotic stress. This could be circumvented in relieving of oxidative exposure by H$_2$O$_2$ through the development of compatible solutes like proline with those rate limiting enzymes (Pavlikova et al. 2008). ROS are produced in plants by reduction of oxygen derived through number of normal metabolic processes. The evaluation of antioxidation system for ROS detoxification adopting both, non enzymatic and enzymatic systems could withstand various abiotic stresses. A number of multiple factors, particularly the existing or even induced or reduced molecules have been provided a fine tuned network. Non enzymatic antioxidants are those compounds which mostly includes ascorbate (AsA) and glutathione (γ-glutamyl-cysteinyl-glycine, GSH) as well as some secondary metabolites mostly polyphenolic in nature (Sharma et al. 2012). Among those, glutathione is the most predominant to furnish its reducing equivalence to detoxify the various ROS with intermediate damaging capacity. H$_2$O$_2$ being an important modulator of plant stress responses can change the cellular state of redox. Glutathione can accomplish the retrieval of the adequate redox required for normal cellular activities (Szalai et al. 2009). In the present case, *Salvinia* plants recorded a steeper increase in GSH to GSSG ratio that is the probable indication for some short fall of redox more towards oxidative states. Glutathione in concerted action of ascorbate could replenish the reducing equivalence of the tissues, particularly, under abiotic stresses (Gill and Tuteja 2010). Ascorbate and glutathione are synchronized in such a way that oxidized state of ascorbate could be refilling by donation of electron from reduced glutathione. Thus, the ascorbate behaves as most predominant antioxidant in plants where cellular system is perturbed with an elevated redox. In general oxidation of the ascorbate in stressed tissue is regenerated by two sequential intermediates, mono dehydroascorbate and dehydroascorbate by two respective enzymes: mono dehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR) (Sharma et al. 2012). Mono dehydroascorbate converted into dehydroascorbate and ascorbate either spontaneously or by enzymatic conversion, whereas, ascorbate is reduced by APX. Dehydroascorbate in turn could be replenished by reductase using GSH as
reducing equivalent or electron donor. Therefore from the earlier literature as well as trend of the glutathione activity in the present experiment clearly suggests that H$_2$O$_2$ can create an elevated oxidized status of the tissues and that could be retrieved by coordination of ascorbate and glutathione (Pekker et al. 2002). On account of Put application, it is quite expected a comeback of cellular redox in the *Salvinia* plants as documented earlier in other plants with PA (Ghosh et al. 2012). Thus, decrease in the ratio of GSH to GSSG is suggestive of the fact that Put might have played a role for donating reducing equivalents to the cellular sites. It could take place possibly by two ways: either diminishing the ROS generation at the most vulnerable sites of sub cellular fraction (Mandal et al. 2014) and induction of some non-enzymatic antioxidant residues to quench the excess energy of ROS (Alcazar et al. 2010; Takahashi and Kakehi 2010). Phenolics in plant system are the most predominant antioxidant residues with its diverse chemical configurations adhered to efficiency in quenching of excess energy of ROS (Mandal et al. 2013a). Thus, oxidative stress in plants is characterized with abundance of phenolics in many plant species and very often acts as an index for adaptive nature under such condition. Supplementation of PAs could interact with phenolics in a dose dependent manner in quenching of ROS has also been reported earlier (Farooq et al. 2009). With this indication, the exercise of Put in determining the ability of plants to regulate the flavanoid contents was studied and showed a steeper up regulation by the *Salvinia* plants in the present experiment. H$_2$O$_2$ being a ROS, however, is not a free radical can also amplify the ROS induced damages by other such moieties. As reported earlier plants under contamination of metal more specifically with those of transition in nature (Cu$^{2+}$/Fe$^{2+/3+}$) could able to generate OH$^-$ ions through Fenton reactions. From the point of peroxidation reaction OH$^-$ happens to be the most detrimental with macromolecules like nucleic acid, protein etc (Sharma et al. 2012). It is the inherent property of plants to over express the poly phenolics like flavonoids which may reduce the OH$^-$ toxicity by direct oxidation with acceptance of electrons from flavanoids. As stated earlier Put might have minimized the ROS generation in its biosynthetic pathways and thereby reduces the activity of flavonoids and other phenolics. This might corroborate the H$_2$O$_2$ induced oxidative damages of plant under PA mediated recovery.
DNA disintegration also does not escaped from the fatal effects of ROS. H$_2$O$_2$ itself or/and hydroxyl radical (OH$^\cdot$) produced by one electron reduction of H$_2$O$_2$, which are most potent ROS for DNA breakage (Rodriguez et al. 2011). In the present experiment, with the increase of H$_2$O$_2$ concentration, there recorded a concomitant effect of DNA disintegration. So for measuring the DNA disintegration we followed comet assay techniques and measured the tail length. Concomitantly, we have tested the mitigation of DNA damages by application of Put and there we found the significant reduction of comet tail length in the Salvinia plants. PA content has been analyzed from the H$_2$O$_2$ treated Salvinia plants and it is interesting to see that though there is linear increasing in PA with exposure of H$_2$O$_2$ still no such direct relationship with individual PA has been obtained. This possibly suggests that it is the total PA or more the bound PA that is responsible to furnish its activity against oxidative stress than a single one.

The emphasis on eradication of reactive oxygen species (ROS) by non enzymatic antioxidants are more established when plants are sufficiently exposed to the conditions of oxidative damages. However, the involvement of H$_2$O$_2$ in plants signals responses demands more attention with closer relationship of redox cascade in chloroplast and mitochondria and other sub cellular fraction of cells harbouring electron transport chain (Sharma et al. 2012). In a steady state condition the permeability of H$_2$O$_2$ is fairly accomplished over the chloroplast membrane more than that in case of overcoming cell membrane of chloroplastic organelle. Chloroplastic bound ascorbate peroxidase is more induced for expression in activities has been reported in some species (Sharma and Dubey 2004; Nakano and Asada 1987). Compared to those references, we distinctly found a linear increase in H$_2$O$_2$ alleviation with the GPX pool of Salvinia plant. PA like Put has reduced the activity for GPX suggesting the probable regulation of H$_2$O$_2$ generation and/or induced oxidative damages. Thus, the activity of GPX had been relieved (Mandal et al. 2014). The increased activity of cytosolic ascorbate peroxidase and glutathione reductase (GR) also found abundantly in expression with H$_2$O$_2$ concentration (Sairam and Srivastava 2000). This is more possibly when the existing non enzymatic antioxidant remains less expressed. Salvinia plants also recorded similar induction in activities for both GPX and GR in the present experiment with regard to
recovery of adequate redox in the tissues and PAs have played significant role as recorded in other crop species also (Mandal et al. 2013b; Ghosh et al. 2012). Thus, the replenishment of glutathione from its oxidized (GSSG) into reduced form (GSH) is now required with the participation of GR. Similar interpretation could be thought in case of Put mediated resumption of GR activity as in the present experiment under H₂O₂ toxicity. Since, ROS produced under the condition of excess illumination through dislocation of electrons from its normal path to molecular oxygen (O₂). Photo inhibition, another bottle neck for proper electron reduction into H₂O in tissues sets a significant contribution for ROS generation which has even been demonstrated under H₂O₂ treatments (H₂O₂ photo inhibition). Induction of photo inhibition with the application of H₂O₂ has also been reported with an associated increase of APX, GR, CAT transcript in Arabidopsis plant (Cho and Seo 2005; Jung S 2004). However, in our experiment CAT activity are found to be down regulated by application of H₂O₂, which has been overcome with Put application. The elevation of tissue concentration of H₂O₂ exogenously applied might supersede with specific reactions (like photo respiration, glycolate metabolism, photosynthesis etc.). This excess toxicity of H₂O₂ may distort the cellular or sub cellular redox and thereby enzyme conformity is denatured that also reflects in its activity (Gill and Tuteza 2010). Still, in most of the cases the antioxidative pathways acts as a physiological component for rescue of the plant from oxidative damages that are signaled with increased of antioxidative enzyme activity (Mandal et al. 2014). Ample references have enriched the feasibility of H₂O₂ as inducer to evoke cellular responses in antioxidation for many plant species and pretreatment with H₂O₂ has been a practice (Wahida et al. 2007; Li et al. 2011). Whether H₂O₂ produced endogenously follows the increase of antioxidants or antioxidative enzymes in plants is still to be ascertained. While it would be rational to consider the modern sophistication for analytical determination of H₂O₂, its standardization through experimental system and a choice of model plant to determine the threshold value of H₂O₂ for its beneficiary or toxicity has to be deduced. Salvinia an aquatic pteridophytic plant with its wider adaptability could accommodate the excess of metals (Al being of those as elucidated in the previous chapter). More so, this study has expected to de-fold the possible interaction with Put which is also a donor of H₂O₂ by oxidative degradation. Admitted well, it is still
uncertain and require extensive studies to decipher the role of H₂O₂ at the molecular level behaving either as a good faith to plant or as a part of other toxic moieties like ROS. Still, this study may decipher the underlying mechanism related to H₂O₂ metabolism in some non angiosperimic plant species like Salvinia (which has hardly been explored under H₂O₂ mediated oxidative stress) (Mandal et al. 2013a). Moreover, illustration with Salvinia and the possible interactive events with PA may also add new insights in such primitive plant species. Moreover, Salvinia plants could be hypothesized as a well responding species with respect to oxidative environment for bio-indication.