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Arsenic bioaccumulation and tolerance in terrestrial fern *Ampelopteris prolifera* (Retz.) Copel and aquatic fern, *Azolla pinnata* R. Br. are related to coordinated but differential responses of their antioxidant defense components

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ABSTRACT

Present study aims to analyze the arsenate bioaccumulation and antioxidant defense responses in fronds of terrestrial fern *Ampelopteris prolifera* (Retz.) Copel and an aquatic fern *Azolla pinnata* R. Br. Both the ferns were exposed to increased concentrations of sodium arsenate (As; 0, 20, 60, 100, and 160 mg L^{-1}) with Hoagland nutrient solution. Shoot dry weight reduced in both ferns at 160 mg As L^{-1} with higher decline in *A. pinnata* than *A. prolifera*. Fronds accumulated considerably higher As than roots but, compared to *A. pinnata*, *A. prolifera* transferred greater amount of As aboveground. Proper coordination among antioxidant defense facilitated high As accumulation in both ferns, but they differed in mechanistic responses. Reduced activities of ascorbate (AsA)-glutathione (GSH) cycle enzymes and increased level of γ-glutamyl transpeptidases (γ-GT) resulted in significant decline in GSH and AsA redox in *A. pinnata* at 160 mg As L^{-1}. Contrastingly, powered by increased antioxidant defense capacity and reduced level of γ-GT, *A. prolifera* prevented As-induced oxidative damage. As triggered oxidative damage in *A. pinnata* through membrane lipid peroxidation and electrolyte leakage at 160 mg As L^{-1}. This consequently inhibited plant growth and As accumulation potential of the aquatic fern.

Key words: *Ampeloptens prolifera, Azolla pinnata,* Aresenate, antioxidant, oxidative damage.

INTRODUCTION

Arsenic (As) is a toxic and carcinogenic metalloid. The impact of irrigation with high As-laced groundwater on soil and crop has currently drawn huge attention due to transfer of As to the food chain via groundwater-soil-plant system, and the worst scenario is found in South and South-East Asian nations (Flora *et al*., 2007). In India, groundwater contamination by As and toxicity of As-contaminated vegetables, cereals and pulses are continuously being reported in lower Gangetic basin and central-north plain (Mishra *et al*., 2008; Talukdar, 2014, 2015).

In plants, as is usually taken up from contaminated soils and waters via the roots and is further translocated to shoots (Zhao *et al*., 2009). While non-hyper accumulating plant species generally contained As in their roots, an ideal hyperaccumulator accumulates and tolerates As in their shoots to levels (at least 1000 mg As kg^{-1} dry weight, DW) far exceeding those present in the soil or in the nearby growing non-accumulating plants (Singh & Ma, 2006; Girdhar *et al*., 2014). After discovery of *Pteris vittata* as an As tolerant and superb hyperaccumulator (Ma *et al*., 2001; Kertulis-Tartar *et al*., 2006; Cao *et al*., 2010), continuous search for more

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hyperaccumulator/high accumulator plant species in contaminated soil and aquatic system identified several fern species with potential As-tolerance (Singh *et al*., 2006; Srivastava *et al*., 2010). Arsenic exists predominantly as arsenate (AsV) in natural waters and under aerobic condition. Being an analog of phosphate, it can easily be transported within cell and is readily converted to highly toxic arsenite (AsIII) which attacks sulfhydryl groups of proteins (Finnegan & Chen, 2012). Coordinated modulation of antioxidant defense comprising both enzymatic and non-enzymatic components is one of the fundamental mechanisms of plants generally takes against As-toxicity (Mishra *et al*., 2008). As induces oxidative stress by producing excess reactive oxygen species (ROS) and if is not effectively scavenged, it perturbs the delicate balance of ROS-homeostasis in favor of oxidative imbalance (Finnegan & Chen, 2012; Talukdar, 2013a, b) and inhibits plant growth predominantly through enhanced membrane lipid peroxidation and impediment through antioxidant defense components. Ascorbate (AsA) and glutathione (GSH) are two prominent non-enzymatic defense shields whereas superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR), glutathione reductase (GR), and glutathione-s-transferases (GSTs) constitute major backbone of enzymatic defense (Noctor *et al*., 2012). GSH is a tripeptide thiol, synthesized by two step processes of which the first step is a rate-limiting catalyzed by γ-glutamylcysteine synthetase (γ-ECS). Phytochelatins (PCs) are major down-stream As-detoxifying thiol peptides which are synthesized by PC synthase (PCS) through oligomerization of GSH (Noctor *et al*., 2012). The enzyme γ-glutamyl transpeptidases (γ-GTs), which is localized outside the cell membrane and in vacuoles (Mishra *et al*., 2008) is supposed to initiate the GSH degradation by catalyzing the hydrolysis of uniquely linked N-terminal Glu from GSH, GSSG, GS-conjugates and probably PCs (Połe´c-Pawlak *et al*., 2005). Reduction of AsV to AsIII, complexation of AsIII with GSH and PCs, and then their vacuolar sequestration are some of major strategies plants take during As detoxification (Mishra *et al*., 2008; Talukdar, 2015; Talukdar & Talukdar, 2014). Alterations/modulations in antioxidant defense components under As stress have been observed in hyper-accumulator, moderately accumulator (Singh et al. 2006; Zhao et al. 2009) as well as in non-hyper-accumulator plants (Srivastava *et al*., 2007; Talukdar, 2013 a,b).

Ferns (Pteridophytes) have excellent phytoremediation potential against metal (loid) toxicity (Sarkar & Jana, 1986). Works on absorption, transport, and storage of As have been extensively done on *P. vittata* (Tu & Ma, 2005; Wang *et al*., 2007) but only preliminary assessment was carried out on aquatic and other ferns (Zhang *et al*., 2008). Furthermore, onset of oxidative stress and mechanism of As tolerance varied greatly in different ferns (Pabby *et al*., 2002; Mehrag, 2003; Srivastava *et al*., 2005, 2010; Singh *et al*., 2006; Sood *et al*., 2011). The terrestrial fern *Ampelopteris prolifera* grows at extremely fast rate in Gangetic basin of South Asia (Patra & Bera, 2007; Talukdar, 2013c). *Azolla*, an aquatic free floating fern, is an ideal biofertilizer and a promising agent for heavy metal phytoremediation (Pabby *et al*., 2004; Rai, 2010; Sood *et al*., 2014). As accumulation has primarily been reported in *Ampelopteris prolifera* and *Azolla pinnata* (Zhang *et al*., 2008; Singh *et al*., 2010). However, intrinsic physoiological and biochemical mechanisms of their bioaccumulation potential and tolerance to as is completely unknown. Underpinning the mechanistic details of As-tolerance is prerequisite to introduce more plants in phytoremediation process of contaminated soil and toxic aquatic environment. Present study was, therefore, focused on i) comparative assessment of bioaccumulation and transfer potential, and ii) to dissect the antioxidant defense response of *A. prolifera* and *A. pinnata* exposed to increasing concentrations of As.

MATERIAL AND METHODS

Plant materials and treatment protocol

Azolla pinnata was collected from nearby ponds at Chakdaha (23° 5' N/88°31'E), West Bengal, India. The plants were washed and cleaned of contaminating organisms. Further, the plants were surface sterilized by keeping them in a solution of 0.1% mercuric chloride for 30 s and then dipped into a large volume of sterile distilled water. Healthy plants picked up gently were cultivated in a greenhouse on plastic trays containing Hoagland solution, ¼ ionic strength, pH 6.5 for acclimatization. Plants used in the experiments were derived from two mother plants, resulting in minimal genotypic and pre-experimental variations, following the protocol standardized in aquatic fern (Outridge & Hutchinson, 1991). For *Ampelopteris prolifera*, fertile fronds were collected from their natural vegetations near Chakdaha, West Bengal, India, and placed in paper bags and maintained in a desiccator until release of spores. After careful separation of spores from sporangia, spores were properly sieved, sterilized in 20% w/v sodium hypochlorite, washed, and allowed to germinate in Petri dishes (5 mg in each) with deep layer of cotton and Whatman filter paper 1. After germination, the gametophytes were transferred to glass jars containing the supplemented MS medium in photoperiod of 16 h light/8 h dark and Photosynthetic photon flux density (PPFD) of 150 µmol of photons m^{-2} s^{-1} illumination without addition of agar for the sporophyte development up to 4-frond stages. Both plant materials were then grown in controlled growth room with temperature 25 ± 2°C, PPFD of 280 µmol of photons m^{-2} s^{-1} illumination, RH 60-70% and photoperiod of 10 h of light/14 h of dark, and kept for 10 d in plastic pots filled with Hoagland nutrient (pH adjusted to 6-7) solution. Nutrient solution was then supplemented with As in the form of sodium arsenate (Sigma-Aldrich, Bangalore, India) at different

concentrations of 20, 60, 100, and 160 mg As L^{-1}, keeping un-supplemented nutrient solution as control with four replicates per treatment in a completely randomized design. Nutrient solution was refreshed every three days. Plants were harvested after 10 d treatment period and washed in 1% nitric acid solution to remove the As adsorbed on their surfaces. The plants were oven-dried at 72° C, 24 h, for dry weight (DW) and estimation of As absorbed.

Estimation of As level and As removal efficiency

As concentration in dried roots and shoots (frond + stipes) was measured by digestion methods (HNO_3–$HClO_4$ mixture at 3:1, v/v) using flow injection-hydride generation atomic absorption spectrophotometer (Perkin-Elmer, FIA-HAAS Analyst 400) and keeping Standard Reference Materials of tomato leaves (item number 1573a, from NIST, USA) for part of the quality assurance/quality control protocol, as detailed earlier (Talukdar, 2013a,b). The translocation factor (TF) is the ratio of the level of As in fronds upon roots. As removal efficiency in solution was estimated through the percentage of As removed by the plants = concentration of As absorbed by the plants (mg g^{-1} dry weight) × dry mass (g $plant^{-1}$) produced during the period of exposure to the As, thus producing the total amount of As removed from the solution. The amount of As in solution was considered as 100% (Guimaraes et al., 2012).

Photopigment estimation

Leaf chlorophyll and carotenoid contents were determined by the method of Lichtenthaler (1987). Leaf tissue (0.5 g) was homogenized in 10 mL chilled acetone (80%). The homogenate was centrifuged at 4000 *g* for 12 min. Absorbance of the supernatant was recorded at 663, 647 and 470 nm for chlorophyll a, chlorophyll b and carotenoids, respectively. The contents were expressed as mg chlorophyll or carotenoids g^{-1} FW.

Estimation of ascorbate and glutathione

Reduced and oxidized form of ascorbate (AsA) and glutathione (GSH) were measured following the methods of Law *et al*., (1983) and Griffith (1980), respectively. AsA and GSH redox was calculated as AsA/(AsA+DHA) and GSH/(GSH+GSSG), respectively.

Assay of antioxidant enzymes and thiol-metabolizing enzymes

Fresh leaf tissue of 250 mg was homogenized in 1 mL of 50 mM K-phosphate buffer (pH 7.8) containing 1 mM EDTA, 1 mM dithiothreitol and 2 % (w/v) polyvinyl pyrrolidone (PVP) using a chilled mortar and pestle kept in an ice bath. The homogenate was centrifuged at 15,000 *g* at 4 °C for 20 min. Clear supernatant was used for enzyme assays. For measuring APX activity, the tissue was separately ground in homogenizing medium containing 2.0 mM AsA in addition to the other ingredients. All assays were done at 25 °C. Soluble protein content was determined using Bovine Serun Albumin as standard (Bradford, 1976).

SOD (EC 1.15.1.1) activity was determined by nitro blue tetrazolium (NBT) photochemical assay (Beyer & Fridovich, 1987) and was expressed as U (unit) min^{-1} mg^{-1} protein. One unit of SOD was equal to that amount causing a 50% decrease in SOD-inhibited NBT reduction. For APX (EC 1.11.1.11) activity, three milliliter of the reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM EDTA, 0.5 mM AsA, 0.1 mM H_2O_2, and 0.1 mL enzyme extract. The H_2O_2-dependent oxidation of AsA was followed by a decrease in the absorbance at 290 nm (ε = 2.8 mM^{-1} cm^{-1}). APX activity was expressed as µmol AsA oxidized min^{-1} mg^{-1} protein (Nakano & Asada, 1981). DHAR (EC 1.8.5.1) activity was measured following the protocol of Nakano and Asada (1981). Enzyme activity was expressed as µmol AsA formed min^{-1} mg^{-1} protein. GR (EC 1.6.4.2) specific activity was determined by monitoring the GSH-dependant oxidation of NADPH (Carlberg & Mannervik, 1985). GR specific activity was expressed as nmol NADPH oxidized min^{-1} mg^{-1} protein. GST (EC 2.5.1.18) activity was assayed in a reaction mixture containing 50 mM phosphate buffer, pH 7.5, 1 mM1-chloro-2,4-dinitrobenzene (CDNB), and the elute equivalent to 100 µg of protein. The reaction was initiated by the addition of 1 mM GSH (Sigma-Aldrich), and formation of S-(2,4-dinitrophenyl) glutathione was monitored as an increase in absorbance at 334 nm to calculate the GST specific activity (Li et al., 1995). CAT (EC 1.11.1.6) extraction was performed in a 50 mM Tris–HCl buffer. The enzyme activity was assayed by measuring the reduction of H_2O_2 at 240 nm (ε = 39.4 $mM^{-1}cm^{-1}$) and 25 °C, as detailed earlier (Talukdar, 2013a). Among thiol-metabolizing enzymes, assay of γ-ECS (EC 6.3.2.2) and PC synthase (PCS; EC 2.3.2.15) activity was done following Seelig and Meister (1984) and Howden *et al*., (1995), respectively, as detailed earlier (Misra *et al*., 2008; Talukdar, 2015). For assays of γ-GT (EC 2.3.2.2), plants were homogenized in 100 mM Tris–HCl (pH 8.0). The reaction mixture (1 mL) containing 100 mM Tris–HCl (pH 8.0), 2.5 mM L-γ-glutamyl *p*-nitroanilide, 20 mM glycylglycine and a suitable aliquot of enzyme extract was incubated for 30 min at 30°C. The reaction was terminated by adding 1mL of 25% TCA. Formation of *p*-nitroaniline (ε = 1.74mM^{-1} cm^{-1}) was monitored at 405 nm (Orlowski & Meister, 1973).

Determination of H_2O_2, superoxide radicals, and membrane damage

H_2O_2 was estimated following the methods of Wang *et al*., (2007). Superoxide radical (SOR; $O^{-\bullet}_2$) was determined using the method of Elstner and Heupel (1976) by monitoring nitrite formation from hydroxylamine in the presence of $O^{-\bullet}_2$contained in the homogenates supernatant. Lipid peroxidation rates were determined by measuring the malondialdehyde (MDA) equivalents (Hodges *et al*., 1999). Membrane electrolyte leakage (EL%) was assayed by measuring

the ions leaching from tissue into deionised water (Dionisio-Sese & Tobita, 1998).

Statistical analysis

Data are mean ± standard error (SE) of at least four replicates. Variance analysis was performed on all experimental data, and statistical significance ($P < 0.05$) of means was determined by Duncan's multiple range tests using SPSS software (SPS Inc., USA v. 10.0). A linear correlation was carried out to understand the relation between different traits with significance at $P < 0.05$.

RESULTS AND DISCUSSION

Plant growth response

Shoot DW increased (1.6 fold) significantly ($P < 0.05$) in *A. pinnata* at 20 mg As L^{-1} over control but reduced by about 4-fold at 160 mg As L^{-1} (Fig. 1a). Compared to control, shoot DW in *A. prolifera* was reduced by about 1.2-fold only at 160 mg As L^{-1} (Fig.1a). Changes were not significant in rest of the cases and for root dry weight in both ferns. Shoot DW gain by *A. pinnata* at 20 mgL^{-1} over control indicates high biomass productivity which confirmed the earlier observations that toxic chemicals often stimulate growth at lower doses through enhancement of phosphorus and potassium in fronds/shoots (Tu & Ma, 2005; Cedergreen, 2008; Talukdar, 2013b). Declining dry mass at 160 mg As L^{-1} in *A. pinnata* suggested inhibition of plant growth at higher As concentration. Remarkably enough, growth of *A. prolifera* was comparable to control across the treatments barring at 160 mg L^{-1} where it declined marginally. As exposure increased dry weight in *Azolla filiculoides* at 5 and 10 µg As mL^{-1} (Zhang *et al*., 2008) but exhibited toxicity at increasing As concentrations (Sánchez-Viveros *et al*., 2015). In *A. caroliniana,* growth was inhibited at 0.5 mg As L^{-1} (Zhang *et al*., 2008). However, Moradi *et al*., (2013) observed declining growth characteristics of *A. caroliniana* at 5 mg L^{-1} As which was further reduced significantly at 160 mg L^{-1} As. Far better dry matter accumulation was observed in the present *A. pinnata* till 100 mg As L^{-1}. Both *A. pinnata* and *A. prolifera* maintained growth traits comparable to control up to 100 mg As L^{-1}, indicating better performances of the two ferns in comparison to their sister taxa under As exposure. Based on growth responses, *A. prolifera* exhibited more biomass accumulation than *A. pinnata* to increasing As concentrations.

As uptake and As-removal efficiency

As accumulation in both ferns was significantly higher in shoots than in roots but the magnitude was markedly higher in terrestrial fern *A. prolifera* than its aquatic counterpart (Fig. 1b). As uptake was 1.6-(20 mg L^{-1}) to 2.8-fold (160 mg L^{-1}) higher in *A. prolifera* frond than that in *A. pinnata*. As level continuously increased in both ferns till 100 mg L^{-1} (r = 0. 813, $P < 0.001$ for *A. prolifera*; 0.678, $P < 0.01$ for *A. pinnata*, n = 12), but became stabilized in *A. prolifera* and declined even below the initial concentration (at 20 mg L^{-1}) in A. *pinnata* at 160 mg L^{-1} (Fig. 1b). As concentration ranged 0.781-0.933 mg g^{-1}DW in *A. prolifera* while it varied between 0.337 mg g^{-1}DW and 0.789 mg g^{-1}DW in *A. pinnata*. Translocation factor (frond/root) was >1.0 in both ferns throughout the treatment regimes, but the factor value was significantly higher in *A. prolifera* compared to *A. pinnata* across the treatments (Fig.1b). As accumulation in both ferns was found considerably higher than other aquatic macrophytes reported earlier (Srivastava *et al*., 2007; Mishra *et al*., 2008; Singh *et al*., 2013; Talukdar & Talukdar, 2015) but marginally lower than Iranian species of *A. caroliniana* (Sufian *et al*., 2013). As concentrations in fronds of 50 strains of Chinese *Azolla* spp varied by about 13.5-fold and the highest uptake (0.397 mg g^{-1} DW) was shown by *A. caroliniana*, followed by *A. microphylla*, *A. pinnata*, *A. maxicana*, and *A. filiculoides* (Zhang *et al*., 2008). However, As accumulation in the present *A. pinnata* was much higher than their Chinese counterpart. The results also suggested efficient transport and accumulation of substantial amount of As in fern fronds and *A. prolifera* was found superior to *A. pinnata* at increasing As concentrations. Efficient transport to the aboveground biomass is important for effective detoxification of As in hyperaccumulators (Singh & Ma, 2006). Present As accumulation and translocation by both ferns was recorded under hydroponics supplemented with Hoagland's nutrient solution while Yoshida nutrient solution was used in case of *A. filiculoides* which accumulated maximum 28 µg As g^{-1} DW at 60 µg As mL^{-1} (Sánchez-Viveros *et al*., 2011). The results revealed inter-specific variation as well as experimental protocol and selection of nutrient solution as factors influencing As uptake system in ferns. Based on the As accumulation in the fronds at hydroponics, Singh *et al*., (2010) categorized ferns into a) high (> 0.500 mg g^{-1}), b) moderate (> 0.250-0500 mg g^{-1}), and c) low accumulator (< 0.250 mg g^{-1}). Considering this observation, present *A. prolifera* may be categorized as high As accumulator across the treatments but not as hyper-accumulator. On the other hand, As accumulation in *A. pinnata* was medium (20 and 160 mg As L^{-1}) to high (at 60 and 100 mg As L^{-1}). However, declining accumulation of As was accompanied by significant growth inhibition (r = 0.788, n =12, $P < 0.001$) and appearance of chlorotic spots and low number of fronds in *A. pinnata* at 160 mg L^{-1}, similar to that found in different other ferns, also (Srivasrava *et al*., 2005, 2010). In contrast, no visual symptoms of toxicity were reported in *A. prolifera*, which agreed with earlier findings in *P. vittata* and *Adiantum capillus-veneris* (Singh *et al*., 2010). Certainly, *A. prolifera* in the present protocol exhibited As accumulation and tolerance even at 160 mg As L^{-1} while *A. pinnata* accumulated As without any visual toxicity till 100 mg As L^{-1}.

As-removal efficiency was comparable between *A. prolifera* (38.19%) and *A. pinnata* (32.64%) at 20 mg As

L^{-1} but the highest (45.62%) capacity in *A. prolifera* and that (33.53%) in *A. pinnata* was observed in 100 mgL^{-1} (Fig. 1c). The As-removal efficiency increased by about 1.6-fold, 1.4-fold and 10.50-fold in *A. prolifera* over *A. pinnata* at 60, 100 and 160 mgL^{-1} respectively (Fig. 1c). Similarly, another water fern, *Salvinia natans* removed As at enhanced rate with increasing As (V) concentrations (Mukherjee & Kumar, 2005). But, inverse relationship between As level in solution and removal efficiency was reported in *A. caroliniana*, *Salvinia minima* and *Lemna gibba* with 2.5%, 18% and 2.24% removal efficiency, respectively, at 0.5 mg L^{-1} (Guimaraes *et al*., 2012). Present results, thus, suggested significantly higher As-removal efficiency by both ferns but *A. prolifera* was far better in the process than *A. pinnata* particularly, at concentrations > 20 $mgAsL^{-1}$.

Effect of As exposure on photopigment levels

Chlorophyll (Chl) a and b level and carotenoid contents in *A. prolifera* increased over control by nearly 1.3-2-fold but did not change significantly at 160 mg L^{-1} (Fig. 1d). In *A. pinnata*, chl a level and carotenoid contents increased significantly at 20 mg L^{-1} but decreased substantially at 160 mg L^{-1} (Fig. 1d). The greater the amount of As in solution, the lower the total chl contents in *A. pinnata* (r = -0.633, n = 12, P < 0.01). The chl a/b ratio did not change markedly in *A. prolifera* but it increased significantly in *A. pinnata* at 20 mg L^{-1} (Fig. 1d). As-induced decrease in chl content was also reported in heavy metal exposed *A. pinnata* (Rai, 2010; Prasad & Singh, 2011), its sister species (Sood *et al*., 2011), and As-exposed *Pteris ensiformis* (Singh *et al*.,2006). However, like the *P. vittata* (Singh *et al*., 2006), As increased photopigment production in the present *A. prolifera*. As in photosynthetic tissues of *A. pinnata* probably damaged the thylakoid membranes, disrupted electron transport processes and photopigment biosynthesis (Singh *et al*., 2010; Talukdar, 2013a). The low total chl, chl a/b ratio and carotenoid content detected in *A. pinnata* at 160 mg L^{-1} could possibly account for the low dry mass gain of the plants exposed to higher As.

Effect of As exposure on superxode radicals, H_2O_2, MDA and EL%

Superoxide radicals increased significantly in both ferns throughout the treatment regimes but H_2O_2 and MDA level became markedly higher only in *A. pinnata*, particularly at 160 mg L^{-1} (Fig. 2a). Similar trend was noticed in case of EL% in both fern fronds (Fig. 2a). Compared to control, changes were not significant in rest of the cases (Fig. 2a).

Effect of As exposure on thiol-metabolisms and antioxidant defense

Significant contrasting responses were observed between two ferns in thiol metabolism, also. The GSH-synthesizing enzyme γ-ECS activity increased significantly over control in *A. prolifera* only at 100 mg L^{-1} but was comparable to control in other treatments (Fig. 2b). Enzyme activity was enhanced by about 2-3-folds over control at 20 and 100 mgL^{-1} in *A. pinnata* but declined significantly at 160 mgL^{-1}(Fig. 2b). PCS activity increased significantly over control throughout the treatments in *A. prolifera* but enhanced in *A. pinnata* till 100 mg L^{-1}. Activity reduced markedly (nearly 3-fold) in *A. pinnata* at 160 mgL^{-1} (Fig. 2b). The γ-GT activity reduced continuously in *A. prolifera* at increasing As concentrations (Fig. 2b). Contrastingly, γ-GT activity in *A. pinnata* increased by 2.7 and 3.9-fold at 100 and 160 mgL^{-1} respectively, despite an initial reduction at 20 mgL^{-1} and comparable value to control at 60 mgL^{-1} (Fig. 2b).

Total GSH (GSH + GSSG) level and GSH-redox in As-exposed *A. prolifera* did not change significantly (P > 0.05) in relation to control (Fig. 2c). GSH redox in the fern hovered around 0.8 across the treatment protocols. Similar situation was found in case of *A. pinnata* till 100 mg L^{-1}, after which redox value declined by nearly 4.2-fold at 160 mgL^{-1} (Fig. 2c). AsA content in *A. prolifera* increased continuously with increasing concentrations of As (r = 0.817, n = 12, P < 0.001) in the medium. AsA redox in the fern continued to hover around 0.8 (Fig. 2d). In contrast, AsA level increased in *A. pinnata* exposed to 60 mgL^{-1} but then reduced by about 3-5-fold at higher As treatments (r = -0.709, P < 0.01; Fig. 2d). The GSSG and DHA level in *A. pinnata* exposed to 100 and 160 mg As L^{-1} increased substantially and both GSH and AsA redox values reduced significantly (Fig. 2d).

The two ferns responded differently for enzymatic antioxidant defense also. From 60 mgL^{-1} onwards, SOD activity exhibited significant up-ward trend in fronds of both ferns but the magnitude of increase was greater in *A. pinnata* than in *A. prolifera* (Fig. 2e). APX, DHAR, GR and GSTs activities increased markedly in *A. prolifera* up to 100 mg L^{-1} but were comparable to control at 160 mgL^{-1} (Fig. 2e, f). In *A. pinnata*, APX and GR level increased over control only at 60 and 100 mgL^{-1} while DHAR activity increased only at 60 mgL^{-1}. GSTs level did not change significantly in *A. pinnata* till 100 mg L^{-1} (Fig. 2e,f). Contrastingly, CAT level remained comparable to control level in *A. prolifera* throughout the treatment regimes. CAT activity exhibited upward trend in *A. pinnata* from 60 mgL^{-1} onwards (Fig. 2f). Barring SOD and CAT, rest of the enzyme activities reduced significantly in *A. pinnata* at 160 mg As L^{-1} (Fig. 2f).

Proper coordination and complementation between thiol metabolisms and antioxidant defense response is necessary to facilitate As uptake, translocation and to confer As tolerance in plants (Mishra *et al*., 2008; Tripathi *et al*., 2013). High γ-ECS activity coupled with normal (close to control) to enhanced GR level in *A. prolifera* facilitated GSH synthesis and regeneration of GSH from GSSG, respectively. GSH-degradation was effectively contained as γ-GT activity in *A. prolifera* decreased continuously, suggesting an inverse relationship (r = - 0.821, n = 12, P < 0.001) between As

concentration and enzyme activity. This in turn significantly propelled high GSH pool, favorable to maintain proper GSH redox in *A. prolifera* even under the highest As concentration. A high GSH/GSSG ratio had also been reported in *P. vittata* and *A. capillus-veneris* in As-spiked hydroponic and pot soil experiment (Singh *et al*., 2006, 2010). Partially similar situation was encountered in the present *A. pinnata*, but at 160 mg L^{-1}, reduction in γ-ECS and GR activities coupled with rise in γ-GT level resulted in significantly lower GSH content and concomitant sharp decline in GSH redox. It is noteworthy here that role of GR-mediated GSH recycling at AsA-GSH cycle presumed to be more important than γ-ECS in maintenance of GSH-redox and prevention of sulfide stress in cell, as was observed earlier in different plants including *P. vittata* frond (Singh & Ma, 2006; Singh *et al*., 2006; Kertulis-Tartar *et al*., 2009; Talukdar, 2014). Also, importance of GSH degradation rate through γ-GT is noteworthy during As exposure (Mishra *et al*., 2008). In the present study, decline in GR activity and elevation in γ-GT level at higher magnitude than γ-ECS seem to be far more influential in regulating GSH pool in *A. pinnata* under increasing As concentrations. Obviously, elevated GSH pool coupled with enhanced PCS activity conferred substantial capability of both ferns to accumulate and sequester high level of As in their above ground parts but the capacity sharply decreased in *A. pinnata* exposed to 160 mg As L^{-1} due to low magnitude of cellular GSH pool and low PCS activity. Considering both ferns, the significant correlation (n = 12) between As uptake and GSH level (r = 0. 711, $P < 0.01$), As uptake and PCS activity (r = 0.727, $P < 0.01$) and between GSH and PCS (r = 0.755, $P < 0.01$) in the present case is not in agreement with *Pteris vittata* where the former two traits inversely co-related with PCs (Zhao *et al*., 2009). This might be one of the prime reasons for fall in As accumulation and transfer potential of *A. pinnata* at 160 mgL^{-1} while that potential was well maintained by *A. prolifera* throughout the treatment regimes. Besides, As-sequestration and building blocks of PCs, GSH is extensively used as a reductant by DHAR, an important AsA-GSH cycle enzyme involved in AsA regeneration from its oxidized state DHA (Noctor *et al*., 2012). Normal (close to control) to increased DHAR activity in *A. prolifera* across the treatments and in *A. pinnata* up to 60 mgL^{-1} regenerated enough AsA, stabilizing the AsA redox around 0.8 in both cases. Although AsA biosynthesis was not studied in the present case, importance of DHAR in regulating AsA redox in stressed plants have been proved in different AsA-deficient mutants of model and crop plants (Veljovic-Jovanovic *et al*., 2001; Talukdar, 2012). The fact was further substantiated once DHAR activity started to decline in the present *A. pinnata* at elevated As treatments. At 100 and 160 mg L^{-1}, low DHAR activity probably led to huge fall in AsA level and concomitant rise in DHA with an obvious detrimental effect on AsA redox in aquatic fern. The cascading effects of changes in DHAR level were also percolated in ROS-scavenging capacity of APX. Over-accumulation of superoxide radicals and increase in SOD activity in both ferns strongly indicate As-induced free radical generation and its dismutation (via SOD) to generate huge H_2O_2. As-induced high SOD activity was also reported in As-accumulating plants (Singh *et al*., 2010 Talukdar, 2015; Talukdar & Talukdar, 2014). However, normal to increased APX activity in the present *A. prolifera* fronds effectively scavenged ROS. Situation was more or less similar in case of *A. pinnata* till 100 mgL^{-1}, but sharp decline in APX and GST activity at 160 mgL^{-1} severely jeopardized ROS scavenging capacity of the aquatic fern. This was despite the fact that CAT activity in *A. pinnata* was maintained high throughout the treatment regimes. Simultaneous rise in SOD and CAT activity as found in *A. pinnata* at higher As treatment contradicts the earlier reports on inverse response of SOD and CAT activity in *A. pinnata* under cadmium stress (Prasad & Singh, 2011). Although CAT was found effective in H_2O_2-scavenging in As-exposed *A. capillus-veneris* and *P. vittata* and in other As-accumulating plants (Kertulis-Tartar *et al*., 2009; Singh *et al*., 2010; Talukdar, 2013b, 2014), present results show necessity of GSH-dependent antioxidant defense in ferns experiencing elevated As level. Certainly, high CAT activity was not enough to detoxify rising H_2O_2 level in *A. pinnata*. Elevated ROS level in *A. pinnata* resulted in membrane damage through lipid peroxidation, as was evident from higher MDA and EL% than control. Lipid peroxidation of unsaturated fatty acids in the membranes is the prime reason for loss of membrane integrity and permeability and is often positively correlated with ROS levels as oxidative stress markers in plants (Singh & Ma, 2006; Mishra *et al*., 2008), as observed in the present case also. This has not only impeded As accumulation potential of *A. pinnata* but also inhibited its growth potential and induced frond chlorosis at 160 mgL^{-1} As. In contrast, As accumulation potential, transport and removal efficiency was not disrupted and no symptom of visual toxicity and oxidative damage was conspicuous in *A. prolifera*, even though there was marginal decrease in DW at 160 mg L^{-1}. Increased photopigment levels in *A. prolifera* fronds confirms occurrence of effective defense strategy of the terrestrial fern against As-induced oxidative stress. Enhanced carotenoid content in *A. prolifera* obviously represent its supportive role against oxidative stress, as also observed in high to hyper As accumulator fern fronds (Singh *et al*., 2010; Srivastava *et al*., 2010). A coordination between thiol metabolisms and antioxidant defense within and outside AsA-GSH cycle enzymes thus conferred better uptake and As-tolerance by preventing ROS over accumulation and oxidative membrane damage in *A. prolifera*.

Figure-1. Changes in (a) frond and root dry weight (DW), (b) arsenate (As) accumulation and transfer factor (TF), (c) As-removal efficiency, and (d) chlorophyll (chl) a, b, a/b ratio and carotenoid contents in terrestrial fern *Ampelopteris prolifera* and water fern *Azolla pinnata* under control (0 mg As L-1) and 20, 60, 100 and 160 mg As L-1. Data are means ± standard error of at least four replicates. Error bars followed by different lowercase letters indicate significant changes ($P < 0.05$) in ANOVA followed by Duncan's Multiple Range Test. FW-fresh weight.

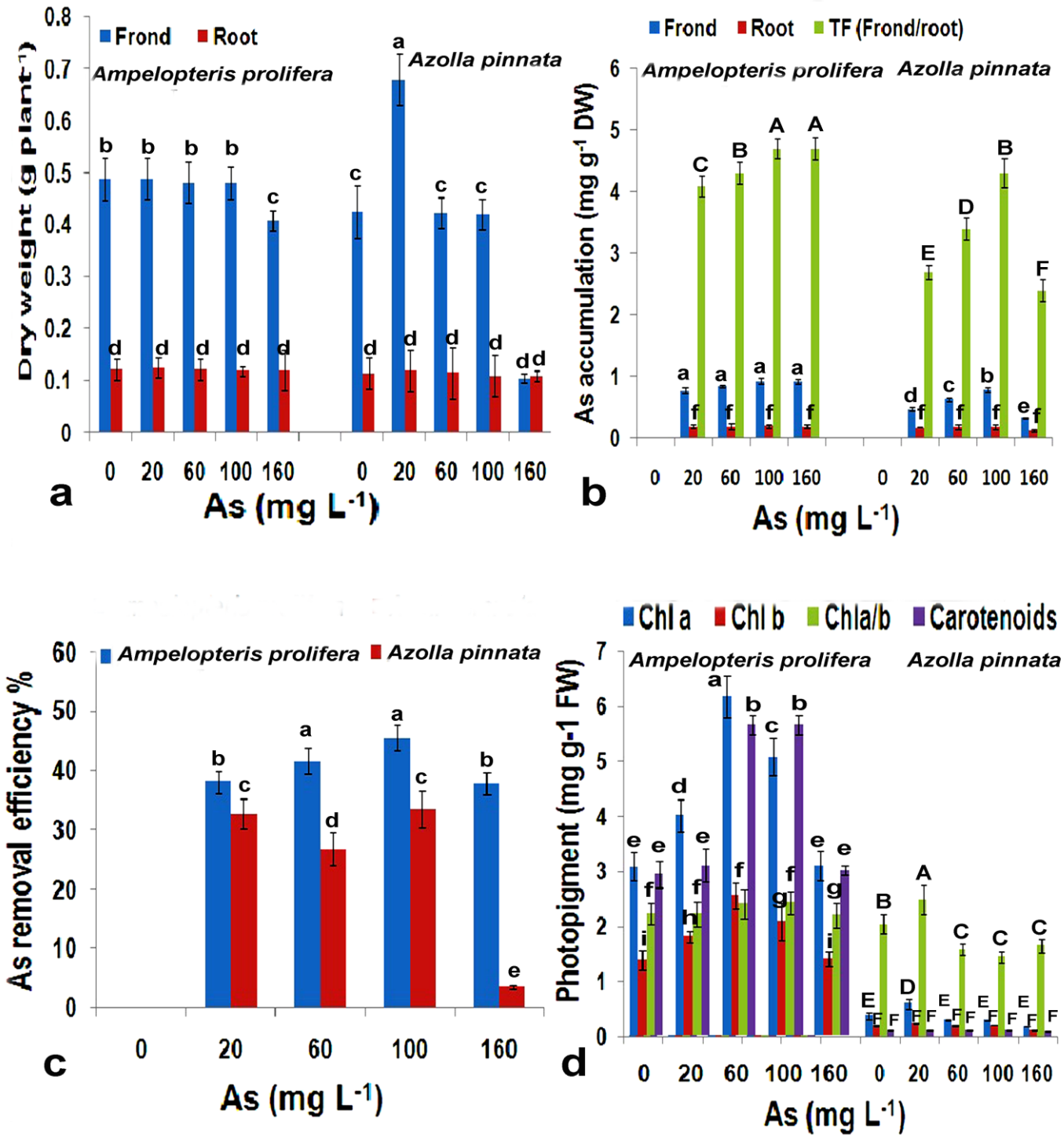
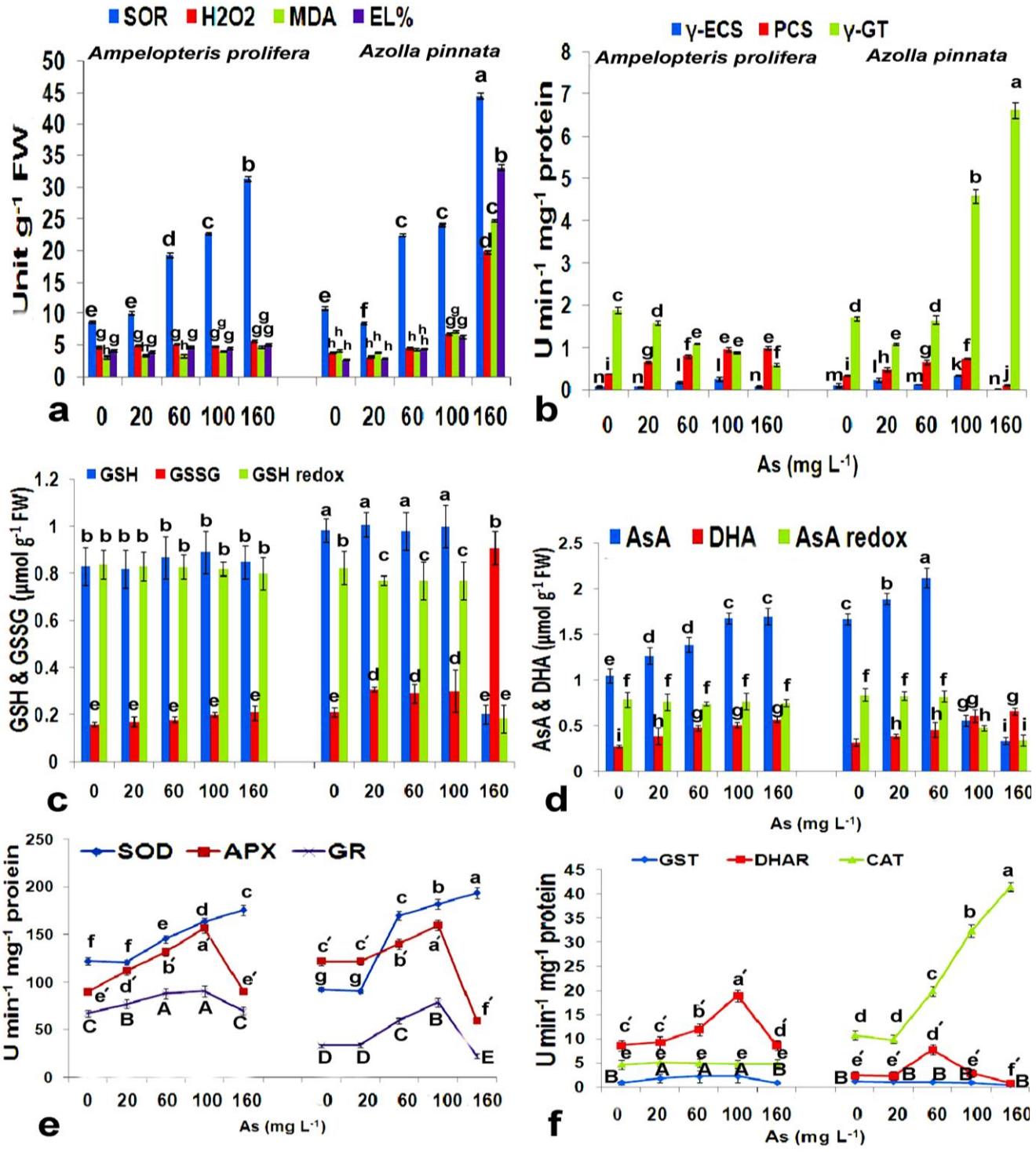


Figure-2. Changes in (a) superoxide radicals (SOR, Unit-nmol), H_2O_2 (Unit-µmol), malondealdehyde (MDA, unit- nmol) and electrolyte leakage (EL %), (b) activities of γ-glutamylcysteine synthetase (γ-ECS; Unit-nmol γ-EC), Phytochelatin synthase (PCS; unit-nmol GSH eq), and γ-glutamyl transpeptidases (γ-GT), (c) levels of GSH, GSSG, and GSH redox (GSH/(GSH+GSSG), (d) contents of AsA, DHA, AsA redox (AsA/(AsA+DHA), (e) activities of superoxide dismutase (SOD), ascorbate peroxidase (APX, Unit-µmol AsA oxidized), glutathione reductase (GR, Unit-nmol NADPH oxidized), and (f) glutatathione-s-transferase (GSTs,), dehydroascorbate reductase (DHAR, µmol AsA formed) and catalase (CAT, nmol H_2O_2) in *Ampelopteris prolifera* and *Azolla pinnata* under control (0 mg As L^{-1}) and 20, 60, 100 and 160 mg As L^{-1}. Data are means ± standard error of at least four replicates. Error bars followed by different lowercase letters indicate significant changes ($P < 0.05$) by ANOVA followed by Duncan's Multiple Range Test. Test was done on respective enzymes in two ferns, and accordingly, they were distinguished by different forms of alphabets (small letters for SOD and CAT, letters with prime for APX and DHAR, and capital letters for GR and GST in fig. 2e and fig. 2f, respectively). FW-Fresh weight.



CONCLUSION

The desirable characteristics for As-remediation purposes are high biomass production, efficient removal of As from source sites (here nutrient solution), efficient transport, and high accumulation of As in over ground parts without induction of any oxidative damage (Tu & Ma, 2005). These characteristics were observed for the two different fern taxa investigated, when grown in nutrient solution with As, and can be used as medium to high As accumulators. However, *A. prolifera* exhibited better fitness in dry mass accumulation, As transport potential and tolerance to As-induced oxidative stress in comparison to those observed in *A. pinnata*. The differences were particularly distinct at 160 mg As L^{-1}, the highest concentration selected in the present study. The results pointed out that thiolic capacity and antioxidant defense worked in fine tune in conferring As accumulation potential and As-tolerance in photosynthetic tissues. The increase in the removal efficiency of As in solution along with normal (control like) to increased dry mass manifested the viability of using the two ferns in As phytoremediation technique, mainly in environments polluted with medium to high metalloid concentration. While *A. prolifera* has the potential to remediate terrestrial environment, *A. pinnata* can clean-up aquatic As-contamination to a considerable extent.

Conflict of Interests

Authors declare that there is no conflict of interests regarding the publication of this paper.

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