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Differential Arsenic-Induced Membrane Damage and Antioxidant Defence in Isolated Pinna Segments of Selected Fern Species from Western Himalaya

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Abstract

A comparative evaluation of arsenic (As)-induced toxicity has been made in selected Western Himalayan ferns belonging to the family Pteridaceae (*Pteris vittata* L. and *P. cretica* L.) and Polypodiaceae (*Polypodiodes microrhizoma* (C. B. Clarke ex Baker) Ching and *Lepisorus contortus* (H. Chirst) Ching) employing a rapid leaf (pinna) slice test. The exposure of pinna segments to As(V) (Na_2HAsO_4; 0–1000 µM), led to a concentration-dependent increase in K^+ leakage into the incubation medium owing to membrane damage. Strong species-specific differences were evident. Thus, the magnitude of K^+ leakage enhancement was much lower in Pteridaceae members (lowest in *P. vittata*) than in those of Polypodiaceae. The As effects were like those of Cu, a redox active element like As, and H_2O_2 suggesting them to be mediated by oxidative stress. Involvement of K^+ channels in As-induced K^+ leakage is unlikely, as no effect of tetraethylammonium chloride (TEA), a K^+ channel inhibitor was evident. Furthermore, the activities of antioxidant enzymes, superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) were markedly altered due to As treatment in a species-specific manner pointing to a role of antioxidant defence in differential response and thereby tolerance of the concerned ferns to As. Findings have implications for phytoremediation.

Keywords Arsenic · Ferns · Pteridaceae · Polypodiaceae · K^+ leakage · Antioxidative defence

Introduction

Arsenic (As) contamination of the environment, owing to natural as well as anthropogenic release, is a matter of serious concern (Bhattacharya et al. 2007; Punshon et al. 2017; Kumar et al. 2018; Thakur et al. 2019). Indeed, the As levels in groundwater are well above the safe limits as per the WHO standards in some Asian countries like Bangladesh as well as in USA (Henke 2009). Arsenic persists in the food chain following its uptake and accumulation by diverse crops such as rice that eventually poses a threat to human health. The increased As intake through food or drinking water is associated with incidences of cancer, skin diseases, kidney problems, diabetes and heart ailments (Meliker et al. 2007).

Phytoremediation, employing metal(loid) hyperaccumulator plants, offers a reasonable means of lowering the toxic heavy metal(loid) burden on environment (Pilon-Smits 2005; Krämer 2010). In the context of As toxicity, identification of Chinese brake fern (*Pteris vittata*) as the first As hyperaccumulator was a breakthrough (Ma et al. 2001). This led to the screening of other fern species for their ability to hyperaccumulate As. Several species e.g., *P. cretica, P. longifolia, P. umbrosa, Pityrogramma calomelanos* and *P. melanocaulon* exhibited substantial As accumulation rates in their fronds (Francesconi et al. 2002; Zhao et al. 2002; Claveria et al. 2019) although they did not match the capabilities of *P. vittata* (Luongo and Ma 2005). In contrast, several other species e.g., *Asplenium nidus, Davillia canarensis, Polypodium aureum,* and *Polystichum tsus-simense* did not have such ability (Zhao et al. 2002). *P. vittata* represents an attractive model system for getting insight into the

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mechanisms of As tolerance (Ma et al. 2001). Despite the identification and availability of a large number of metal hyperaccumulators, implementation of phytoremediation in the field remains limited (Gerhardt et al. 2017). Interestingly, intercropping of *P. vittata* was reported to reduce As accumulation in certain cash crops including maize (Ma et al. 2018) and *Morus alba* (Wan and Lei 2018).

Arsenic is taken up by *P. vittata* as As(V), the predominant form in aerobic environments, through specific inorganic phosphate (Pi) uptake systems in competition with Pi (Meharg and Hartley-Whitaker 2002; Tu and Ma 2003). Subsequently, As(V) is converted to As(III) in the roots by arsenate reductases (Ellis et al. 2006) and is accumulated as the major As form in the fronds (Bleeker et al. 2006). In contrast, the uptake of As(III), abundant in reducing environments (pH < 8) such as in the paddy fields occurs via nodulin26-like intrinsic proteins (NIPs) (Ma et al. 2008; Lindsay and Maathuis 2017). PvTIP4;1, an aquaporin localized to plasma membrane, is involved in As(III) uptake in *P. vittata* (He et al. 2016). Vacuolar compartmentalization is an important component of cellular detoxification of As (Sharma et al. 2016). A tonoplast localized As(III) transporter, PvACR3, has been reported in *P. vitatta* (Indriolo et al. 2010). As(III) is complexed by phytochelatin (PC) and As(III)-PC complex is transported into the vacuole via two ABC transporters, AtABCC1 and AtABCC2, in *Arabidopsis thaliana* (Zhao et al. 2003; Song et al. 2010). Recently, a novel mechanism of arsenic detoxification in *P. vittata* has been reported. Here, the glyceraldehyde 3-phosphate dehydrogenase PvGAPC1 converts arsenate into 1-arseno-3-phosphoglycerate that is transported by PvOCT4 into the As metabolizing vesicles where the released As(V) is reduced by PvGSTF1 to As(III) that is stored in the vacuoles (Cai et al. 2019). Apparently, As hyperaccumulation is linked to the mechanisms of tolerance. The toxicity due to metal(loid)s including As could inter alia be ascribed to the cellular redox imbalance; conversely, the antioxidant defence contributes to As tolerance (Sharma and Dietz 2009). Arsenic altered activities of antioxidant enzymes like superoxide dismutase (SOD) and catalase (CAT) in *P. vittata* and other ferns (Srivastava et al. 2005). Also, the biological membranes are involved in determining the tolerance to metal(loids) through selective uptake and efflux e.g. in *Agrostis tenuis* in response to Cu and Zn (Wainwright and Woolhouse 1977) and *Mimulus guttatus* in response to Cu (Strange and Macnair 1991).

The As hyperaccumulation, associated with elevated As tolerance ability in fronds, is a constitutive trait in *Pteris vittata*. Nevertheless, As uptake and accumulation rates vary among *P. vittata* populations originating from distinct geographical locations (Srivastava et al. 2010). *P. vittata* populations differed in As decreasing efficiency when grown as intercrops with *Morus alba* (Wan and Lei 2018). Such ecotypic variations likely occur in case of other species of Pteridaceae and related families that might influence the response to arsenic as well as toxic heavy metals (HMs). To exploit this genetic and functional potential, it is of significance to screen the ferns for species- as well as population-specific differences in tolerance to metal(loids). In view of the cross tolerance to metals with common toxicity pathways (Schat and Vooijs 1997), it is interesting to examine the responses of As-hyperaccumulator species to toxic HMs. For example, *P. melanocaulon*, besides being an As hyperaccumulator, also accumulates and tolerates high concentrations of Cu (Claveria et al. 2019). In the present study, we compared the As-induced toxicity in Western Himalayan populations of *P. vittata* and *P. cretica* (Pteridaceae) with that in *Polypodiodes microrhizoma* and *Lepisorus contortus* (Polypodiaceae) in terms of membrane integrity and antioxidant defence parameters. Besides, in order to get an idea whether As-imposed toxic effects were a consequence of cellular redox imbalance, they were compared with those of Cu, a redox active metal, and H_2O_2, a strong oxidant.

Materials and Methods

Plant Source

Pteris vittata L. and *Pteris cretica* L. (Pteridaceae) and *Polypodiodes microrhizhoma* (C. B. Clarke ex Baker) Ching and *Lepisorus contortus* (H. Christ) Ching (Polypodiaceae) were collected from the wild populations in Western Himalayan region; the first from Hamirpur (Himachal Pradesh; 700 m asl) and all others from Shimla hills (Himachal Pradesh; 2300 m asl). The fronds were used in the described experiments.

Determination of the Effects of As, Cu and H_2O_2 on Membrane Integrity

Membrane integrity of the selected ferns was assessed in a short-term pinna segment assay adopted from a rapid leaf slice test introduced by Cho et al. (2003) for comparing the tolerance of *Arabidopsis halleri* and *A. thaliana* to certain HMs. The pinnae from uniform fern fronds were sliced into 1–2 mm wide segments (~ 100 mg), that were vacuum-infiltrated for 5 min with solutions containing As (Na_2HAsO_4; 0–1000 µM), Cu (50 µM; $CuSO_4$) or H_2O_2 (50 mM) and incubated in glass Petri plates at 25 °C under constant illumination (PAR; 40 µmol photons m^{-2} s^{-1}). K^+-leakage into the incubation medium and activities of antioxidant enzymes namely, catalase, ascorbate peroxidase and superoxide dismutase in tissue extracts were measured after the stated durations.

Determination of the Effects of Phosphate and TEA on As-Induced K^+ Leakage

The effects of simultaneously applied Pi, a structural analogue of As, and tetraethylammonium chloride (TEA), a K^+ channel blocker, were examined on As-induced K^+ leakage from pinna segments of *P. microrhizoma*. The pinna segments were vacuum-infiltrated with As (250, 500 µM), $NH_4H_2PO_4$ (100, 500 and 1000 µM;) and TEA (10 mM) individually or in the stated combinations. K^+-leakage was assessed after 18 h of incubation.

Determination of K^+ Leakage

K^+ content in the pinna segment incubation medium was estimated after 3, 6 and 18 h of treatment using a flame photometer (Corning 400, UK) calibrated using KCl (0–20 ppm).

Antioxidant Enzyme Assays

The fern pinna segments (~ 100 mg) were homogenised with 100 mM HEPES–NaOH buffer (pH 7.6, supplemented with 5 mM ascorbate) and the obtained homogenate centrifuged at 8000×*g* for 5 min at 4 °C. The supernatant was used for catalase (CAT) and ascorbate peroxidase (APX) activity measurement. CAT activity was assayed polarographically using a Clark-type O_2 electrode (Hansatech, UK), calibrated using the signal difference between air- saturated water and water deprived of O_2 using sodium dithionite. The reaction mixture contained 890 µl HEPES–NaOH buffer (100 mM, pH 7.6), 10 µl of enzyme extract and 100 µl H_2O_2 (100 mM). The enzyme activity was calculated from the slopes recorded on chart paper using the value of dissolved O_2 at 25ºC (Goldstein 1968; Sharma et al. 2004). APX activity was assayed according to Hossain and Asada (1984). The reaction mixture contained 50 µl ascorbate (5 mM), 1 ml 50 mM HEPES–NaOH buffer (pH 7.6), 50 µl enzyme extract and 100 µl H_2O_2 (3 mM). Following the addition of H_2O_2, change in absorbance was monitored at 290 nm and enzyme activity determined by using the extinction coefficient 2.8 mM^{-1} cm^{-1} for ascorbate. Superoxide dismutase (SOD) activity was measured using method modified after Beauchamp and Fridovich (1971). Extraction buffer used was 100 mM K-phosphate (pH 7.0). The reaction mixture contained 680–695 µl assay buffer (100 mM K-phosphate, pH 7.8), 100 µl riboflavin (130 µM), 100 µl methionine (13 mM), 100 µl nitro-blue tetrazolium chloride (NBT, 1.26 mM) and 5–20 µl enzyme extract. The reaction mixture was illuminated for 10 min. and the absorbance read at 560 nm. A 50% reduction in absorbance was taken as one-unit activity. Specific activity for all the antioxidant enzymes was calculated from the protein content determined with the Bradford reagent (Bradford 1976).

Tissue Staining for Superoxide ($O_2^{-\cdot}$) and H_2O_2 Accumulation

In order to ascertain the role of ROS in As-induced toxicity, accumulation of $O_2^{-\cdot}$ and H_2O_2 in As treated pinna segments was monitored by nitro blue tetrazolium (NBT) and diaminobenzidine (DAB) staining, respectively. The pinna segments treated with As and Cu for 6 h were incubated with NBT (0.05% in PBS buffer, pH 7.8) and DAB (0.05% in 0.1 N HCl) staining solutions overnight in dark. They were then shifted to de-staining solution (ethanol and chloroform in a ratio of 1:5 containing 0.15% trichloroacetic acid) to make stained areas visible through chlorophyll extraction. The stained pinna segments were fixed in PBS-glycerol (1:1). The results are shown in the form of representative images from two biological replicates.

Statistics

Data are presented as arithmetic means ± SE for all parameters except CAT and SOD activities that are presented in box plots with each hollow dot representing a data point (showing different data points available). Data points that are not connected represent outliers. All data were tested for statistical significance using one-way ANOVA followed by Tukey's test or *t*-test as described in respective figure legend.

Results

As-Induced K^+ Leakage from Pinna Segments

Changes in K^+ leakage from fern pinna segments, an indicator of membrane damage, were monitored in response to As(V), Cu(II) and H_2O_2 treatments (Figs. 1, 2, 3). Species-specific differences were evident which were concentration and treatment time-dependent. Arsenic did not markedly change the K^+-leakage from pinna segments of *Pteris vittata*, *P. cretica* and *Polypodiodes microrhizoma* after 3 and 6 h except a marginal increase of 13% after 3 h in *P. vittata* and 19% after 6 h in *P. microrhizoma,* at 1000 µM As (Fig. 1). However, As-induced K^+ leakage was substantial after 18 h with strong species-specific differences. In *P. vittata,* an increase of 27% at 1000 µM was observed while in *P. cretica* this was 52 and 59% at 500 and 1000 µM, respectively. In contrast, *P. microrhizoma* showed a massive K^+ leakage with 3.3-, 4-, and 5-fold increase at 250, 500 and 1000 µM As, respectively. Apparently, *P. microrhizoma* was much more sensitive to As than the two *Pteris* species. The

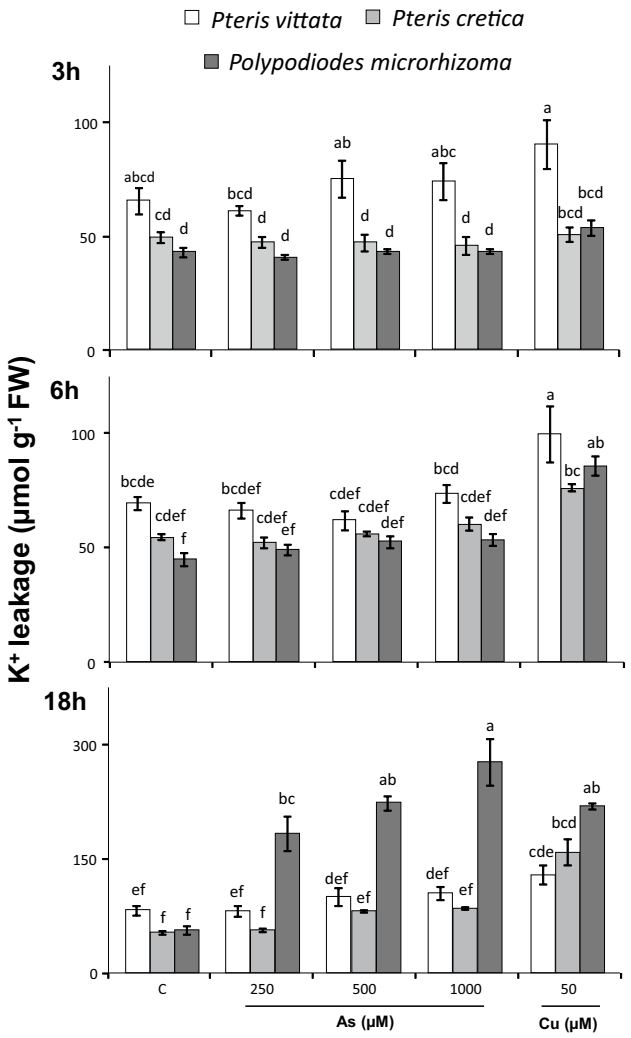

Fig. 1 As- and Cu-induced K^+-leakage from pinna segments of selected fern species into the incubation medium. The pinna segments (1–2 mm width, ~ 100 mg) of *Pteris vittata, P. cretica* and *Polypodiodes microrhizoma* were vacuum-infiltrated for 5 min. with desired treatment solutions and were incubated under continuous light (40 µmol photons m^{-2} s^{-1}) in the treatment solutions (10 ml) for 3, 6 and 18 h. K^+ leakage was measured in the incubation medium by a flame photometer (Corning, 400). Values are means ± SE, $n = 6$ (all means compared for treatment effects; one-way ANOVA, Tukey's test $p \leq 0.05$)

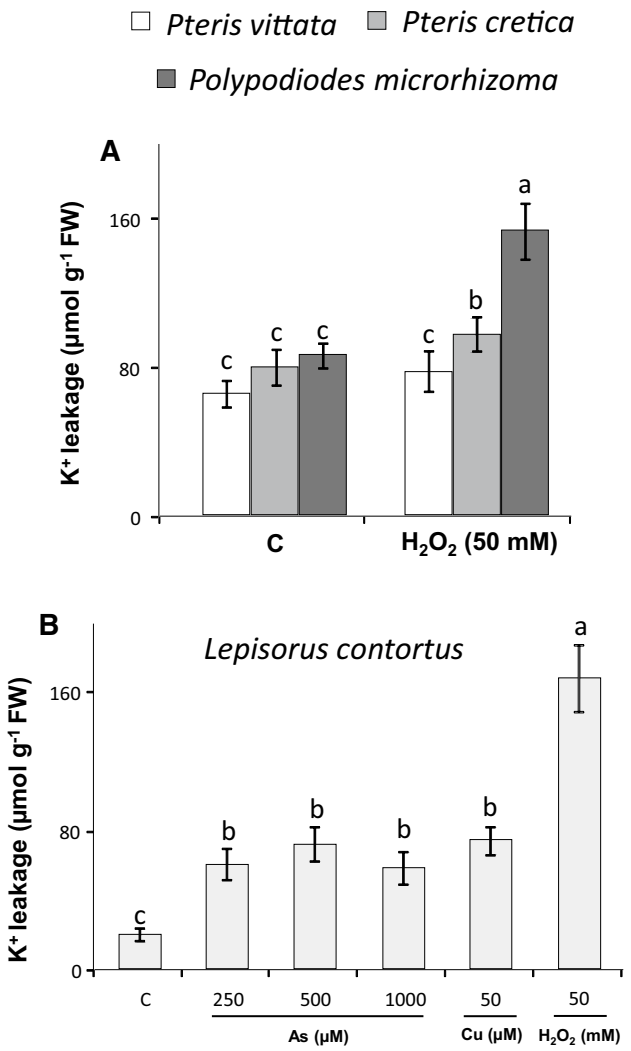

Fig. 2 H_2O_2-induced K^+-leakage from the pinna segments of selected fern species. K^+-leakage measured in the incubation medium from the pinna segments of *P. vittata, P. cretica* and *Polypodiodes microrhizoma* (**A**) and *Lepisorus contortus* (**B**) after 3 h of incubation in H_2O_2 (50 mM). Also, given is the K^+-leakage from the pinna segments of *Lepisorus contortus* as affected by As and Cu (**B**) after 18 h of vacuum infiltration. Values are means ± SE, $n = 6$ (*t*-test results are given with different letters which represent significantly different means)

control values for K^+ contents in incubation medium correspond to basal leakage and cell damage occurred during pinna segment preparation, which also depends on the pinna texture and thickness. As-induced K^+-leakage enhancement was paralleled by a similarly increased electrical conductivity of incubation medium (data not shown).

To get an idea whether As effects are a consequence of oxidative stress, they were compared with those of Cu, a redox active element, and H_2O_2. As in case of As(V), Cu (50 µM) treatment of 18 h enhanced the K^+ leakage from pinna segments by a magnitude of 1.56-, 3.01- and 3.9-fold in *P. vittata*, *P. cretica* and *P. microrhizoma*, respectively (Fig. 1). In *P. microrhizoma,* Cu effect could be observed as early as 3 h after start of the treatment (24% increase). This value increased to 91% after 6 h. Overall, the Cu-sensitivity after 18 h was more alike among different ferns than the As response at any concentration; however, the maximum change was observed for *P. microrhizoma*. An entirely different response of K^+ leakage was seen when fern pinna segments were treated with 50 mM H_2O_2 for 3 h (Fig. 2A). *P. vittata* showed a slight increase of 18%, *P. cretica* lost 21% more K^+ to the medium than under control

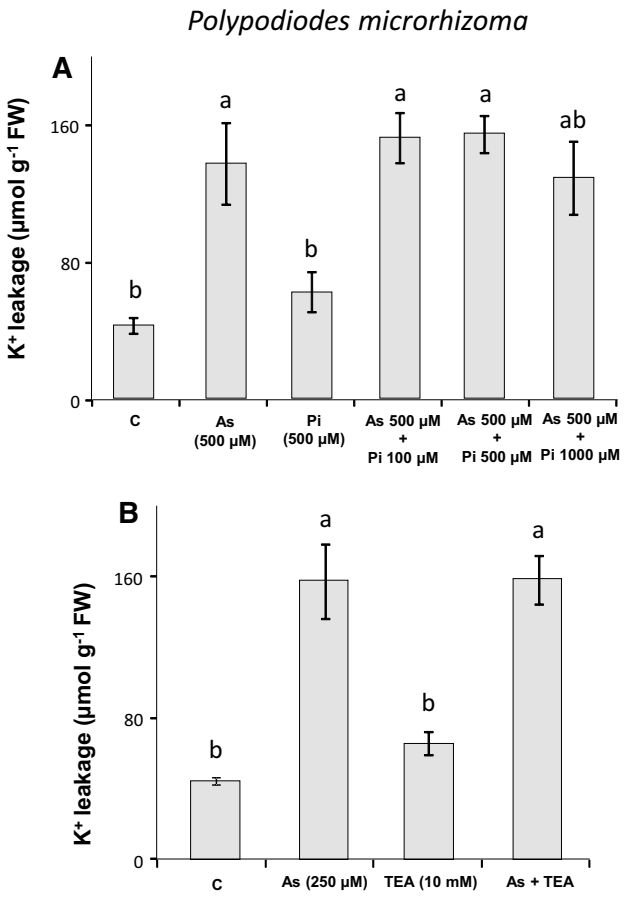

Fig. 3 Effect of Pi and TEA (a K^+-channel blocker) on As-induced K^+-leakage from pinna segments of *Polypodiodes microrhizoma*. K^+-leakage was measured in incubation medium from pinna segments of *P. microrhizoma* as indicated, **A** effect of Pi, **B** effect of tetraethylammonium chloride (TEA). Measurements were done after 18 h of vacuum infiltration. Values are means ± SE, $n = 3$ (*t*-test, different letters represent significantly different means)

conditions, and only *P. microrhizoma* showed a substantially increased (77%) K^+ leakage. This H_2O_2-induced pattern after 3 h tentatively reminded of the K^+ leakage pattern seen with 50 µM Cu after 18 h, but was poorly related to the As response pattern, apart from the fact that the highest value was denoted for *P. microrhizoma*. To substantiate the hypothesis that a major difference in membrane susceptibility to As stress exists between Pteridaceae and Polypodiaceae species, another fern from the family Polypodiaceae, namely *Lepisorus contortus*, was tested. Its pinna segments were exposed to As and Cu for 18 h and to H_2O_2 for 3 h, and K^+ losses recorded (Fig. 2B). The *L. contortus* pinna segments showed a sharp increase in K^+ leakage under As (250, 500 and 1000 µM), Cu (50 µM) and particularly in response to 50 mM H_2O_2 with 3.13-, 3.73-, 3.04-, 3.85- and 8.73-fold increases, respectively, as compared to controls.

As-Induced K^+ Leakage as Influenced by Phosphate and K^+ Channel Blocker

Since As(V) uptake occurs through phosphate uptake transporters, phosphate could be expected to suppress As-uptake and thereby its toxic effects. However, co-supplementation of phosphate did not affect the magnitude of As-induced K^+ leakage in *P. microrhizoma* (Fig. 3A). The K^+ leakage induced by arsenate was similar in the presence and absence of phosphate. Phosphate alone at 500 µM enhanced K^+ leakage by 45% as compared to control, but the change was not statistically significant (Fig. 3A). To investigate the involvement of K^+ channels in As-induced increase in K^+ leakage, the pinna segments of *P. microrhizoma* were treated with As in presence of tetraethylammonium chloride (TEA), a frequently used K^+ channel blocker. The unchanged K^+ leakage in the presence of TEA revealed no effect of K^+ channel blocker on As (250 µM)-induced K^+ leakage (Fig. 3B).

As-Induced Oxidative Stress

The fern pinna segments were analysed for metal(loid)-induced oxidative stress by in-vivo localization of the reactive oxygen species $O_2^{-\cdot}$ and H_2O_2 after 6 h of treatment. NBT staining showed strong accumulation of $O_2^{-\cdot}$ in pinna segments of *P. microrhizoma* after 6 h of As treatment (Fig. 4). The NBT staining in As-treated *P. vittata* and *P. cretica* pinna segments was faint (Fig. 4). Cu also induced higher accumulation of $O_2^{-\cdot}$ in pinna segments of *P. microrhizoma* as compared to those of *P. vittata* and *P. cretica*. DAB staining showed As-induced enhanced H_2O_2 accumulation in *P. microrhizoma* pinna segments but not in case of *P. vittata* and *P. cretica.* Exposure to Cu induced H_2O_2 accumulation in *P. microrhizoma* (Fig. 4). The observed species-specific differences in As-induced ROS accumulation prompted us to analyse the activities of antioxidant enzymes namely, CAT, SOD and APX after 3, 6 and 18 h of treatment.

CAT Activity

The basal CAT activity in pinna segments was higher in *P. cretica* and *P. vittata* than in *P. microrhizoma*. In response to As treatment, CAT activity in *P. vittata* tended to increase after 3 and 6 h of treatment although statistical significance was not evident. CAT activity in *P. cretica* increased after 6 h of As treatment by 67 and 51% at 500 and 1000 µM As, respectively (Fig. 5). However, after 18 h of As treatment, CAT activity decreased in *P. vittata* compared to control while no change was observed in *P. cretica*. Arsenic also induced an increase in CAT activity in the pinna segments of *P. microrhizoma* at 500 and 1000 µM after 3 and 6 h of exposure; however, the activity

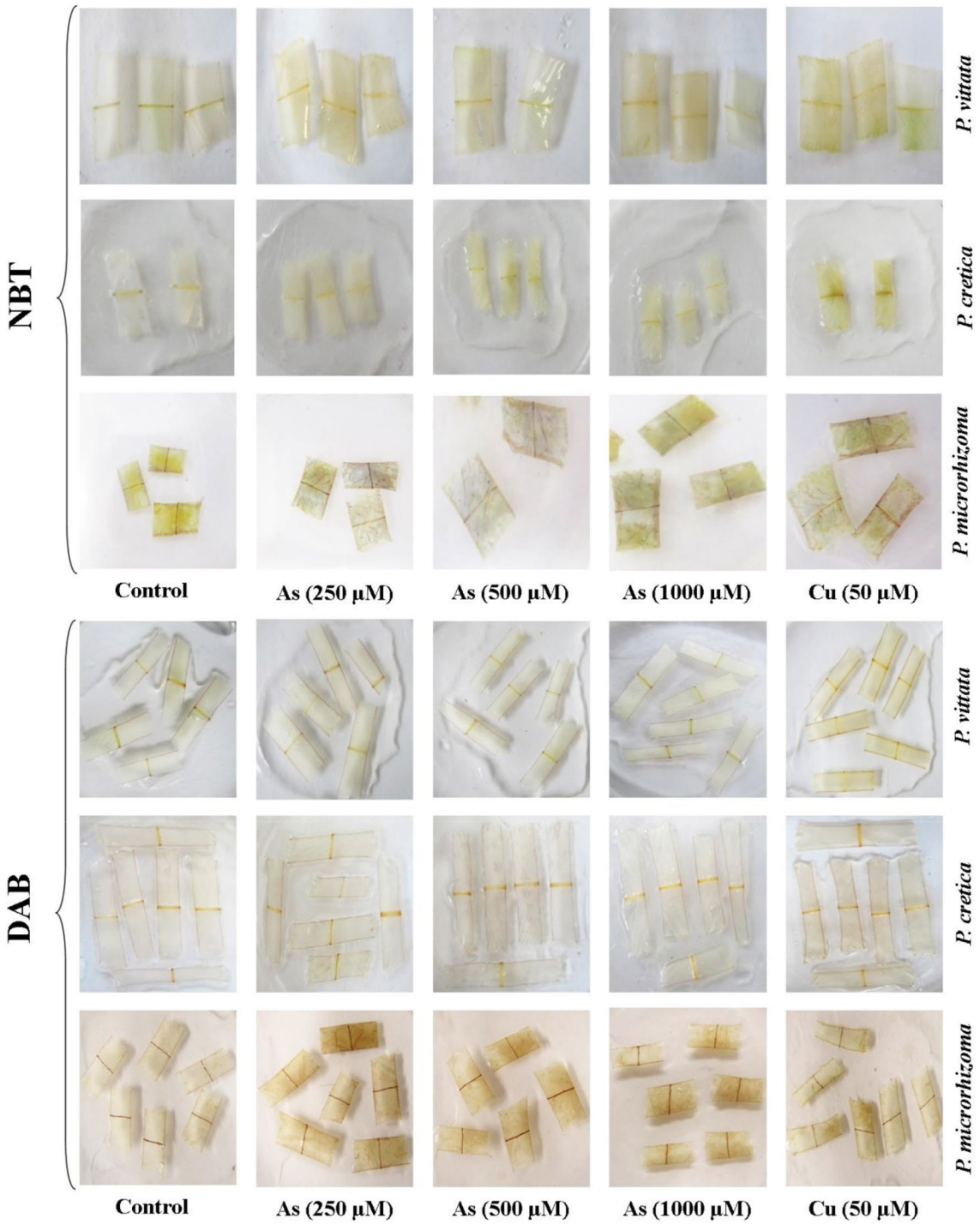

Fig. 4 *In-vivo* localization of superoxide radicals and hydrogen peroxide in the fern pinna segments. Effect of different treatments was observed on the generation of $O_2^{\cdot-}$ and H_2O_2 in differentially treated pinna segments of *Pteris vittata, P. cretica* and *Polypodiodes microrhizoma* by NBT and DAB staining, respectively, after 6 h of vacuum infiltration. The images are representative of two independent experiments

was significantly lower than that of respective treatments in *P. cretica* and *P. vittata* (Fig. 5). For example, after 6 h of As (500 and 1000 µM) exposure, the CAT activity was 2.70-, 2.74-fold and 3.37-, 3.45-fold higher in *P. vittata* and *P. cretica*, respectively, as compared to that in *P. microrhizoma*. After 18 h of treatment, the As-induced increase was only marginal. Due to Cu (50 µM) treatment, CAT activity was inhibited in the pinna segments of *P.*

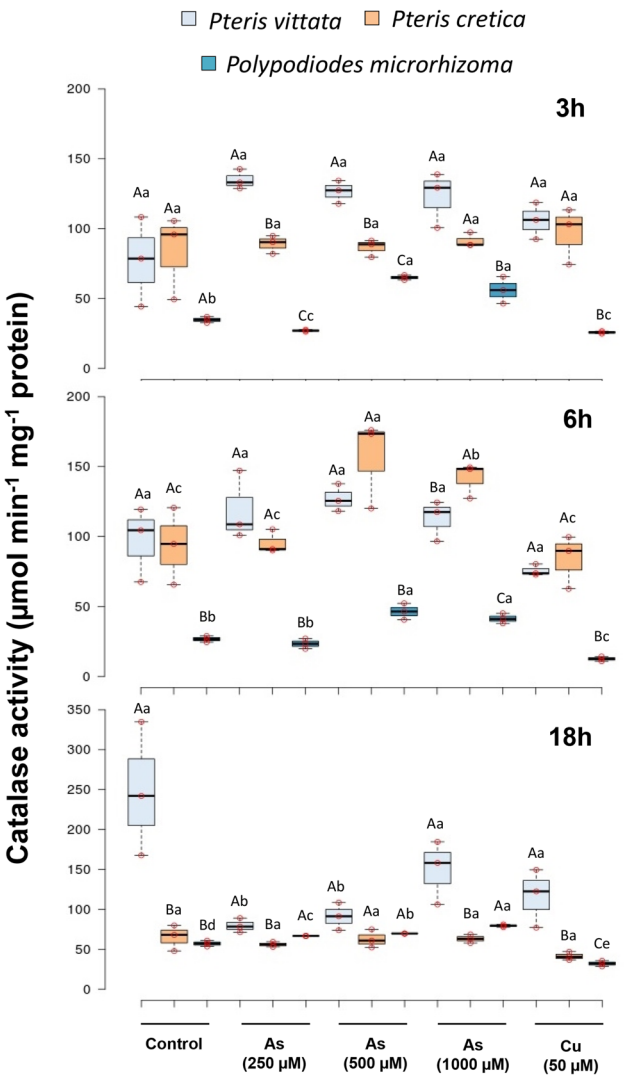

Fig. 5 Catalase activity in differentially treated pinna segments. The activity of catalase was measured in the pinna segments of *P. vittata, P. cretica* and *P. microrhizoma* treated with different concentrations of As and Cu after 3, 6 and 18 h of vacuum infiltration. The data are presented in box plots with each hollow dot showing an available data point. In the box plot, centre lines show the medians; box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles; not connected data points represent outliers, $n=3$ (*t*-test, different letters represent significantly different means; A, B, C… for significant differences between values for different fern species at a specific treatment, while a, b, c… for significant differences among different treatments for a specific fern species). The box plots were generated using the online tool shiny.chemgrid.org/boxplotr

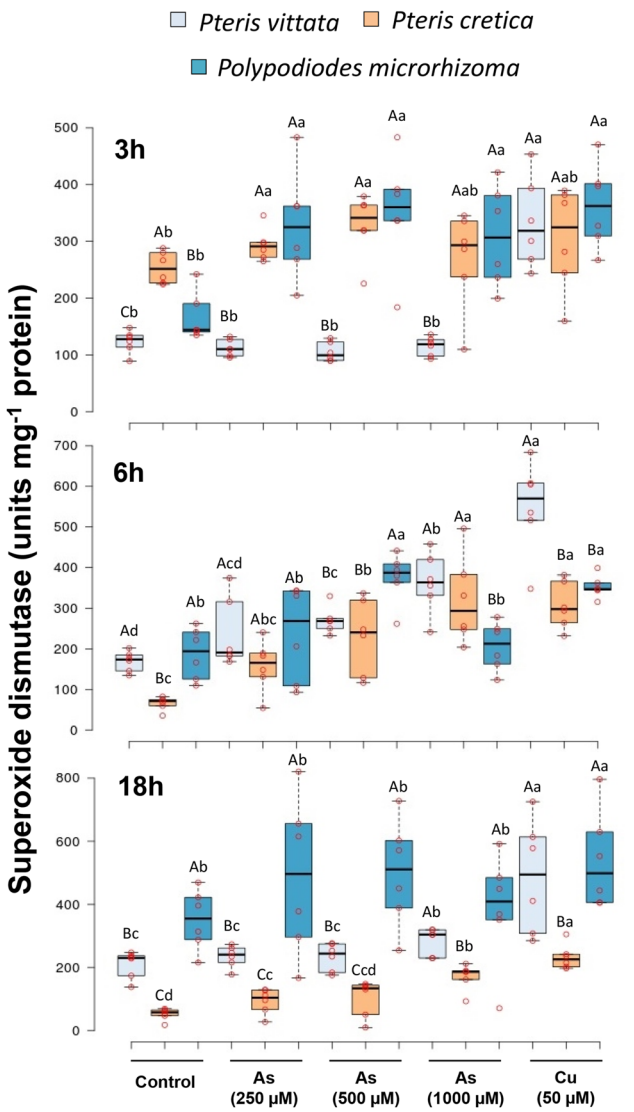

Fig. 6 Superoxide dismutase activity in differentially treated pinna segments. The activity of superoxide dismutase (SOD) was measured in pinna segment extracts of *P. vittata, P. cretica* and *P. microrhizoma* treated with As and Cu as indicated after 3, 6 and 18 h of vacuum infiltration. The data are presented in box plots with each hollow dot showing an available data point. In the box plot, centre lines show the medians; box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles; not connected data points represent outliers. Values are means ± SE, $n=6$ (*t*-test, different letters represent significantly different means; A, B, C… for significant differences between values for different fern species at a specific treatment, while a, b, c… for significant differences among different treatments for a specific fern species)

microrhizoma but remained unchanged in those of *P. vittata* and *P. cretica* (Fig. 5).

SOD Activity

In response to As treatment, SOD activity increased in *P. microrhizoma* pinna segments as early as after 3 h although the magnitude of increase did not correspond with As concentrations. The increased activity was maintained until 6 h except at 1000 µM As (Fig. 6). SOD activity was not affected by As after 18 h. In *P. vittata*, SOD activity was not affected by As after 3 h of treatment; however, it increased in a concentration-dependent manner after 6 h; a 1.9-fold

increase was evident at 1000 µM. SOD activity was marginally enhanced by As in *P. cretica* after 3 h particularly at 250 and 500 µM As. The As-induced activity enhancement was substantial and concentration-dependent after 6 h with a maximal increase of 7.13-fold at 1000 µM. After 18 h, As-induced increase in SOD activity was observed only at 1000 µM both in *P. vittata* and *P. cretica*. Interestingly, SOD activity was higher in *P. microrhizoma* than in *P. vittata* and *P. cretica* after 18 h As-exposure. Cu strongly increased the SOD activity at all time points. The magnitude of increase was higher in *P. vittata* after 6 and 18 h of treatment (Fig. 6).

APX Activity

Constitutive APX activity was higher in pinna segments of *P. cretica* and *P. microrhizoma* than that in *P. vittata*. It changed with the lapse of incubation period particularly in *P. vittata* and *P. microrhizoma* with a substantial increase or decrease, respectively (Table 1). In response to As, APX activity increased after 3 h in *P. vittata* by a factor of 2.39, 7.67 and 6.94 at 250, 500 and 1000 µM As, respectively. A similar pattern with a lower magnitude of increase was observed in *P. cretica* after 3 h. Thus, the activity was 1.34-, 1.77- and 2.27-fold higher at 250, 500 and 1000 µM As, respectively. Subsequently, however, no definite pattern of change was observed. APX activity tended to increase at higher As concentrations (500 µM in *P. cretica* and 1000 µM in *P. vittata*) after 6 h. After 18 h, As suppressed the APX activity in *P. cretica* at all concentrations and at 1000 µM in *P. vittata* (Table 1). As suppressed the APX activity in *P. microrhizoma* after 3 h particularly at 500 µM. The activity was not affected by As after 6 h but was promoted after 18 h by 66, 84 and 50% at 250, 500 and 1000 µM, respectively (Table 1). In response to Cu (50 µM) treatment, APX activity increased in *P. vittata* after 3 and 6 h by 1.46- and 4.81-fold, respectively and then decreased by 50% after 18 h. In *P. cretica*, 84% increase in APX activity was observed after 3 h that was followed by a decline of 22 and 50% after 6 and 18 h, respectively. APX activity increased due to Cu after 6 and 18 h in *P. microrhizoma* by 99 and 40%, respectively (Table 1).

Discussion

Screening for As Tolerance

The initial steps in developing phytoremediation strategies based on hyperaccumulation traits need to ascertain the metal tolerance levels of the species under consideration. In the best case, one should also understand the underlying mechanisms of metal(loid) accumulation. In view of the extraordinary root-to-shoot metal(loid) translocation efficiency of hyperaccumulators the tolerance of shoot organs, in particular the leaves, to the metal(loid)s is of utmost importance. To shorten the process of tolerance screening, Cho et al. (2003) introduced an easy and rapid leaf slice test for deciphering the HM-specific tolerance of *Arabidopsis halleri* and *A. thaliana*. On similar lines, in present study, As- tolerance of *P. vittata*, an As hyperaccumulator, and some related ferns from Western Himalayan region has been evaluated in terms of membrane integrity parameters by direct application of As to the leaf cells using pinna slices. Quick screening through such simple assays could be expected to reveal novel As hyperaccumulator species and the tissue-specific tolerance strategies. As such, a rich fern diversity from the region awaits physiological and ecological scrutiny.

Species Specificity of As-Induced Membrane Damage

Damage to the cell membranes, especially plasma membrane, constitutes one of the primary events in development of HM toxic effects in plants (Janicka-Russak et al. 2008). Conversely, the improved plasma membrane integrity has

Table 1 Ascorbate peroxidase activity in As- and Cu-treated pinna segments of selected fern species

Ascorbate peroxidase activity (µmol min^{-1} mg^{-1} protein)									
Treatment (µM)	*Pteris vittata*			*Pteris cretica*			*Polypodiodes microrhizoma*		
	3 h	6 h	18 h	3 h	6 h	18 h	3 h	6 h	18 h
0	$0.30^{Ac} \pm 0.04$	$0.96^{Ba} \pm 0.30$	$1.04^{Ba} \pm 0.15$	$3.37^{Ab} \pm 0.42$	$3.13^{Aa} \pm 0.41$	$3.74^{Aa} \pm 0.80$	$7.58^{Aa} \pm 0.84$	$2.91^{Aa} \pm 0.26$	$3.33^{Ab} \pm 0.50$
As (250)	$0.65^{Bb} \pm 0.14$	$1.13^{Ba} \pm 0.41$	$1.25^{Ca} \pm 0.18$	$4.53^{Ab} \pm 0.32$	$3.37^{Aa} \pm 0.26$	$3.20^{Ba} \pm 0.27$	$6.89^{Aa} \pm 1.44$	$3.32^{Aa} \pm 0.19$	$5.52^{Aa} \pm 0.44$
As (500)	$2.48^{Ba} \pm 0.71$	$0.87^{Ba} \pm 0.36$	$1.28^{Ca} \pm 0.39$	$4.57^{Ab} \pm 0.59$	$5.36^{Aa} \pm 1.04$	$2.52^{Ba} \pm 0.24$	$3.51^{Ab} \pm 0.60$	$3.24^{Aa} \pm 0.03$	$6.12^{Aa} \pm 0.61$
As (1000)	$2.28^{Ba} \pm 0.58$	$2.03^{Aa} \pm 0.56$	$0.81^{Ca} \pm 0.09$	$5.55^{Ab} \pm 1.16$	$3.82^{Aa} \pm 0.48$	$2.20^{Bb} \pm 0.35$	$6.53^{Aa} \pm 0.86$	$2.99^{Aa} \pm 0.63$	$5.01^{Aa} \pm 0.30$
Cu (50)	$0.61^{Bb} \pm 0.11$	$1.30^{Ba} \pm 0.45$	$0.52^{Cb} \pm 0.08$	$5.54^{Aa} \pm 0.40$	$2.99^{Aa} \pm 0.17$	$2.59^{Ba} \pm 0.44$	$2.50^{Bb} \pm 0.48$	$5.80^{Aa} \pm 0.57$	$4.64^{Ab} \pm 0.36$

Activity was measured after 3, 6 and 18 h of vacuum infiltration. Values are means ± SE, n = 3 (*t*-test, different letters represent significantly different means; A, B, C… used for significant differences between values for different fern species at a specific treatment, while a, b, c.. signify significant differences among different treatments for a specific fern species)

an adaptive value, in protecting membrane functions and in turn cell integrity. Strong species-specific differences in As-induced K^+ leakage, a measure of damage to the membrane permeability barrier, were evident. Arsenic was applied through vacuum infiltration of pinna segments to ensure rapid entry into the intercellular air space and equilibration with the apoplastic fluid. Whereas As induced a massive K^+ leakage in case of *P. microrhizoma* and *L. contortus*, both members of Polypodiaceae, the magnitude of the same was much lower in case of Pteridaceae ferns *P. vittata* and *P. cretica*. The findings clearly point to substantially elevated As tolerance of latter species where the membrane damage was negligible even at 1000 µM As. Indeed, the membrane stability index in *P. vittata* under As exposure exceeds that in arsenic-sensitive *P. ensiformis* (Singh et al. 2006). Metal(loid)s cause membrane damage via generation of ROS and subsequent lipid peroxidation or activation of enzymes like lipoxygenases (Sharma and Dietz 2009). In order to get insight into the possibility of mediation of As toxicity by oxidative stress, the As effects were compared with those of Cu, a redox active element like As, on the one hand and H_2O_2, a strong oxidant, on the other. That both Cu and H_2O_2 produced species-specific effects resembling qualitatively to those of As strengthens an assumed role of ROS and in turn oxidative stress in the observed As-induced damage to membrane integrity. Cu-induced damage to the root cell plasma membrane was reported in *Silene cucubalus* (De Vos et al. 1989) and *Mimulus guttatus* (Strange and Macnair 1991). A stronger metal(loid) tolerance capability of *P. vittata* and *P. cretica* as compared to *P. microrhizoma* and *L. contortus* might be due to common tolerance strategies for As and Cu, including generally strengthened plasma membrane and/or some specific ecological adaptations. As an example of ecological adaptation, multiple tolerance and co-tolerance to HMs in *Silene vulgaris* has been reported (Schat and Vooijs 1997). Also, in *P. vittata* (ecotypes from Kerala, India), Cr tolerance and hyperaccumulation along with that of As was shown (Kalve et al. 2011). There is a need to screen *P. vittata* as well as other related ferns for tolerance to different HMs.

K^+ ions are involved in several cellular functions in plants including activation of enzymes and ribosomes, counterbalancing of ions, turgor adjustment and maintenance of membrane potentials (Maathuis et al. 1997). Indeed, cytosolic K^+-contents work as "master switches" in the transition of plant metabolism from the normal to 'adjusted' state under abiotic stresses (Shabala and Pottosin 2014). Electrolyte leakage is considered a constituent of plant stress response (Demidchik et al. 2014). The stress mediated K^+-efflux provides an adaptive mechanism, e.g. in guard cells in order to initiate stomatal closure. There is a possibility that As, in addition to causing membrane damage via ROS generation, might have stimulated the K^+ channels as was suggested in case of Cu treated *A. thaliana* (Murphy and Taiz 1995). The K^+ channels include voltage-dependent, slow activating, outward rectifying K^+-selective 'Shakers' channels encoded by guard cell outward rectifying K^+-channel (GORK), selective K^+-outward rectifying channel (SKOR) and annexin genes. Incidentally, exposure to HMs stimulates the generation of ROS such as $O_2^{-\cdot}$ and H_2O_2 (Sharma and Dietz 2009) that in-turn could activate GORK, SKOR and annexins catalysing K^+-efflux (Demidchik 2010). Besides, a hypothetical possibility of involvement of voltage-independent, instantaneously activating, cation non-selective 'Shaker like' cyclic nucleotide-gated channels (CNGC) and ionotropic glutamate receptors (GLRs) channels is suggested (Demidchik et al. 2014). To examine the possible involvement of K^+ channels in the leakage of K^+ ions, TEA, a specific K^+-channel blocker for voltage-dependent K^+-selective 'Shakers' (Thompson and Begenisich 2003), was applied simultaneously with As (250 µM) in *P. microrhizoma*. However, TEA did not alter the As-induced K^+ leakage. A slight increase in K^+ leakage due to TEA alone might be a result of TEA toxicity. It is pertinent to add that for different categories of voltage-dependent K^+-selective 'Shakers', the required TEA concentration for effective blocking could vary several fold owing to the differences in the amino acid composition of TEA binding site (Kavanaugh et al. 1991). The likely involvement of other K^+ permeable channels in As-dependent K^+ leakage has not been examined in this study.

The possible alleviation of As-inflicted membrane damage by the hypothesized competition between Pi and As for uptake pathway was also investigated in *P. microrhizoma*. Mesophyll cells show a rapid Pi uptake from IWF using proton motive force generated by H^+-ATPase (Mimura et al. 1990). The As-induced effect on K^+ leakage was not altered by the presence of Pi. However, there is a need to check actual As uptake into the cell. Besides, there is also a possibility of cell wall binding and apoplastic sequestration of As in these ferns as a strategy to avert toxicity as was demonstrated for barley for certain HMs (Brune et al. 1995). Arsenic likely affected the membranes by acting on the apoplastic surface.

Altered Antioxidative Defence System

The stress induced electrolyte leakage is often accompanied by stimulated generation of ROS (Demidchik et al. 2014). Plasmalemma-bound NAD(P)H oxidases as well as cell wall-associated peroxidases are the main $O_2^{-\cdot}$ and H_2O_2 producing apoplastic enzymes induced by HMs (Sagi and Fluhr 2006; Sharma and Dietz 2009). The As-induced increased SOD activity in *P. vittata* (6 h) and *P. cretica* (6 and 18 h) pinna segments seems adequate for scavenging of $O_2^{-\cdot}$ as is also evident from NBT staining. CAT and peroxidases likely dealt with the subsequently produced H_2O_2. In accordance, both *Pteris* species exhibited high

constitutive CAT activity vis-à-vis the other fern that was further enhanced due to As. In addition, the higher As concentrations slightly enhanced the APX activity during initial phase (3 h) of treatment. This might be particularly significant for degradation of low concentrations of H_2O_2 in the stroma, matrix and cytosol in view of the lower K_m of APX. Efficient scavenging of the H_2O_2 in these two ferns was evident from DAB staining.

Low doses of $O_2^{-\cdot}$ and H_2O_2 are known to induce acclimation responses against oxidative and abiotic stresses (Gechev et al. 2002; Mittler 2017). The increased activities of the antioxidant enzymes in *Pteris* species might be responsible, in a large part, for lower K^+ leakage in response to As. The activities of antioxidant enzymes have been reported to increase in response to different HMs. For example, SOD, CAT and guaiacol peroxidase (POD) increased in response to Pb, Cd and Hg in metal tolerant *Kandelia candel* (Zhang et al. 2007) and those of SOD and CAT in *P. vittata* due to As (Srivastava et al. 2005). The decrease in enzyme activity after 18 h of As treatment (SOD in *P. vittata*) and (CAT in *P. vittata* and *P. cretica*) might reflect a stabilised cell metabolic state. A similar Cu-dependent enhancement of SOD and APX activities in *P. vittata* and *P. cretica* is consistent with the role of these enzymes in imparting tolerance against membrane damage.

The responses of antioxidant enzymes to As in *P. microrhizoma* differed strongly from those in *P. vittata* and *P. cretica*. Thus, the SOD activity increased abruptly at lower As concentrations followed by a decline. This was apparently not sufficient as is clear from the accumulation of $O_2^{-\cdot}$ in the As-treated pinna segments. The conversion of $O_2^{-\cdot}$ to H_2O_2 as well as the As-indifferent CAT activities possibly led to the accumulation of damaging levels of H_2O_2 concentrations. The DAB staining results support the assertion. The increased activity of APX after 18 h in As-treated segments of *P. microrhizoma* was obviously inadequate in coping with H_2O_2. The elevated H_2O_2 levels trigger alterations in Ca^{2+} fluxes and activities (Harper et al. 2004) which have rapid and short-term effects. The stress-specific transient Ca^{2+} oscillations can stimulate enzymes as NAD(P)H oxidase through specific Ca^{2+}-interacting proteins (Harper et al. 2004). The high doses of $O_2^{-\cdot}$ and H_2O_2 were shown to trigger cell death (Gechev et al. 2002; Stone and Yang 2006). The present findings are in agreement with those of Mascher et al. (2002) who linked the As sensitivity to oxidative stress resulting from cellular damage due to enhanced lipid peroxidation, H_2O_2 accumulation and up-regulation or deactivation of several scavenging enzymes. Taken together, the differential As tolerance of the ferns belonging to Pteridaceae and Polypodiaceae, assigned based on the rapid leaf segment assay, could be ascribed to the difference in As-índuced oxidative stress and antioxidant defence abilities. The findings have implications for selection of As tolerant and hyperaccumulator ferns for possible inclusion in phytoremediation applications.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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