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Metabolic adaptations to arsenic-induced oxidative stress in *Pteris vittata* L and *Pteris ensiformis* L

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Abstract

This study examined the metabolic adaptations of *Pteris vittata* L, an arsenic hyperaccumulator, under arsenic stress as compared to *Pteris ensiformis*, a non-arsenic hyperaccumulator. Both plants were grown hydroponically in 20% Hoagland medium in controlled conditions and were treated with 0, 133 or 267 μM arsenic as sodium arsenate for 1, 5 or 10 d. The fern fronds were analysed for differences in oxidative stress and antioxidant capacities after arsenic exposure. Upon exposure to 133 μM arsenic, concentrations of chlorophyll, protein and carotenoids increased in *P. vittata* whereas they decreased in *P. ensiformis*. The H_2O_2 and TBARs concentrations were greater in *P. ensiformis* than *P. vittata* in all treatments, indicating greater production of reactive oxygen species (ROS) by *P. ensiformis*. The levels of ascorbate and glutathione, and their reduced/oxidized ratios in the fronds of *P. vittata* of the control was much greater than *P. ensiformis* indicating that *P. vittata* has an inherently greater antioxidant potential than *P. ensiformis*. The lower levels of antioxidant compounds (ascorbate, carotenoids and glutathione) in *P. ensiformis* than *P. vittata* are consistent with its greater exposure to ROS and lower scavenging ability. The results together indicate that protection from oxidative damage by a greater level of ascorbate–glutathione pool is involved in the arsenic-tolerance in arsenic-hyperaccumulator *P. vittata*.

Keywords: Arsenic; Ascorbate; Glutathione; Hyperaccumulator; *Pteris ensiformis* L; *Pteris vittata* L; Thiobarbituric acid-reactive substances

1. Introduction

Arsenic is ubiquitous in the environment. Arsenic contamination in soils often leads to groundwater contamination and arsenic toxicity in plants, humans and animals. Remediation of arsenic-contaminated soils has become a major environmental issue. Phytoextraction, a plant based technology for the removal of toxic contaminants from soil and water is an attractive approach [1,2]. Ma et al. [3] reported the first known arsenic hyperaccumulator *Pteris vittata* L (Chinese brake fern), which can accumulate large amounts of arsenic (up to 2.3% dry wt.) in its aboveground biomass. Several other fern species have recently been reported to hyperaccumulate arsenic [4–6].

Under environmental stresses, plants often produce reactive oxygen species (ROS) such as superoxide, hydrogen peroxide and hydroxyl radicals, causing damage to DNA, proteins and lipids. There is significant evidence that arsenic exposure leads to the generation of ROS through the conversion of arsenate to arsenite, a process that readily occurs in plants [7,8]. To minimize the harmful effects of ROS, plants have evolved an effective scavenging system composed of antioxidant molecules and antioxidant enzymes [9].

Large genotypic differences in arsenic-tolerance have been reported within fern species [6]. A previous study comparing two fern species, *P. vittata* and *Nephrolepis exaltata* (Boston fern, a non-arsenic-hyperaccumulator), demonstrated that *P. vittata* displayed a greater arsenic-uptake influx rate than *N. exaltata* when subjected to arsenic [10]. Recently, *Pteris* species—*P. ensiformis* and *P. tremula* have been shown to be non-arsenic-hyperaccumulator [11,12], making it possible to compare the behaviours of two fern species of the same genus. In the present work, we tested the hypothesis that arsenic hyperaccumulator *P. vittata* has evolved metabolic adaptations to cope with arsenic-induced oxidative stress, compared to the non-hyperaccumulator *P. ensiformis*. Therefore, we assessed the differences in the oxidative damage, concentrations and nature of the antioxidant compounds between the two species

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of *Pteris*. To our knowledge, this is the first time non-enzymatic antioxidant capabilities have been compared between As-sensitive (non-hyperaccumulator) and As-tolerant (hyperaccumulator) species of the same genus.

The objectives of this study were (i) to compare the extent of arsenic-induced oxidative stress in *P. vittata* and *P. ensiformis* and (ii) to evaluate the reaction systems involved in ascorbate and glutathione metabolism to overcome oxidative stress in the two plants. The first objective was achieved by determining the changes in the concentrations of chlorophyll, protein, membrane stability index, TBARs, H_2O_2 and carotenoid in the plants, while the second objective by determining the changes in the concentrations of reduced and oxidized glutathione (GSH and GSSH), and ascorbate and dehydroascorbate (AsA and DasA) in the plants.

2. Materials and methods

2.1. Plant materials and treatments

Four-month-old *P. vittata* and *P. ensiformis* were obtained from a nearby nursery (Milestone Agriculture, Inc., FL, USA). The plants were acclimatized in a hydroponic system to promote root growth. After acclimatization in 0.2 strength Hoagland nutrient solution [13] for 2 weeks, the plants were transferred into 0.2-strength Hoagland nutrient solution containing 0, 133 or 267 μM arsenic as $Na_2HAsO_4{\cdot}7\ H_2O$ (Sigma Chemical Company). The solution was aerated continuously and renewed twice a week during the experiment. The plants were kept in a controlled room with 14-h light period at light intensity of 350 μmol m^{-2} s^{-1}, 25 °C/20 °C day/night temperature and 60–70% relative humidity.

The plants were harvested at three intervals, i.e. 1, 5 and 10 d after arsenic treatment. Since most of the arsenic was present in the fronds [3], all the analyses were performed using fronds. All the analyses were performed using fresh or flash-frozen fronds in liquid nitrogen and stored at −80 °C except for total arsenic where air-dried (65 °C for 2 d) samples were used. The experiment was replicated three times and arranged in a completely randomised design.

2.2. Arsenic concentration determination

Air-dried fern samples (0.5 g) were digested with nitric acid on a temperature-controlled digestion block (Environmental Express, Mt. Pleasant, S.C.) using USEPA Method 3050A. Analysis was performed with a transversely heated, Zeeman background correction equipped graphite furnace atomic absorption spectrophotometer (Perkin-Elmer SIMAA 6000, Norwalk, CT).

2.3. Chlorophyll and total carotenoids determination

Fresh fronds (0.5 g) were homogenised in 80% ice-cold acetone in dark and centrifuged at 10,000 × *g* for 10 min. For carotenoids the acetone extract was treated with ether and the acetone was completely removed by washing with water. The absorbance of the supernatant was measured using a Shimadzu UV160U UV–visible recording spectrophotometer. The chlorophyll and carotenoids were estimated by the formula from Lichtenthaler and Wellburn [14].

2.4. Protein and H_2O_2 determination

Protein estimation was carried out by the method of Bradford [15] using bovine serum albumin as the standard. Hydrogen peroxide levels were determined according to Velikova et al. [16]. Fresh fronds (0.5 g) were homogenised in ice bath with 5 ml 0.1% (w/v) tri chloro acetic acid (TCA). The homogenate was centrifuged at 12,000 × *g* for 15 min and 0.5 ml of the supernatant was added to 0.5 ml 10 mM potassium phosphate buffer (pH 7.0) and 1 ml of 1 M KI. H_2O_2 concentration was estimated based on the absorbance of the supernatant at 390 nm.

2.5. Membrane stability index

Membrane stability index (MSI) of the fronds was measured according to the method of Sairam et al. [17]. Fresh fronds (0.5 g) were cut into 20 mm segments, rinsed with distilled water and placed in tubes containing 15 ml of distilled water. Two sets were made. One set was subjected to 40 °C temperature for 30 min and conductivity of water ($C1$) in the tubes was determined using a Fischer Scientific Accument Model 20 pH/conductivity meter. The other set was placed in a boiling water bath for 20 min to kill the tissue completely, cooled to 24 °C and the conductivity ($C2$) was again measured to determine the ion concentration after complete membrane disintegration. The MSI was calculated as $[1 - (C1/C2)] \times 100$.

2.6. Determination of lipid peroxidation

Lipid peroxidation was measured as the amount of thiobarbituric acid reacting substances (TBARs) determined by the thiobarbituric acid (TBA) reaction, following the methods of Groppa et al. [18]. Fresh fronds (0.2 g) was cut into small pieces and homogenised, using a cold mortar and pestle in an ice bath, using 1 ml of 5% (w/v) trichloroacetic acid (TCA) solution. The homogenate was transferred into fresh tubes and centrifuged at 10,000 × *g* for 15 min at room temperature. To 1 ml of the aliquot of the supernatant, 1 ml of 20% (w/v)TCA containing 0.5% (w/v) TBA and 100 μl 4% butylated hydroxytoluene in ethanol were added. The mixture was heated at 95 °C for 30 min and then quickly cooled on ice. The concentrations were centrifuged at 10,000 × *g* for 15 min and the absorbance was measured at 532 nm. The value for non-specific absorption at 600 nm was subtracted. The concentration of TBARs was calculated using extinction coefficient of 155 mM^{-1} cm^{-1}.

2.7. Ascorbate determination

Reduced ascorbate (AsA), dehydroascorbate (DasA) and total ascorbate (ASC, AsA + DasA) were determined following

the method of Gossett et al. [19]. This assay is based on the reduction of ferric ion to ferrous ion with ascorbic acid in acid solution followed by formation of red chelate between ferrous ion and dipyridyl. Over ice, freeze-dried fronds (1 g) was ground with inert sand and 10 ml of 5% (v/v) *m*-phosphoric acid using a mortar and pestle. The homogenate was centrifuged at 22,000 × *g* for 15 min. Total ascorbate was determined in a reaction mixture consisting of 200 μl of supernatant, 500 μl of 150 mM KH_2PO_4 buffer (pH 7.4) containing 5 mM EDTA and 100 μl of 10 mM dithiothreitol (DTT) to reduce DasA to AsA. After 10 min at room temperature, 100 μl of 0.5% (w/v) *N*-ethylmaleimide was added to remove excess DTT. AsA was assayed in a similar manner except that 200 μl of deionized H_2O was substituted for DTT. Color was developed in both reaction mixtures with the addition of 400 μl of 10% (w/v) TCA, 400 μl of 44% (v/v) *o*-phosphoric acid, 400 μl of a,á-dipyridyl in 70% (v/v) ethanol and 200 μl of 30 g l^{-1} $FeCl_3$. The reaction mixtures were incubated at 40 °C for 1 h and quantified spectrophotometrically (Shimadzu UV160U UV–visible recording spectrophotometer) at 525 nm. Ascorbate standards were between 1 and 50 μmol ascorbate in 5% (v/v) *m*-phosphoric acid. DasA was estimated from the difference between total ascorbate and AsA.

2.8. Glutathione determination

Oxidised glutathione (GSSG), reduced glutathione (GSH) and total glutathione (GSSG + GSH) (TGSH) were determined by a method adapted from Gossett et al. [19]. The procedure utilized the enzymatic recycling method, using glutathione reductase (GR), for the quantification of GSH. The sulfhydryl group of GSH reacts with DTNB (5,5′-dithiobis-2-nitrobenzoic acid) to produce a yellow colored 5-thio-2-nitrobenzoic acid (TNB). The mixed disulfide (between GSH and TNB) that is concomitantly produced, is reduced by GR to recycle the GSH and produce more TNB. The rate of TNB production is directly proportional to this recycling reaction, which is in turn directly proportional to the concentration of GSH in the sample.

Over ice, flash-frozen fronds (1 g) was ground with inert sand and 5 ml of ice-cold 6% (v/v) *m*-phosphoric acid (pH 2.8) containing 1 mM EDTA in a cold mortar and pestle. The homogenate was centrifuged at 22,000 × *g* for 15 min and the supernatant removed and then filtered through a 0.45-μm filter. Two solutions were then prepared. Solution A consisted of 110 mM $Na_2PO_4{\cdot}7H_2O$, 40 mM $NaH_2PO_4{\cdot}H_2O$, 15 mM EDTA, 0.3 mM 5,5′-dithiobis-(2-nitrobenzoic acid) and 0.4 ml l^{-1} BSA. Solution B consisted of 1 mM EDTA, 50 mM imidazole, 0.2 ml l^{-1} BSA and an equivalent of 1.5 units GR activity (Baker's yeast, Sigma Chemical Company). Total glutathione was measured in a reaction mixture consisting of 400 μl of solution A, 320 μl of solution B, 400 μl of a 1:50 dilution of the extract in 5% (w/v) Na_2HPO_4 (pH 7.5) and 80 μl of 3 mM NADPH. The reaction rate was measured spectrophotometrically by following the change in absorbance at 412 nm for 4 min. For GSSG, 1 ml of the 1:50 extract dilution was initially incubated with 40 μl of 2-vinylpyridine at 25 °C for 1 h and assayed as described above. A standard curve was developed by preparing solutions of 0.5–16 μM GSH and analyzing them in the same manner as the extracts. GSH was estimated as the difference between total glutathione and GSSG.

2.9. Statistical analysis

Antioxidant compounds and chlorophyll assays were based on the means of three replicate samples of three independent experiments. The effects of plant species, arsenic concentration and exposure time on antioxidant compounds and chlorophyll concentrations were analysed by a three-factor completely randomised ANOVA. Both control and arsenic treatment results were expressed as mean ± S.D. where $n = 3$. Duncan's Multiple Range Test was employed to compare the changes within the plant species and in same species with changing arsenate dose [20].

3. Results

Pteris vittata (an arsenic-hyperaccumulator) and *P. ensiformis* (a non-arsenic-hyperaccumulator) were exposed to three concentrations of arsenic as arsenate (0, 133 or 267 μM) for 1, 5 or 10 d. The impacts of plant species, arsenic concentrations and exposure times were evaluated to assess the arsenic-induced oxidative damage by measuring the changes in the concentrations of chlorophyll, protein, membrane stability index, TBARs, H_2O_2 and carotenoid. Further, we tested the hypothesis that the antioxidant molecules ascorbate and glutathione were involved in the detoxification of arsenic-induced stress in *P. vittata*. Since most of the arsenic taken up by *P. vittata* is concentrated in the fronds [3], arsenic detoxification in the fronds is thus critical for understanding arsenic detoxification mechanisms in *P. vittata*. Therefore, this research focuses only on the fronds.

To evaluate the extent of arsenic-induced oxidative stress and the associated arsenic detoxification systems in the two plant species, it is important to know plant arsenic concentrations. Compared to *P. vittata*, arsenic concentrations in *P. ensiformis* were much lower, the differences were more pronounced at greater arsenic concentration and longer exposure time (Fig. 1). This is because arsenic concentrations in *P. vittata* increased steadily and significantly ($p < 0.01$) with both arsenic concentration and exposure time whereas they were mostly unchanged in *P. ensiformis*. *Pteris vittata* accumulated 2.4–2.8 times more arsenic than *P. ensiformis* in the fronds after 1 d exposure, and the difference increased to 10–19 times after 5- and 10-d exposures.

3.1. Chlorophyll contents

Exposure to 133 μM arsenic did not significantly reduce the concentrations of chlorophyll *a*, *b* and total chlorophyll in *P. vittata* regardless of the exposure time (Table 1); however, it reduced those in *P. ensiformis* after 10-d exposure. After 5-d exposure to arsenic, the fronds of *P. ensiformis* showed

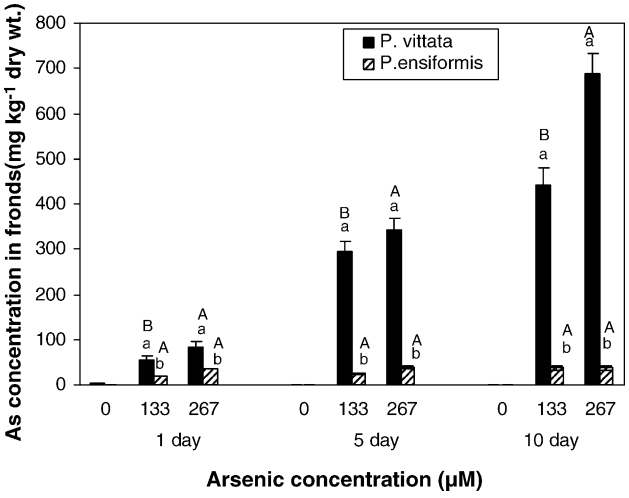

Fig. 1. Total arsenic concentrations (mg kg^{-1} dry wt.) in the fronds of *Pteris vittata* and *Pteris ensiformis* after exposing to 0, 133 or 267 μm arsenic as Na_2HAsO_4 for 1, 5 or 10 d. Vertical bars indicate mean ± S.D. of three separate experiments having three replicates each. Means followed by the same letter were not significantly different at $p < 0.05$ ('A–C' denote significance within a species at different doses of arsenic at a particular day; 'a and b' denote significance within the two species at a particular treatment).

interveinal necrotic symptoms and reduction in plant biomass (data not shown), typical of arsenic toxicity, which were not observed in *P. vittata*. However, after exposing to 267 μM arsenic, the concentrations of total chlorophyll in both ferns were significantly reduced ($p < 0.001$) compared to that of the controls. The decline in the chlorophyll concentration implies arsenic-induced stress in both species.

3.2. Total soluble protein, membrane stability index and carotenoids

After exposure to 133 or 267 μM arsenic for 1 d, no change was observed in the total soluble protein in either species (Fig. 2A). Similar to chlorophyll (Table 1), however, plant protein concentrations decreased significantly after exposure to 276 μM arsenic for 5 or 10 d in both species, although in *P. ensiformis* even 133 μM arsenic was enough to decrease the soluble protein. The change in the total soluble protein was highly significant ($p < 0.001$) on species basis. The reduction was more pronounced in *P. ensiformis* than *P. vittata*.

The extent of membrane damage was evaluated indirectly by analysing solution conductivity, which measures electrolyte leakage from cells. Membrane stability index (MSI) decreased significantly ($p < 0.05$) with increasing arsenic doses in *P. ensiformis* for all three exposure times (Fig. 2B). After exposure to 267 μM arsenic for 10 d, *P. vittata* showed 78.8% MSI compared to the control, whereas *P. ensiformis* had only 22.3% MSI.

Carotenoids are a class of natural fat-soluble pigments found in plants, where they play a critical role in the photosynthetic process as well as membrane-associated antioxidant activity. Arsenic exposure for 1-d reduced the carotenoids concentration in *P. vittata* ($p < 0.05$) but not in *P. ensiformis*. However, after 5 or 10 d exposure to arsenic, those in *P. vittata* remained unchanged whereas those in *P. ensiformis* decreased significantly at higher (267 μM) arsenic concentration (Fig. 2C). Similar to chlorophyll (Table 1) and protein (Fig. 2B), concentrations of carotenoids were significantly lower in *P.*

Table 1
Concentration of chlorophyll (mg g^{-1} fr. wt.) in the fronds of *P. vittata* and *P. ensifo*rmis after exposing to 0, 133 or 267 μm arsenic as Na_2HAsO_4 for 1, 5 or 10 d

Exposure time (d)	Arsenic exposure (μM)	Plant species	Chlorophyll *a*	Chlorophyll *b*	Total chlorophyll
1	0	PV	3.25 ± 0.22*a A	1.30 ± 0.11a A	4.55 a A
		PE	1.89 ± 0.09 b A	0.67 ± 0.03b A	2.56 b A
	133	PV	2.33 ± 0.14 a A	1.52 ± 0.05 a A	4.85 a A
		PE	1.54 ± 0.09 b AB	0.54 ± 0.03 b AB	2.09 b AB
	267	PV	2.16 ± 0.09 a A	0.89 ± 0.10 a B	3.04 a B
		PE	1.14 ± 0.12 b B	0.41 ± 0.04 a B	1.55 b B
5	0	PV	3.33 ± 0.08 a A	1.14 ± 0.05 a A	4.46 a A
		PE	2.41 ± 0.31 a A	0.86 ± 0.17 b A	3.27 b A
	133	PV	3.64 ± 0.14 a A	1.36 ± 0.05 a A	4.99 a A
		PE	2.02 ± 0.09 a A	0.76 ± 0.09 b A	2.77 b A
	267	PV	2.80 ± 0.30 a B	1.06 ± 0.13 a B	3.86 a B
		PE	1.58 ± 0.33 a B	0.55 ± 0.12 b B	2.12 b B
10	0	PV	3.73 ± 0.03 a A	1.45 ± 0.08 a A	5.17 a A
		PE	2.12 ± 0.54 b A	0.87 ± 0.11 b A	2.98 b A
	133	PV	3.59 ± 0.11 a A	1.51 ± 0.04 a A	5.09 a A
		PE	1.58 ± 0.09 b B	0.69 ± 0.08 b B	2.27 b AB
	267	PV	2.41 ± 0.61 a B	0.97 ± 0.25 a B	3.38 a B
		PE	1.17 ± 0.11 b B	0.66 ± 0.11 b B	1.81 b B

PV: *Pteris vittata*; PE: *Pteris ensiformis*. Means followed by the same letter were not significantly different at $p < 0.05$ ('A–C' denote significance between different arsenic concentrations for a given plant species at a given arsenic exposure time; 'a and b' denote significance between two plant species for a given treatment).
* Expressed as mean ± S.D. of three replicates.

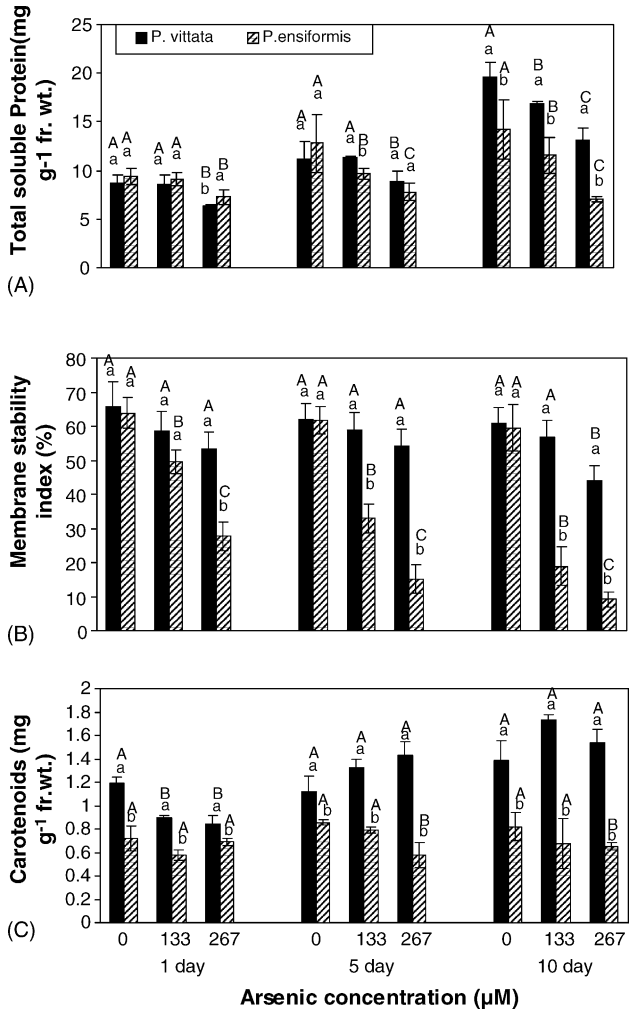

Fig. 2. Changes in (A) total soluble protein concentration (mg g^{-1} fr. wt.); (B) Membrane stability index (%); (C) carotenoids concentration (mg g^{-1} fr. wt.) in the fronds of *Pteris vittata* and *Pteris ensiformis* after exposing to 0, 133 or 267 μm arsenic as Na_2HAsO_4 for 1, 5 or 10 d. Vertical bars indicate mean ± S.D. of three separate experiments having three replicates each. Means followed by the same letter were not significantly different at $p < 0.05$ ('A–C' denote significance within a species at different doses of arsenic at a particular day; 'a and b' denote significance within the two species at a particular treatment).

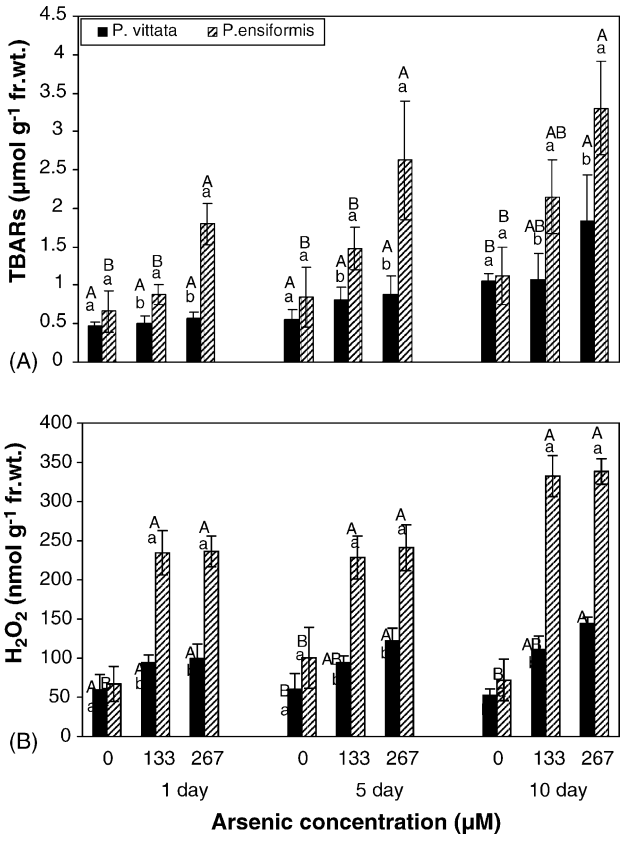

Fig. 3. Effect of arsenic on (A) TBARs (μmol g^{-1} fr. wt.) and (B) H_2O_2 (nmol g^{-1} fr. wt.), in the fronds of *Pteris vittata* and *Pteris ensiformis*. Vertical bars indicate mean ± S.D. of three separate experiments having three replicates each. Means followed by the same letter were not significantly different at $p < 0.05$ ('A–C' denote significance within a species at different doses of arsenic at a particular day; 'a and b' denote significance within the two species at a particular treatment).

ensiformis relative to those of *P. vittata* with or without arsenic (Fig. 2C).

3.3. Lipid peroxidation and H_2O_2 concentrations

Thiobarbituric acid-reactive substances (TBARS) formation in plants exposed to adverse environmental conditions is a reliable indicator of free radical formation in the tissues, and it is currently used as an index of lipid oxidation in biological systems. Membrane lipid peroxidation of two *Pteris* species was assessed by measuring TBARs (μmol malondialdehyde g^{-1} fr. wt.) in the extracts of fronds. Lipid peroxidation was significantly ($p < 0.001$) greater in *P. ensiformis* than in *P. vittata* in all treatments (Fig. 3A). After exposure to 133 and 276 μM arsenic for 10 d, the rate of increase was 102 and 177% in *P. vittata* compared to 132 and 203% in *P. ensiformis*.

H_2O_2 is a product of superoxide dismutase reaction, with greater levels indicating greater stress. Greater H_2O_2 concentrations in *P. ensiformis* were observed at 133 and 276 μM arsenic exposure compared to the control (Fig. 3B). The magnitude of difference in H_2O_2 concentrations between the two species increased significantly ($p < 0.001$) when exposure time increased from 5 to 10 d (Fig. 3B). After exposure to 267 μM arsenic, the H_2O_2 concentration in *P. ensiformis* reached 220% of the control after 1-d compared to 175% after 10-d exposure in *P. vittata*.

3.4. Ascorbate pool

Ascorbate is the most abundant antioxidant in plants and has important roles in plant growth and development. Compared to the control, the reduced ascorbate (AsA) concentrations in *P. vittata* were significantly ($p < 0.05$) increased except for arsenic exposure at 133 μM for 1 d (Fig. 4A). However, such an increase was observed in *P. ensiformis* only for arsenic exposure at 133 μM for 5 or 10 d. Similar to chlorophyll (Table 1), and carotenoids concentrations (Fig. 2C), the AsA concentrations in *P. ensiformis* were 67–88% lower than *P. vittata* in the absence of arsenic (Fig. 4A).

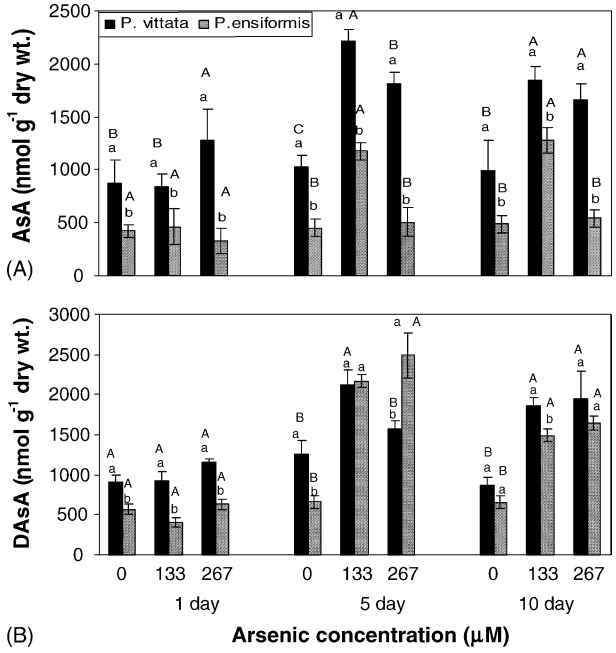

Fig. 4. Concentrations of (A) reduced (AsA) and (B) oxidized (DasA) ascorbate (nmol g^{-1} dry wt.) in fronds of *Pteris vittata* and *Pteris ensiformis* after exposing to 0, 133 or 267 μm arsenic as Na_2HAsO_4 for 1, 5 or 10 d. Vertical bars indicate mean ± S.D. of three separate experiments having three replicates each. Means followed by the same letter were not significantly different at $p < 0.05$ (A–C denote significance within a species at different doses of arsenic at a particular day; a–b denote significance within the two species at a particular treatment).

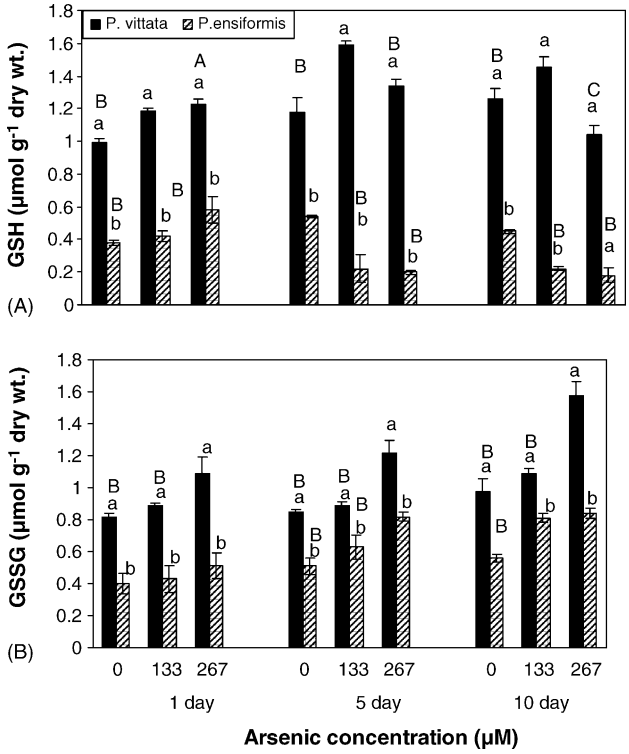

Fig. 5. Changes in the concentrations of (A) reduced (GSH) and (B) oxidized (GSSG) glutathione (μmol g^{-1} dry mass) in the fronds of *Pteris vittata* and *Pteris ensiformis* after exposing to 0, 133 or 267 μm arsenic as Na_2HAsO_4 for 1, 5 or 10 d. Vertical bars indicate mean ± S.D. of three separate experiments having three replicates each. Means followed by the same letter were not significantly different at $p < 0.05$ (A–C denote significance within a species at different doses of arsenic at a particular day; a and b denote significance within the two species at a particular treatment).

Similar to AsA, the oxidized ascorbate (DAsA) in both plants increased significantly ($p < 0.05$) after arsenic exposure for 5- and 10-d compared to the control (Fig. 4B). However, such an increase was more pronounced in *P. ensiformis* than in *P. vittata*. In the absence of arsenic, the DAsA concentrations in *P. vittata* were 33.6–89.9% greater than those in *P. ensiformis* (Fig. 4B).

The ratios of AsA/DAsA in *P. vittata* were invariably greater than those in *P. ensiformis* in all treatments except for arsenic exposure of 133 μM for 1 d.

3.5. Glutathione pool

Glutathione maintains the cellular redox status and also serves as substrate for phytochelatin synthesis. The reduced glutathione (GSH) increased upon arsenic exposure at 133 μM in *P. vittata.* Whereas, in *P. ensiformis*, it decreased significantly except for 1 d (Fig. 5A). Unlike GSH, upon arsenic exposure, the oxidized form of glutathione (GSSG) increased significantly in both plants though the magnitude of increase was greater in *P. vittata* than in *P. ensiformis* (Fig. 5B). Similar to the ratios of AsA/DAsA, the ratios of GSH/GSSG were greater in *P. vittata* in comparison to *P. ensiformis* in all treatments.

In the absence of arsenic, the concentrations GSH and GSSG in *P. vittata* were 119–291 and 67–105% greater than those in *P. ensiformis* (Fig. 5A and B).

4. Discussion

Arsenic is known to induce oxidative stress in plants by generating various ROS [21], resulting in a range of responses in plants, including readjustment of transport and metabolic processes and growth inhibition [11,22]. Like other environmental stress, arsenic may create conditions in the thylakoids where the energy level exceeds the amount that can be dissipated by the metabolic pathways of the chloroplast [23]. As a consequence, the electron transport processes in the thylakoid membranes are impeded and toxic symptoms develop. Several toxic intermediates (superoxide anion, hydroxyl radicals, hydrogen peroxide and lipid peroxide) are generated in the cell wall region as well as inside the cell during this process, which affects membrane permeability, enzyme activity, metabolic pool, photosynthetic activity, plant biomass and leaf chlorosis and necrosis [11,24,25].

It is conceivable that greater arsenic concentrations in plants cause greater oxidative stress [26,27]. Based on arsenic loading in the plant (Fig. 1), arsenic-induced oxidative stress in *P. vittata* should be at least 2 and 10 times greater than that in *P. ensiformis* after 1 and 5–10 d arsenic exposure. However, *P. ensiformis* showed necrotic symptoms of injury after arsenic exposure of 5 or 10 d, whereas no toxicity symptoms were observed in *P. vittata.* Therefore, as an efficient arsenic

hyperaccumulator, *P. vittata* must also have developed an efficient defence system to mitigate arsenic-induced oxidative stress.

4.1. Oxidative stress

In this research, the arsenic-induced oxidative stress in plants was evaluated in the fronds of two *Pteris* species after arsenic exposure. It is clear that arsenic induced oxidative stress in both species, arsenic hyperaccumulator *P. vittata* and non-arsenic-hyperaccumulator *P. ensiformis*, especially at arsenic exposure of 267 μM (Fig. 3). However, significant differences existed between the two plants species in their responses to arsenic stress.

In comparison to *P. ensiformis*, with and without arsenic exposure, *P. vittata* had greater concentrations of chlorophyll, soluble protein and carotenoids (Table 1; Fig. 2A and C). It is therefore possible that *P. vittata* was constitutively equipped with a better defence system than *P. ensiformis* and this system functioned efficiently even under arsenic exposure to protect the plants against oxidative stress. Greater concentrations of H_2O_2 concentrations (Fig. 3B) and TBARs (Fig. 3A) in *P. ensiformis* in comparison to *P. vittata* indicated that *P. vittata* was able to maintain homeostatic control of the photosynthetic light reactions upon arsenic exposure. Therefore, *P. vittata* did not produce ROS as readily as *P. ensiformis*.

Oxidative stress also induces the degradation of a variety of biologically important molecules such as amino acids, protein and carbohydrates, with the consequent release of malondialdehyde [28,29]. Therefore, the increase in TBARs concentration in *P. ensiformis* is more precisely an indicator of arsenic-induced oxidative damage due to the impairment of cell defense system. Srivastava et al. [27] have also observed an increase in the level of TBARs in the fronds of ferns with increasing concentration of arsenate, indicating that arsenic induces oxidative stress in fern plants. High lipid peroxidation coupled with high H_2O_2 might have damaged chloroplast, decreased plant biomass and inhibited chlorophyll synthesis, leading to lower chlorophyll concentration (Table 1) and protein concentration (Fig. 2A) in *P. ensiformis*.

The chlorophyll concentration was lower in *P. ensiformis* as compared to *P. vittata* in all treatments (Table 1). Arsenic has been reported to reduce chlorophyll biosynthesis in maize [30] and red clover [8]. The chlorophyll concentration in the leaves of *Pisum sativum* L increased, but the ratio of chlorophyll *a*/*b* decreased after exposing to arsenic [31]. Chlorophyll in *P. vittata* increased after exposing to 133 μM arsenic as compared to the control. The treatment at 267 μM arsenic did not effect the total chlorophyll concentration in *P. vittata* as much as in *P. ensiformis*. The chlorophyll data suggest that a combination of antioxidant compounds and/or enzymes resulted in a greater protection of the photosynthetic systems of arsenic-tolerant *P. vittata* against ROS.

4.2. Defense system

Plants have several mechanisms to defend themselves against arsenic-induced stress. Compartmentation and translocation of arsenic are reported to be basic mechanisms involved in plant arsenic-tolerance [22,32,33]. When these mechanisms become insufficient, biochemical processes can become involved in arsenic detoxification [34,35]. There are in theory three mechanisms that a tolerant plant might use to survive or even grow in stress situation [36]. The tolerant plant may minimize the production of ROS; it may protect itself from the deleterious degradative reactions associated with ROS by efficiently scavenging ROS; or it might repair the injury after the degradation has occurred.

Glutathione and ascorbate both act as antioxidants in either enzymatic or non-enzymatic way. Ascorbate is an essential compound in plant tissues and has been the focus of numerous studies in relation to enzymatic and non-enzymatic oxidation reactions in the biological system [37,38]. It can react directly by reducing superoxide, hydrogen peroxide and hydroxy radical or quenching singlet oxygen. Ascorbate also functions as a co-substrate of plant oxidases, such as the ascorbate peroxidase system, which produces dehydroascorbate [39]. Dehydroascorbate is reduced to ascorbic acid in a GSH-dependent reaction catalysed by dehydroascorbate reductase [40]. Glutathione, a non-enzymatic antioxidant, is a low molecular weight thiol implicated in a wide range of metabolic processes and constitutes an important plant defense system against environmental stress including heavy metals [41]. Glutathione levels are constitutively higher in plants adapted to stress conditions [42,43]. The increased GSH pool apparently renders them substantially more resistant to different stresses. The reactive cystein residue in GSH enables it to keep thiol group containing proteins in their native state during stress condition. Glutathione metabolism is also tied into other defense pathways. For example, different sources have described the role of GSH in ASC regeneration, both in chloroplast and in cytosol [44]. The ascorbate–glutathione cycle does not occur only in the chloroplast. Components of the ASC–GSH cycle have been reported in the cytoplasm, mitochondria and peroxisomes, and represent an important antioxidant protection system against oxidative molecules generated in these organelles [45]. Besides ASC and GSH, carotenoids are also known to have functional role in quenching singlet oxygen and scavenging free radicals because of their capability to transfer energy in photosynthesis and photoprotection [46].

In this study, changes in ascorbate (AsA and DasA) and glutathione (GSH and GSSG) were determined upon arsenic exposure in the fronds of two *Pteris* species (Figs. 4 and 5). While ascorbate has been demonstrated to play a direct role in reducing ROS in plants [39], glutathione's role in arsenic reduction is still unclear. Glutathione has been shown to serve as a source of reducing potential during arsenic reduction in bacteria under arsenic stress [47]. Since one of the detoxification mechanisms in *P. vittata* is via arsenic reduction in the fronds [22], it is possible that glutathione may play a similar role in *P. vittata*. If this is the case, then the amounts of reduced ascorbate (AsA) and glutathione (GSH) in the plants are better indicators of a plant's ability to protect itself against arsenic stress than total ascorbate and glutathione.

Glutathione is a precursor of the metal binding phytochelatins (PC). Zhao et al. [48] have demonstrated that PC_2 is found in *P. vittata* but the concentrations of PCs are considerably lower (up to 7 μmol g^{-1} dry wt.) in comparison to that reported for *Silene vulgaris* [49,50], *Holcus lanatus* [21] and *Rauwolfia serpentina* cell cultures [51] under arsenic exposure. According to Zhao et al. [48], *P. vittata* has a rather limited capacity to accumulate PCs in response to arsenic exposure. The results suggest that the arsenic-induced increase in the levels of ascorbate and glutathione in As-hyperaccumulator *P. vittata* may represent a defense system that is not as direct as the primary defense response such as phytochelatins and vacuole compartmentalisation [35]. The high ascorbate–glutathione pool would help *P. vittata* to some extent in minimizing the adverse effects caused by arsenic in cytoplasm and enhance the metabolic process of plant to tolerate arsenic.

Under arsenic exposure, initially total ascorbate and glutathione concentrations increased in both plants in comparison to the control (Figs. 4 and 5) perhaps to protect them from oxidative stress [43,52]. In addition, the increase in oxidized ascorbate DAsA in *P. ensiformis* (almost linear increase) was greater than *P. vittata* (increased initially then decreased) with higher arsenic exposure (Fig. 4B), which were reflected by greater ratios of AsA/DAsA in *P. vittata* than in *P. ensiformis*. In other words, upon high arsenic exposure, more DAsA was formed in *P. ensiformis*, in comparison to AsA, which reduced its power for quenching ROSs. Even though *P. vittata* had greater GSSG concentrations than *P. ensiformis*, it also had greater GSH/GSSG ratios, i.e. relatively greater reducing power. A high GSH/GSSG ratio helps plants in several physiological functions. This includes activation and inactivation of redox-dependent enzyme systems [53] and regeneration of the cellular antioxidant ascorbic acid under oxidative condition [54].

Metal tolerance has been associated with the homeostatic control of activated oxygen metabolism involving a balance between the formation of activated oxygen and the termination of the peroxidation chain reaction [55]. This is supported by the data in the present study on two *Pteris* plants under arsenic stress. In *P. vittata* arsenic-tolerance apparently involves minimization of ROS production (Fig. 3) with more antioxidant (Figs. 4 and 5) than *P. ensiformis*. The stimulation of carotenoid (Fig. 2C), ascorbate and glutathione synthesis during arsenic exposure is an acclimation response of *P. vittata* and enhances its ability to withstand arsenic-induced oxidative stress.

Thus, the plant redox status of the ascorbate and glutathione pools highly respond to arsenic phytotoxicity in *P. ensiformis*. This has also been observed for other sensitive species to various stress factors, e.g., copper toxicity [38], senescence [56] and heat acclimation [57,58]. Future studies of arsenic impact on the biosynthesis of these metabolites are required to elucidate the molecular basis of the metal-imposed anti-oxidative defense mechanism.

Considering the data obtained on various parameters studied as antioxidant indices, it is clear that *P. vittata* was inherently more tolerant to ROS in the presence or in the absence of arsenic stress, than *P. ensiformis*. It is possible that greater arsenic resistance of *P. vittata* was associated with its ability to maintain a coordinated increase in the concentration of chlorophyll, carotenoids, glutathione and ascorbate resulting in lower H_2O_2 production, lipid peroxidation and higher membrane stability. Further studies are required to investigate whether this coordinated increase is due to enhanced expression of the genes controlling the biosynthesis of these antioxidant compounds in *P. vittata*.

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