



Effects of arsenic on nitrate metabolism in arsenic hyperaccumulating and non-hyperaccumulating ferns

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Arsenic reduced the activity of nitrate and nitrite reductase more in *Pteris ensiformis* than *Pteris vittata*.

ARTICLE INFO

Article history:

Received 29 December 2008

Received in revised form

19 March 2009

Accepted 26 March 2009

Keywords:

Arsenic

Nitrate metabolism

Pteris vittata

Arsenic hyperaccumulator

Pteris ensiformis

Nitrate reductase

Nitrite reductase

ABSTRACT

This study investigated the effects of arsenic on the *in vitro* activities of the enzymes (nitrate reductase and nitrite reductase) involved in nitrate metabolism in the roots, rhizomes, and fronds of four-month old *Pteris vittata* (arsenic – hyperaccumulator) and *Pteris ensiformis* (non-arsenic-hyperaccumulator) plants. The arsenic treatments (0, 150, and 300 μM as sodium arsenate) in hydroponics had adverse effects on the root and frond dry weights, and this effect was more evident in *P. ensiformis* than in *P. vittata*. Nitrate reductase and nitrite reductase activities of arsenate-treated plants were reduced more in *P. ensiformis* than in *P. vittata*. This effect was accompanied by similar decreases in tissue NO_3^- concentrations. Therefore, this decrease is interpreted as being indirect, i.e., the consequence of the reduced NO_3^- uptake and translocation in the plants. The study shows the difference in the tolerance level of the two *Pteris* species with varying sensitivity to arsenic.

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1. Introduction

Arsenic (As) is distributed widely in the environment, and results from weathering of soil parent material and human activities (Azcue and Nriagu, 1994; Frankenberger and Arshad, 2002). It is a known human carcinogen, with cancers related to arsenic in drinking water being reported in Taiwan, Argentina, Chile, Bangladesh, and India (Rahman et al., 2008; Zhu et al., 2008; Zavala and Duxbury, 2008; Stone, 2008).

In aerobic soils, arsenate (AsV) is the most stable and dominant species. The uptake of arsenate by plants has been studied extensively (Srivastava et al., 2005; Singh and Ma, 2006; Singh et al., 2006). In an anaerobic environment under reducing conditions, arsenite (AsIII) is the dominant species. Both inorganic arsenic species are highly toxic to plants. Arsenate is a phosphate analog, and therefore it can compete with phosphate in the cytoplasm, replacing phosphate in ATP, leading to the disruption of energy flows in cells. On the other hand, AsIII is highly toxic to plants

because it reacts with sulfhydryl groups ($-\text{SH}$) in enzymes, their cofactors and tissue proteins (Tripathi et al., 2007).

In spite of extensive investigations on the phytotoxic effects and mechanisms of arsenic on plants, further studies are necessary to understand various metabolic activities associated with arsenic stress in plants. Changes in the activity of several enzymes of intermediary metabolism in plants, which were exposed to high arsenic concentrations, have been reported (Cao et al., 2003; Srivastava et al., 2005). Cellular protection against toxic levels of arsenic in As-hyperaccumulators depends upon various mechanisms (Bleeker et al., 2003; Srivastava et al., 2005; Singh et al., 2006). In this respect, evidence exists for the involvement of arsenic-binding peptides (Sneller et al., 1999; Pickering et al., 2000; Schmöger et al., 2000; Zhao et al., 2003).

Qualitative analysis of arsenic-binding peptides in plants exposed to arsenic stress have demonstrated the existence of various families of γ -glutamyl cysteine synthetase (γ -ECS, EC 6.3.2.2) (Schmöger et al., 2000; Hartley-Whitaker et al., 2002; Schat and Kalf, 2002), but cellular levels of glutathione (GSH) also depend upon the availability of glutamate and cysteine (Strohm et al., 1995). Despite the substantial contribution of glutamate in the biosynthesis of the above mentioned arsenic-binding peptides (γ -ECS), a clear understanding of the relative importance of various

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processes that regulate the supply of glutamate and other amino acids is lacking.

In plants, three major reactions are involved in the assimilation of nitrate into amino-acids. Nitrate is first reduced to ammonium sequentially by nitrate reductase (NR, EC 1.6.6.1) and nitrite reductase (NiR, EC 1.6.6.4), which is a key regulatory step of N-NO₃⁻ conversion to organic nitrogen (Campbell, 1999; Kaiser et al., 1999). The ammonium is then incorporated into glutamine and glutamate primarily by the glutamine synthase–glutamate synthase cycle (GS/GOGAT cycle) (Mifflin and Lea, 1982) and then assimilated into amino acids, nucleic acids, proteins, chlorophylls, and other metabolites (Marschner, 1995; Stitt et al., 2002).

This study was undertaken to determine the activities of the enzymes involved in nitrate reduction in arsenic hyperaccumulator *Pteris vittata* and non-arsenic-hyperaccumulator *Pteris ensiformis*, both were treated with arsenate. Analyses of nitrate and nitrite in arsenic-exposed plants also were included to gain a better insight into the sequence of various metabolic reactions. Relationship between these parameters provides an overall view of the possible role of inorganic nitrogen assimilation pathways in plants under arsenic – stress conditions.

2. Materials and methods

2.1. Plant materials and plant growth condition

Two ferns – Chinese brake fern (*P. vittata*) and Slender brake fern (*P. ensiformis*) were used in this study. Four-month-old ferns procured from a nursery (Milestone Agriculture, Inc., FL, USA) were used in this experiment. The plants were transferred to a hydroponic system in a growth room with temperature 23–28 °C and relative humidity 70%. A 14-h photoperiod with a daily photosynthetic photon flux of 350 μmol m⁻² s⁻¹ at plant canopy was supplied by an assembly of cool-white and warm-white fluorescent lamps. Both ferns were allowed to grow for 2 weeks in 500 ml of nutrient solution to initiate new root growth. The nutrient solution used was half-strength Hoagland-Arnon solution (Hoagland and Arnon, 1938) with pH adjusted to 6–7. The solution was aerated continually and replenished twice a week.

A factorial completely randomized design (3 × 2, arsenic concentration × plant species) was used to study the arsenic accumulation and nitrogen metabolism as influenced by fern species (*P. vittata* and *P. ensiformis*) treated with three arsenic levels of (0, 150, and 300 μM) sodium arsenate. Each treatment was conducted in triplicate. Water losses via transpiration were replenished by frequent additions of deionized water to maintain a constant volume of solutions in the containers.

The plants were harvested after 7 days of arsenic treatment. The roots of harvested plants were washed with ice-cold phosphate buffer to ensure desorption of arsenic from root surface and root free space. The plants were separated into fronds, rhizome, and root, and were frozen in liquid N₂ to stop enzyme activity. They were then freeze-dried (Freeze Zone 12, LABCONCO, Kansas City, MO), ground and stored at –80 °C until enzyme analysis.

2.2. Quantification of arsenic concentration

Total arsenic was determined in air-dried fern samples (0.5 g). The plant material was digested with nitric acid on a temperature-controlled digestion block (Environmental Express, Mt. Pleasant, SC) using a modified USEPA Method 3050 A (USEPA, 1986). Total arsenic was determined by a graphite furnace atomic absorption spectrophotometer (GFAAS; Perkin–Elmer SIMMA 6000, Norwalk, CT) with a detection limit of 2 μg L⁻¹ arsenic. The standard reference material (NIST, SRM 2709) was carried through the extraction (digestion using HNO₃/H₂O₂) and analyzed as a part of the quality assurance/quality control protocol (showing recovery range of 91.3–96.8% of As).

2.3. Preparation of cell-free extracts

Frozen samples of roots, rhizome and fronds were homogenized in chilled mortar and pestle with four volumes (v/w) of the extraction buffer. The extraction buffer contained 0.05 M Tris–HCl (pH 8.5), 5 mM (dithiothreitol), 10 μM flavin adenine dinucleotide, 1 μM Na₂ MoO₄, 1 mM EDTA, 10 μM leupeptin, 1% (w/v) insoluble poly vinyl pyrrolidone and 1 mM cysteine (Modified after Kuo et al., 1982; Boussama et al., 1999). The addition of 2% (w/v) bovine serum albumin to the standard extraction medium resulted in increased recovery and stability of nitrate reductase (NR) and nitrite reductase (NiR) (Hageman and Reed, 1980). The homogenate was filtered through four layers of cheesecloth, and the filtrate was centrifuged for 15 min at 30,000 g. The supernatant fluid was then was decanted through glass wool and used for assays of NR, NiR, NO₃⁻ and NO₂⁻.

2.4. Enzyme assays

Nitrate reductase (NR; EC 1.6.6.1) activity was performed by measuring the amount of NO₂⁻ formed in NR assay buffer (Wray and Filner, 1970). The assay medium for NR contained 0.5 ml potassium phosphate buffer (100 mM, pH 7.5), 0.1 ml KNO₃ (100 mM), 0.1 ml NADH (0.15 mM) and 0.1 ml extract in a final volume of 2.0 ml. The assays were conducted at 30 °C for 30 min. The reaction was terminated by addition of 0.1 ml of 1 M zinc acetate, and excess NADH was oxidized by phenazine methosulfate (Scholl et al., 1974). The NO₂⁻ produced was assayed after diazotization with 1 ml of sulfanilamide (1 g l⁻¹ dissolved in 1.5 N HCl) and 1 ml N-naphthyl-ethylene-diamine-dichloride (NED, 0.2 g l⁻¹ in double distilled water). The solution mixture was centrifuged at 12,000 g for 5 min, and the absorbance of the supernatant was determined at 540 nm. The enzyme activity was measured as μmol of nitrite produced g⁻¹h⁻¹.

Nitrite reductase (NiR; EC 1.6.6.4) activity was measured by NO₂⁻ consumption during the assay (Losada and Paneque, 1971). The assay mixture contained 0.5 ml potassium phosphate buffer (100 mM, pH 7.5), 0.2 ml potassium nitrite (15 mM), 0.3 ml methyl viologen (5 mM), and 0.1 ml extract in a total volume of 1.1 ml. The reaction was initiated by adding 0.2 ml sodium dithionite (Na₂S₂O₄) [86.15 mM sodium dithionite dissolved in 290 mM sodium bicarbonate (NaHCO₃)]. A blank without sodium dithionite also was run simultaneously. After incubation for 30 min at 30 °C, the reaction was stopped by vigorously mixing the content of the assay tube on vortex mixer until the methyl viologen was oxidized completely (10–15 s). The residual NO₂⁻ in the reaction mixture was determined colorimetrically as described above. The enzyme activity was measured as μmol of nitrite reduced g⁻¹h⁻¹.

2.5. NO₃⁻ and NO₂⁻ analysis

Nitrite (NO₂⁻) concentration was determined colorimetrically as described above. Nitrate (NO₃⁻) concentration was determined by automated analysis via hydrazine reduction (Kamphake et al., 1967). Nitrate was reduced to NO₂⁻ with hydrazine sulfate. The reaction mixture consisted of 50 μl NaOH (1N), 50 μl copper sulfate (5 g l⁻¹) and 100 μl hydrazine sulfate (1 g l⁻¹). To this was added 2.5 ml of sample. This mixture was incubated at 45 °C for 5 min. The NO₂⁻ thus formed (reduced NO₃⁻) plus originally present NO₂⁻ was determined by diazotization with sulfanilamide and coupling with NED to form a highly colored azo dye that was measured colorimetrically as described earlier. The difference between total N estimation (NO₃⁻ and NO₂⁻) and actual NO₂⁻ gave the concentration of NO₃⁻.

2.6. Protein content

Protein estimation was carried out by the method of Bradford (1976) using bovine serum albumin as standard.

2.7. Statistical analysis

A two-way analysis of variance in complete randomized block design involving two plant species and three treatments was performed. For each data point, the mean of three replicates were obtained from two independent experiments (*n* = 6). Comparisons between means of the different treatments were done by Duncan's multiple range test (Gomez and Gomez, 1984).

3. Results

The significance level for the measured parameters, including As accumulation, plant biomass, concentrations of nitrate, nitrite and protein, and activities of nitrate and nitrite reductase for the two *Pteris* species in response to As treatment is summarized in Table 1. This table will be used for statistical evaluation in conjunction with

Table 1

Results of Analysis of variance of species (S), arsenic concentration (A), plant parts (P) effects and their interactions (S × An × P) on arsenic accumulation, phytomass, protein content, and levels of NO₃⁻, NO₂⁻, NRA and NiRA. The numbers are *F*-values.

Dependent variable	Independent Variable			
	Species (S)	Arsenic conc. (A)	Plant parts (P)	S × An × P
As accumulation	40.8**	17.8**	16.4**	8.4**
Phytomass	12.4**	3.4*	16.8**	5.7*
Protein content	3.2*	9.3*	1.7 ^{NS}	0.6 ^{NS}
Nitrate concentration	5.2*	44.8**	24.6**	8.2**
Nitrite concentration	1.9 ^{NS}	3.2*	2.7 ^{NS}	1.5 ^{NS}
Nitrate reductase activity	46.1**	16.8**	26.3**	9.4**
Nitrite reductase activity	21.7**	14.3**	12.6**	8.7**

p* ≤ 0.05; *p* ≤ 0.01; NS not significant.

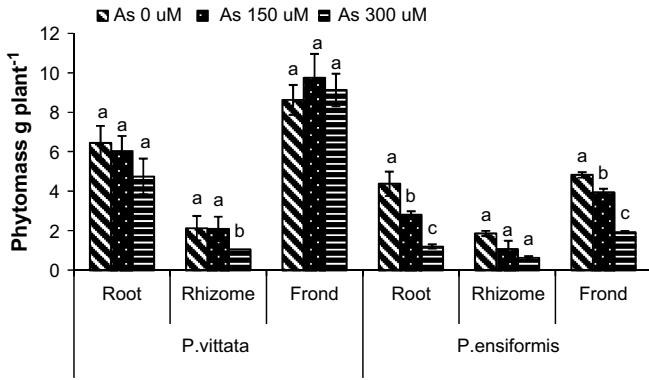


Fig. 1. Changes in phytomass of different plants after 7 days of exposure of *P. vittata* and *P. ensiformis* to arsenic (As). Values are mean ($n = 6$) \pm SD. Different letters above bars denote significant differences ($p = 0.05$) between different exposure concentration in a plant part according to DMRT (Duncan's multiple range test).

the presentation and discussion of the measured parameters as shown in Figs. 1–7.

3.1. Plant growth

Exposure of *Pteris* species to increased concentration of arsenic over a period of 7 days resulted in inhibition of growth, particularly in case of *P. ensiformis* (Fig. 1). At the higher arsenic concentration, i.e., 300 μ M, *P. ensiformis* showed symptoms (necrosis) of injury in the fronds and roots. Arsenic treatments resulted in substantial suppression (>60%) in the dry weight of roots and fronds in case of

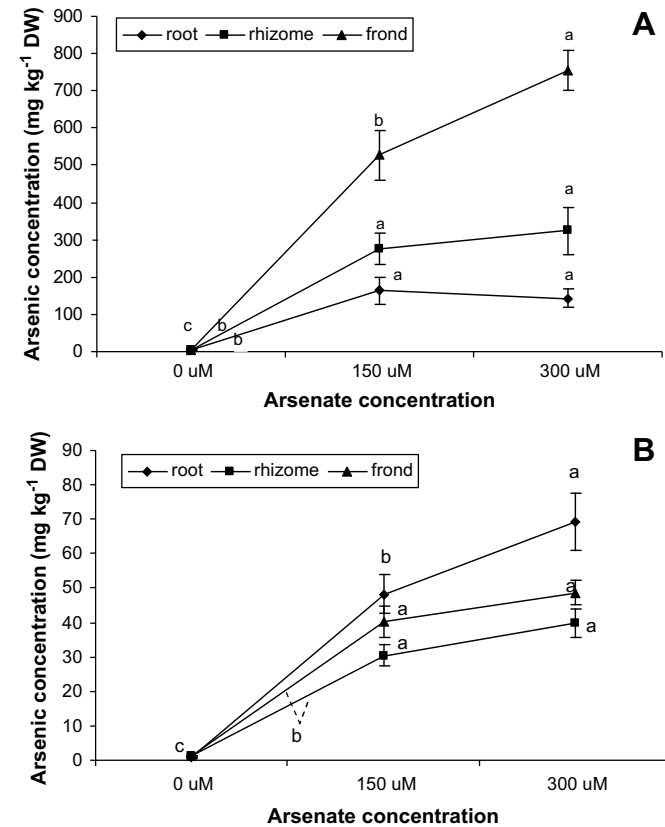


Fig. 2. Changes in arsenic concentration of different plant parts after 7 days of exposure to arsenate in (A) *P. vittata* and (B) *P. ensiformis* to arsenic (As). Values are mean ($n = 6$) \pm SD. Different letters at data points denote significant differences ($p = 0.05$) between different exposure concentration in a plant part according to DMRT.

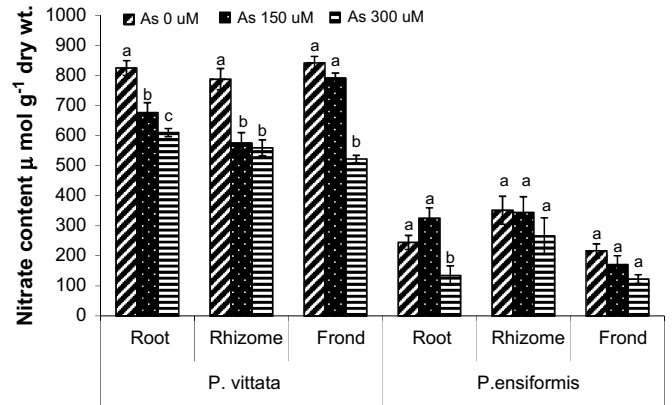


Fig. 3. Changes in NO_3^- concentrations of the roots, rhizomes and fronds of *P. vittata* and *P. ensiformis* after 7 days of exposure to arsenic (As). Each value represents the mean ($n = 6$) \pm SD. Different letters above the bars denote significant differences ($p = 0.05$) between different exposure concentration in a plant part according to DMRT.

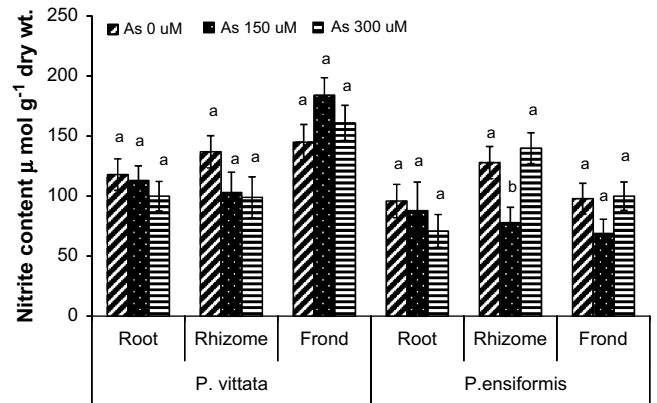


Fig. 4. Changes in NO_2^- concentrations of the roots, rhizomes and fronds of *P. vittata* and *P. ensiformis* after 7 days of exposure to arsenic (As). Each value represents the mean ($n = 6$) \pm SD. Different letters above bars denote significant differences ($p = 0.05$) between different exposure concentration in a plant part according to DMRT.

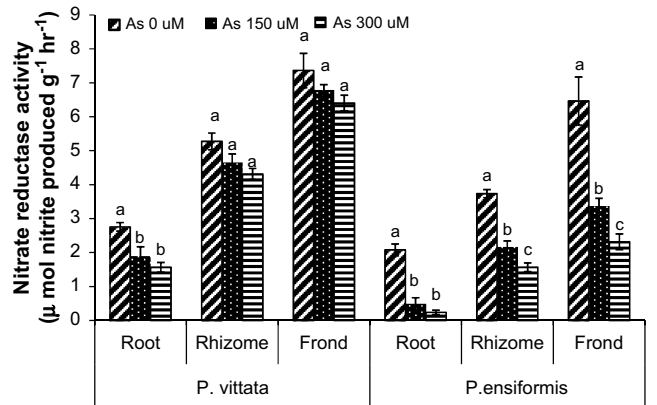


Fig. 5. Changes in NR activity of the roots, rhizomes and fronds of *P. vittata* and *P. ensiformis* after 7 days of exposure to arsenic (As). Each value represents the mean ($n = 6$) \pm SD. Different letters above bars denote significant differences ($p = 0.05$) between different exposure concentration in a plant part according to DMRT.

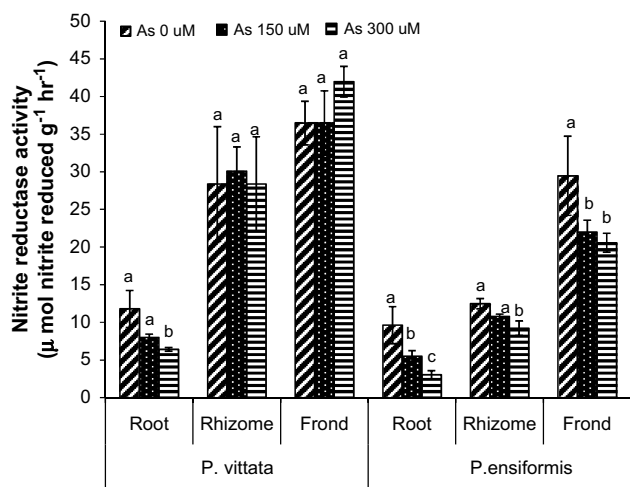


Fig. 6. Changes in NiR activity of the roots, rhizomes and fronds of *P. vittata* and *P. ensiformis* after 7 days of exposure to arsenic (As). Each value represents the mean ($n = 6$) \pm SD. Different letters above bars denote significant differences ($p = 0.05$) between different exposure concentrations in a plant part according to DMRT.

P. ensiformis, whereas, the reduction in *P. vittata* was $<20\%$ in the frond weight. No significant change in the root biomass of *P. vittata* occurred with arsenic exposure.

3.2. Arsenic concentrations in plant parts

Arsenic concentrations in the roots and rhizomes were not influenced by increasing arsenic concentrations from 150 to 300 μM in *P. vittata*. In *P. ensiformis*, however, the arsenic concentrations were increased significantly ($P = 0.001$) in the roots. The total arsenic concentrations in the fronds increased significantly with arsenic treatment in *P. vittata* (Fig. 2). In the case of *P. ensiformis*, about 70–80% of the absorbed arsenic was concentrated in the roots, whereas in *P. vittata* the maximum arsenic was sequestered in the fronds regardless of the arsenic concentration used in the treatments.

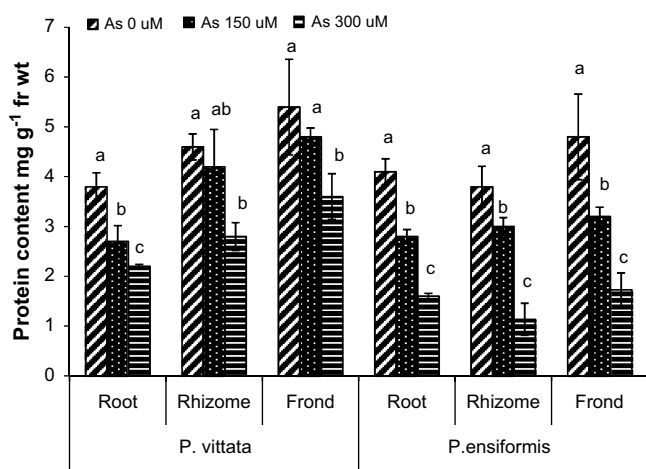


Fig. 7. Changes in total protein concentrations of different plant parts after 7 days of exposure of *P. vittata* and *P. ensiformis* to arsenic (As). Values are mean ($n = 6$) \pm SD. Different letters above bar denote significant differences ($p = 0.05$) between different exposure concentration in a plant part according to DMRT.

3.3. Nitrate and nitrite concentration in plants

The effects of arsenic exposure on NO_3^- and NO_2^- contents of the roots, rhizomes and fronds of *P. vittata* and *P. ensiformis* are represented in Figs. 3 and 4. For both species, the nitrate content in the roots, rhizomes and fronds decreased with increasing arsenic concentration (Fig. 3). Compared to the controls, arsenic exposure resulted in the low nitrate content in the roots of both plants. Nitrate content was depressed to a larger extent in *P. ensiformis* than in *P. vittata* plants, particularly in the roots at high arsenic concentrations. The decrease in nitrate contents in *P. ensiformis* roots at 150 and 300 μM A were 85% and 91%, respectively, compared to that of the control. At high As concentration (300 μM), NO_3^- content in *P. ensiformis* was $<50\%$ of that in *P. vittata* (Fig. 3). The trend was the same for all other parts as well, but the reduction percentage was less than that of roots.

For all arsenic concentrations tested, the nitrite content in the roots of either species was unaffected. However, the NO_2^- levels in the rhizome and fronds of either plant decreased upon arsenic exposure, but the change was significant only in *P. ensiformis* (Fig. 4).

3.4. Nitrate reductase and nitrite reductase in plants

The presence of arsenic in the culture solution resulted in a decrease of nitrate reductase (NR) activity in the roots, rhizome, and fronds of *P. ensiformis*, and the effect became more pronounced as arsenic concentration was increased (Fig. 5). The effect was more pronounced in the roots of *P. vittata*, with no significant change being observed in the rhizomes and fronds. The inhibition of NR activity ranged from 10 to 40% in *P. vittata* and from 25 to 85% in *P. ensiformis*, when arsenic concentration was increased from 150 to 300 μM in the culture medium.

Similarly, the activity of nitrite reductase (NiR) in *P. ensiformis* was decreased by arsenic, and the effect was again more pronounced in the roots than in rhizomes and fronds (Fig. 6). NiR activity in *P. vittata* was decreased significantly ($P = 0.01$) only in the roots exposed to arsenic at high concentration (300 μM). However, the results obtained for these enzymes show that, NiR activity was not as affected as that of NR in either plants by arsenic treatments.

3.5. Protein concentration in plants

The protein concentration decreased in either plant after arsenic exposure for 7 days (Fig. 7). In *P. vittata* the decreases in the roots and fronds were significant ($P = 0.01$) but in the rhizomes, the difference between the control and plants exposed to 150 μM A was not significant. In *P. ensiformis* protein was the least in the rhizomes in comparison to other parts, although the difference with the roots was not significant. The protein content decreased significantly ($P = 0.01$) in all plant parts (Fig. 7).

4. Discussion

To our knowledge, this report is the first to describe the effects of arsenic stress on nitrate acquisition in plants and assimilation in the arsenic hyperaccumulator *P. vittata* and non-arsenic-hyperaccumulator, *P. ensiformis*. Generally, plant resistance to metal toxicity is based on its tolerance (Verkleij et al., 1991; Meharg and Macnair, 1990, 1992) to excess of internal metal concentrations. In the present study, differences in arsenic accumulation in the roots, rhizomes and fronds between *P. vittata* and *P. ensiformis* were observed (Fig. 2). The roots of *P. ensiformis* accumulated higher concentration of arsenic than the other plant parts, whereas in

P. vittata the arsenic accumulation was more in the fronds (Ma et al., 2001; Tu et al., 2003). These data suggest that in *P. ensiformis* the roots limit arsenic translocation towards the rhizomes and fronds, whereas in *P. vittata* arsenic translocation was straight to the fronds without retention in the roots. The amount of arsenic accumulated in *P. ensiformis* fronds was independent of the external arsenic concentrations, but that of *P. vittata* fronds increased with arsenic concentrations (Fig. 2).

Growth inhibition is the most common symptom observed in plants subjected to stress factors, including excess concentrations of heavy metals. Exposure to arsenic in *P. ensiformis* led to rapid accumulation of arsenic in the root with a concomitant reduction in phytomass and necrosis in the fronds.

Nitrate reductase, the rate-limiting enzyme in nitrogen assimilation (Campbell, 1999), is well known to be sensitive to metal stress (Vajpayee et al., 1999, 2000; Rai et al., 2004; Kumar and Joshi, 2008). In this study, a marked inhibition of NR activity by arsenic was recorded in *P. ensiformis*. In *P. vittata*, the endogenous levels of arsenic, which caused 50% inhibition of NR activity in the fronds and roots, were 60–40-fold higher, respectively, than those in the fronds and roots of *P. ensiformis* (Fig. 5). Differential compartmentalization of arsenic in the cells of the two species might protect the enzyme against the inhibition by absorbed arsenic. Earlier studies (Tu et al., 2003; Singh and Ma, 2006) have shown that with arsenic exposure, arsenic is present mostly as AsIII in the fronds and rhizomes of *P. vittata*, whereas it is accumulated mostly as AsV in those of *P. ensiformis*. Thus arsenite exclusion from cytosol or arsenite detoxification by binding to ligands – phytochelatin (Zhao et al., 2003), may be quite efficient in *P. vittata* frond and rhizome cells compared to *P. ensiformis*. In control plants, the NR activity in the fronds and rhizomes was much higher in *P. vittata* than in *P. ensiformis* (Fig. 5). Boussama et al. (1999), studying the effect of cadmium on *Zea mays* L. plants, have shown a greater production of organic acids (mainly malate, citrate, oxalate and pectate) as a consequence of high NO_3^- reduction. One may suggest that a greater production of these organic acids occur in *P. vittata* fronds and rhizome than in *P. ensiformis* as a consequence of the high NO_3^- reduction. It is conceivable that these organic acids may minimize the inhibitory effect of arsenic on NR activity in *P. vittata* because of As inactivation in the symplast as organic acid complexes (Verkleij et al., 1991; Wagner, 1993).

The activity of NR was determined with added NADH and nitrate at optimal levels, and therefore there was no limitation of electron donor during the *in vitro* assay. The observed inhibition in NR activity in *P. ensiformis* from arsenic exposure may be due to the effect of arsenic on the enzyme protein synthesis and/or activity. Since sulfhydryl (SH) groups are required for NADH binding and catalytic activity of NR (Solomonson and Barber, 1990), arsenic might affect the NR activity by binding to functional –SH groups present in the active sites of this enzyme (Sharma and Dubey, 2005; Xiong et al., 2006). Arsenic has been shown to interact with functional sulfhydryl (SH) groups (Schmöger et al., 2000; Hartley-Whitaker et al., 2002; Schat and Kalf, 2002). In addition, NR has also been demonstrated to be very sensitive to H_2O_2 (Sharma and Dubey, 2005). The As-induced accumulation of endogenous H_2O_2 was previously detected in *P. ensiformis* (Singh et al., 2006). Therefore, enhancement of this reactive oxygen species content could have also contributed to the reduced NR activity found in *P. ensiformis*. Furthermore, a decrease in internal concentration of NO_3^- in plants and more so in the roots of *P. ensiformis* upon arsenic exposure may also result in the inhibition of NR activity following restricted availability of nitrate (Solomonson and Barber, 1990; Redinbaugh and Campbell, 1991).

The second step in nitrate assimilation is the conversion of NO_2^- to NH_4^+ by the action of NiR. In this study, the NiR activity decreased in both plant species with arsenic exposure (Fig. 6). Both enzymes,

NR and NiR, are co-regulated on the induction side (Redinbaugh and Campbell, 1991; Migge et al., 1997). After 7 days of exposure to arsenic, NiR activity was relatively less affected than NR activity in the *Pteris* tissues (Figs. 5 and 6). Several lines of evidence indicate that NO_3^- rather than NO_2^- induces NiR: (a) no correlation was found between NO_2^- accumulation and NiR induction, whereas NO_3^- accumulation and NiR had positive correlation ($r = 0.9415$; $P = 0.001$), and (b) change in NiR occurred even if there was no change in NO_2^- concentration. The main regulator of NiR activity seems to be the *in vivo* activity of NR, which is induced by NO_3^- (Aslam and Huffaker, 1989; Dguimi et al., 2009).

Protein synthesis is related closely to the production of new tissues, which are principal sink for N compounds. It is not surprising that growth of *Pteris* was disturbed when arsenic stress inhibited activity of NR and NiR. Due to greater decrease in the activity of NR and NiR in *P. ensiformis*, the protein content was also less in this plant in comparison to *P. vittata* (Fig. 7).

From this study, it appears that the root system of both species merits particular attention. This root response to arsenic stress may also be significant in explaining the hypersensitivity of *P. ensiformis* to arsenic. In earlier experiments (Singh et al., 2006), it is shown that at the same arsenic exposure *P. ensiformis* expresses more lipid peroxidation than *P. vittata* in the root system. Perhaps in such a way arsenic depresses the functioning of ion carrier proteins. If this hypothesis were correct, the differential effect of arsenic on intracellular NO_3^- and NO_2^- contents in *P. vittata* and *P. ensiformis* might be due to differences in response of plasmalemma components. The preservation of membrane integrity and the maintenance of the integrity of ion transport systems may have a role in arsenic tolerance in *P. vittata* as in other higher plants with heavy metals (Hernández et al., 1997; Meharg, 1993).

5. Conclusion

The study shows that for both *P. vittata* and *P. ensiformis*, which display differences in arsenic susceptibility, differences occurred for arsenic accumulation as well as the effects of arsenic on growth, protein, nitrate and nitrite contents in the plants. Reduction in the root growth was more pronounced in *P. ensiformis* than in *P. vittata* plants. This difference in root growth retardation may account partially for the difference in nitrate uptake. It is the concentration of nitrate that limits the activity of NR and NiR. The higher activity of NR and NiR may lead to more NH_4 assimilation in *P. vittata*. The enzyme NADH-GDH, in the presence of substantial level of NH_4 in tissues, is directly involved in the formation of glutamate, which is considered as the principal precursor of proline biosynthesis (Oaks, 1994) as well as participating in transamination and providing substrate in the formation of many other aminoacids. This phenomenon could be viewed as a biochemical adaptive feature of *P. vittata*, which possibly played a protective role under arsenic stress conditions. Although, more work is needed for further information towards the subcellular accumulation of arsenic and its effect on other enzymes of nitrogen metabolism and the accumulation of ammonium and proline in the organs.

Acknowledgements

This research was supported in part by the National Science Foundation (Grant BES-0132114). Nandita Singh is thankful to the U.S. Department of States for the Fulbright Scholarship, and CSIR, India for granting deputation to work in UF. Nandita Singh and Anshita Raj are thankful to the Director, NBRI for his support and encouragement. We gratefully acknowledge the analytical assistance provided by Mr. Thomas Luongo.

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