Available online at www.sciencedirect.com
SCIENCE DIRECT®
Environmental and Experimental Botany 51 (2004) 121–131
Environmental and Experimental Botany
www.elsevier.com/locate/envexpbot

Effects of arsenic species and phosphorus on arsenic absorption, arsenate reduction and thiol formation in excised parts of *Pteris vittata* L.

S. Tu [a], L.Q. Ma [a,*], G.E. MacDonald [b], B. Bondada [a]

[a] *Soil and Water Science Department, University of Florida, Gainesville, FL 32611-0290, USA*
[b] *Department of Agronomy, University of Florida, Gainesville, FL 32611-0290, USA*

Received 4 March 2003; received in revised form 18 August 2003; accepted 20 August 2003

Abstract

Understanding the arsenic (As) detoxification mechanisms employed by the newly discovered As hyperaccumulator Chinese Brake fern (*Pteris vittata* L.) is important to optimize As accumulation capability. The present experiment was carried out to determine the location of As reduction and thiol formation in Chinese Brake fern and the ability of excised plants to absorb As under P influence. Live Chinese Brake ferns were separated into three parts: pinnae (leaflets), fronds (aboveground biomass, de-rooted), and roots (belowground biomass, de-topped). The excised plants were then exposed to 0.5-strength Hoagland nutrient solutions containing 667 μM As(III), As(V), or monomethylarsonic acid (MMA) and 0 μM P or 500 μM P for 1 day. Arsenate and arsenite were separated using an As speciation cartridge and As was determined by graphite furnace atomic absorption spectrophotometry (GFAAS). The pinnae absorbed the greatest amounts of As followed by fronds and roots. In the presence of P, the phytoavailability of As species was As(III) > MMA > As(V) for pinnae and roots and As(V) > As(III) ≅ MMA for fronds. The fact that As(III) was the predominant form in excised aerial tissues whereas As(V) was the main form in excised roots clearly demonstrated that As reduction occurred mostly in the fronds, mainly in the pinnae. Absorption of As species resulted in formation of thiol, with MMA causing the greatest level of formation. Although addition of P to the solution suppressed As(V) accumulation in excised pinnae and roots, it enhanced As(V) reduction, and reduced thiol production. The results suggested that the ability to efficiently reduce As(V), facilitated by P, and to quickly produce thiols might have both contributed to the capability of Chinese Brake fern to hyperaccumulate As.

Keywords: Arsenate; Arsenite; Hyperaccumulation; Monomethylarsonic acid; Speciation; Phytoavailability

1. Introduction

The levels of arsenic (As), a toxic and carcinogenic metalloid, have become elevated in the environment owing to a myriad of anthropogenic activities. Phytoremediation is fast emerging as a remediation technology to decontaminate As polluted sites as a result of recent discoveries of several As hyperaccumulating ferns (Francesconi et al., 2002; Ma et al., 2001; Visoottiviseth et al., 2002; Zhao et al., 2002). The first known As hyperaccumulator *Pteris vittata* L., commonly known as Chinese Brake fern, can

* Corresponding author. Tel.: +1-352-392-1951x208; fax: +1-352-392-3902.
E-mail address: lqma@ufl.edu (L.Q. Ma).

0098-8472/$ – see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/j.envexpbot.2003.08.003

accumulate as much as 2.3% As in fronds (above-ground biomass) from an As-spiked soil (Ma et al., 2001). Even in uncontaminated soils, this fern can accumulate 744 mg kg^{-1} As, compared to other plants that normally take up <10 mg kg^{-1} (Matschullat, 2000), clearly demonstrating both an efficient As uptake and a detoxification system.

Although both organic and inorganic As species are present in the environment, only inorganic forms, arsenite–As(III) and arsenate–As(V), have currently been found in Chinese Brake fern (Tu and Ma, 2002; Wang et al., 2002; Zhang et al., 2002). Organoarsenic compounds, such as monomethylarsonic acid (MMA), dimethylarsinic acid (DMA), tetramethylarsonium ion, trimethylarsine oxide, arsenobetaine, and arseno-sugars, have been detected in a range of marine and terrestrial plant tissues (Meharg and Hardey-Whitaker, 2002; Nissen and Bensun, 1982), but not in the fern after exposure to inorganic As (Zhang et al., 2002). However, the ability of Chinese Brake fern to efficiently reduce As(V) to As(III) has been clearly demonstrated, and is considered one of the As detoxification mechanisms (Tu and Ma, 2002).

In addition to inducing As reduction in the plant, As also triggers the production of phytochelatins (PCs) in both As-sensitive plants (Sneller et al., 1999) and As-tolerant plants (Hartley-Whitaker et al., 2001). Formation of As–PC complexes is believed to be a key mechanism of As detoxification in many plants (Schmöger et al., 2000). Thiol content is closely related to PCs since the latter are thiol-rich peptides, which are synthesized with glutathione as a building block. It has been speculated that Chinese Brake fern may be equipped with an As detoxification mechanism involving thiols (Cai and Ma, 2003; Meharg and Hardey-Whitaker, 2002).

Arsenate and phosphate are chemical analogues. Their interactions have been investigated in both higher and lower plants. A hydroponic study investigating interactions between As and P in alfalfa (*Medicago sativa* L.) shows that As content is strongly suppressed by P (Khattak et al., 1991). It has been well demonstrated that arsenate absorption into cytoplasm is mediated by phosphate carrier in plasma membrane (Asher and Reay, 1979). Wang et al. (2002) have also reported that As uptake by Chinese Brake fern is through P transporters. Tu and Ma (2003) examined the interactions of As and P in Chinese Brake fern in a 20-week soil pot experiment. Phosphorus has little effect on As uptake and plant growth when the soil is spiked with As <2.67 mmol kg^{-1}; however, it increases As uptake and plant growth when the As is 5.34 mmol kg^{-1} (high As). On the other hand, moderate amount of As (<2.67 mmol kg^{-1}) increases plant P uptake but not at As level of 5.34 mmol kg^{-1} probably due to plant As toxicity. This result clearly demonstrates the importance of P in As detoxification of Chinese Brake fern. Thus, examining the effect of P on As uptake by plant is a good means to understand the mechanism of As detoxification by Chinese Brake fern.

Since different biochemical reactions occur in different parts of a plant (Marschner, 1995), excised plant tissues, such as shoots, stems, leaves and roots, have been widely used to characterize the absorption and metabolism of nutrients and chemicals as well as heavy metals in plants (Facanha and Okorokova-Facanha, 2002; Waldrop et al., 1996; Zhang and Taylor, 1991). We have examined the uptake of different As species (organic/inorganic and arsenate/arsenite) by Chinese Brake fern and As speciation in plant biomass (Lombi et al., 2002; Ma et al., 2001; Tu and Ma, 2002). However, there are many questions that still remain unanswered, such as where As reduction occurs in the plant, i.e., roots, fronds, or both, and how P affects plant As uptake and reduction. We tested the hypothesis that effects of As species on As absorption, As speciation and thiol formation in different parts of Chinese Brake fern may be influenced by P because arsenate and phosphate are chemical analogues. The overall objectives of this experiment were to (1) examine the effects of As species and P on As absorption and speciation as well as thiol formation in excised parts of Chinese Brake fern; and (2) determine the location of plant As reduction and thiol formation. It was expected that use of excised parts of Chinese Brake fern to characterize As absorption, speciation and thiol formation would shed light on mechanisms of As hyperaccumulation.

2. Materials and methods

2.1. Plant materials

Stock Chinese Brake fern plants were grown under greenhouse conditions (natural photoperiod;

temperature was roughly controlled at 20–30 °C) and 4-month-old plants were used in this experiment. Plants were first transferred to a hydroponic system in a growth room to promote new root growth. The growth room was climate-controlled with a temperature range 23–28 °C and relative humidity ~70%. A 16 h photoperiod with an average photon flux of 350 μmol m^{-2} s^{-1} was supplied by an assembly of cool-white fluorescent lamps. Hoagland–Arnon nutrition solution at 0.5-strength (Hoagland and Arnon, 1938) with vigorous aeration and replenishment twice a week was used to grow the plants.

2.2. Experimental design and implementation

A complete randomized three-factor experiment design was used to understand how As species [As(III), As(V), MMA] influenced As absorption and triggered As speciation and thiol formation in excised parts of Chinese Brake fern in absence or presence of P. All treatments (3 plant parts × 3 As species × 2 P rates) were applied using Hoagland nutrient solution (0.5-strength, with 0 μM P or 500 μM P) amended with 667 μM of As(III), As(V) or MMA added as $NaAsO_2$ (ACROS, NJ), $Na_2HAsO_4{\cdot}7H_2O$ (J.T. Baker, NJ) and $CH_3AsNa_2O_3{\cdot}6H_2O$ (Supelco, Bellefonte, PA), respectively. In addition, 2 controls (without As and with or without P) were included for each excised parts of plants. Each treatment was replicated three times and the replicates were blocked. The solution was buffered by 5 mM MES [2-(*N*-morpholino) ethanesulfonic acid] (ACROS ORGANICS, NJ) and the pH was adjusted to 6.0 with dilute HCl or NaOH. Pinnae (leaflets), fronds (pinnae + rachis), and roots were excised from intact fern plants after growing in the hydroponic systems for 2 weeks. The excised parts were washed with tap water followed by deionized water before treatments. For each treatment, two grams of excised pinnae, or fronds, or whole roots of a plant were placed in a 250 ml glass containers containing 200 ml control or As-amended solution. The excised plants were placed in the solution such that As absorption was only through leaflet surface (pinnae), root (de-topped roots), and rachis (de-rooted fronds). Pinnae were floated in the solution so the whole pinnae were exposed to the solution; the de-topped roots were placed in the solution such that the cut surfaces were not exposed to the solution, whereas the cut surfaces of the fronds were exposed to the solution. The containers were randomly placed in blocks in a temperature controlled water bath at 25 °C. A photosynthetic photon flux of 350 μmol m^{-2} s^{-1} was supplied by an assembly of cool-white fluorescent lamps to plants during the experiment, except containers with the roots, which were kept in the dark. To provide oxygen to the excised parts and minimize As (III) oxidation in the solution, a limited aeration (5 min every 4 h) was applied to the plants during the experiment. After exposing to As for 1 day, all the plant materials were washed with tap water followed by rinsing in ice-cold phosphate buffer containing 1 mM Na_2HPO_4, 10 mM MES and 0.5 mM $Ca(NO_3)_2$ to ensure desorption of As from material surface and the root free space (Asher and Reay, 1979). The plant materials were again washed with tap water followed by deionized water, and frozen in liquid nitrogen to stop enzyme activity, freeze–dried (FreezZone 12, LABCONCO), ground, and stored in −80 °C for analysis of As speciation and total thiols. The solutions were sampled for immediate analysis of As speciation.

2.3. Determination of As speciation

Arsenic speciation was performed by extracting plant samples ultrasonically in 10 ml of methanol/water mixture (1/1 (v/v)) two times for 4 h at 60 °C (Zhang et al., 2002). The two extracts were decanted into a 100 ml volumetric flask and diluted to 100 ml with water. Arsenate and arsenite were separated using an As speciation cartridge (Metal Soft Center, Highland Park, NJ), which retains arsenate (Meng et al., 2001). Since this cartridge does not remove organic As, As speciation in plants treated with MMA was not conducted. Total As and arsenite were determined by a graphite furnace atomic absorption spectrophotometer (GFAAS; Perkin-Elmer SIMMA 6000, Norwalk, CT). The standard reference material was carried through the extraction and analyzed as part of the quality assurance–quality control protocol. Reagent blanks and internal standards were used where appropriate to ensure accuracy and precision in the analysis of As.

To check the reliability of As speciation cartridge, prior to this experiment, As speciation of Chinese Brake fern plant samples from various resources was

analyzed by using both As cartridge-GFAAS method and HPLC–ICP–MS method (unpublished data). The concentrations of As species determined by the two methods were in good agreement for the majority of the samples. Thus, the As speciation cartridge could be used to analyze As speciation in the conditions of this experiment. However, it is expected that cartridge-GFAAS method may overestimate As(III) concentration if As(V) in the samples is present as organic form and hence passes through the column. Fortunately, there are little organic As compounds found in Chinese brake fern using methanol/water extractant as used in this experiment (Zhang et al., 2002).

2.4. Extraction and assay of total thiols

For total thiol assay, the method of Ric De Vos et al. (1992) was used. Extraction was carried out by grinding 20 mg of freeze–dried plant materials (using a mortar, pestle, and quartz sand) in 2 ml 5% (w/v) sulfosalicylic acid (ACROS ORGANICS, NJ) with 6.3 mM diethylenetriaminepentaacetic acid (DTPA) (J.T. Baker, NJ) (pH < 1) at 0 °C. After centrifugation at 10,000 $\times$ g for 15 min (4 °C), the supernatants were immediately assayed by Ellman's reagent (Ellman, 1959). Supernatant (300 μl) was mixed with 630 μl of 0.5 M K_2HPO_4 and cultured in a water bath at 30 °C for 5 min. Absorbance was measured at 412 nm using a Shimadzu 160 U spectrometer. After adding 50 μl of 5,5′-dithiobas(2-nitrobenzoic acid) (DTNB) (ACROS ORGANICS, NJ) solution (10 mM DTNB, 0.143 M K_2HPO_4, 6.3 mM DTPA, pH 7.5), the absorbance was measured again after 2 min. The increase in absorbance was corrected for the absorbance of DTNB. Values were calculated using the molecular extinction coefficient $\varepsilon_{412} = 13,600\,M^{-1}\,cm^{-1}$ (Anderson, 1985).

2.5. Statistical analysis

Data were analyzed with a three-way ANOVA procedure using SAS Software. Data in percentage were transformed using arsine[sqrt(x)] method before ANOVA to achieve normality (Clewer and Scarisbrick, 2001). The Duncan multiple range test at $P < 0.05$ was used to separate treatment means. Regression analysis was carried out and all the figures and trend curves were drawn using Sigma Plot 7 Software.

3. Results

3.1. Absorption of As species by excised plant parts

Excised tissues of Chinese Brake fern effectively accumulated all three species of As. However, the degree of As accumulation varied with excised parts, As species and P status, and were significantly influenced by their interactions based on the three-way ANOVA (Table 1 and Fig. 1). Excised pinnae displayed the greatest accumulation of As followed by excised fronds and roots. Arsenic absorption by pinnae was in the order of As(III) $>$ As(V) $\cong$ MMA in the absence of P, which were 3.4-, 1.8-, 1.5-fold greater than the control (10.7 ± 0.9 mmol kg^{-1} dry weight). In the presence of P, As uptake by the pinnae was 5.4-, 1.7-, 2.5-fold greater than the control (8.5 ± 1.0 mmol kg^{-1} dry weight). In other words, P increased As(III) and MMA uptake by 28 and 37%, whereas it decreased As(V) uptake by 25% in the pinnae, resulting in a phytoavailability order of As(III) $>$ MMA $>$ As(V).

Compared to excised pinnae, As uptake by excised fronds (de-rooted) was much less; possibly due to reduced ion uptake by the excised fronds as only the cut rachis was immersed in the solution. In the absence of P, As absorption by excised fronds was in the order of As(V) $\cong$ As(III) $>$ MMA, which was 1.6-, 1.5-, and 0.8-fold greater than the control (4.9 ± 0.4 mmol kg^{-1} dry weight). Addition of P increased plant uptake of As(III), As(V) and MMA, by 45, 78, and 183%, which was 2.3-, 3.0-, and 2.5-fold greater than the control (4.7 ± 0.3 mmol kg^{-1} dry weight), with the order of As(V) $>$ As(III) $\cong$ MMA.

Accumulation of As(III), As(V), and MMA by excised roots was the lowest of all, only 10, 11, and 4% of those by excised pinnae and 47, 26, and 14% of those by excised fronds. However, excised roots accumulated 7.6-fold As(III), 4.3-fold As(V), and 2.2-fold MMA greater than the control (0.5 ± 0.1 mmol kg^{-1} dry weight). Addition of P did not significantly influence the absorption of As(III) and MMA, but decreased As(V) by 50%, with the order of As(III) $>$ MMA $>$ As(V).

Table 1
Results of the three-way ANOVA and Duncan tests for the effects of excised parts, As species and P on As absorption, As speciation and thiol formation in Chinese Brake fern

Source of variation	As absorption (mmol g^{-1} dry weight)	Plant As(III) % of total As[a]	Solution As(III) % of total As[a,b]	Thiol concentration (mmol g^{-1} dry weight)[c]
ANOVA *F*-values				
Replication	0.960 ns[d]	0.08 ns	0.230 ns	2.41 ns
Excised parts	795***	510***	74.5***	358***
As species	138***	305***	2197***	540***
P	42.3***	122***	9.44**	327***
Excised parts × As species	109***	49.4***	155***	115***
Excised parts × P	12.8***	17.0***	25.6***	421***
As × P	9.57***	71.3***	7.36*	21.6***
Excised parts × As × P	10.3***	39.9***	0.470 ns	170***
Duncan multiple range test				
Excised parts				
Pinnae	25.2 a[e]	91.8 (73.4) a[f]	58.3 (49.8) a	2.54 a
Fronds	9.41 b	85.1 (67.3) b	–	1.43 b
Roots	2.27 c	47.1 (43.4) c	39.3 (38.8) b	–
As species				
As(III)	17.9 a	86.5 (68.4) a	92.5 (74.1) a	1.37 b
As(V)	9.65 b	65.9 (54.3) b	6.28 (14.5) b	1.25 b
MMA	9.31 b	–	–	3.35 a
P				
Without P	10.7 b	70.1 (56.9) b	45.4 (42.3) b	2.52 a
With P	13.9 a	83.2 (65.8) a	52.2 (46.2) a	1.46 b

[a] As speciation were not carried out for MMA treatment since the As speciation cartridge does not remove organic As.
[b] As speciation was not determined for the solutions in the presence of fronds.
[c] Thiols in the excised roots were not determined due to low concentrations of thiol.
[d] ns: not significant *F* ratio ($P < 0.05$); *, **, and *** mean significant at $P < 0.05$, 0.01, and 0.001, respectively.
[e] Treatment means for the ANOVA test. Values followed by the same letter, within the same source of variation, are not significantly different at $P < 0.05$ based on Duncan multiple range test.
[f] Data in percentage was transformed using arsine[sqrt(*x*)] before analysis of variance. Data in parentheses were the transformed data.

3.2. As speciation in excised plant parts

Previous experiments demonstrated that As(III) and As(V) are the predominant species in Chinese Brake fern, which were analyzed in this experiment. As expected, plants treated with As(III) generally displayed greater percentage of As(III) of the plant compared to that of As(V) (Table 2). However, plant As speciation varied with plant parts and was influenced by external As species and P rates (Tables 1 and 2). In the absence of P, for instance, As(III) consisted of 92 and 95% of the total As in the excised pinnae and fronds, treated with As(III), 6.5 and 50% greater than corresponding plant parts treated with As(V). For the As(V) treatment, As(III) was predominant species in the pinnae whereas it was significantly decreased in the fronds. In the presence of P, As(III) consisted of 90–94% of plant As in all treatments. It was noteworthy that addition of P significantly increased the percentage of As(III) of total As in excised plants treated with As(V) as well as the control.

Excised roots contained significantly lower percentage of As(III) than the excised fronds and pinnae (Table 2). Arsenic(III) accounted for 34 and 24% of total As with and without P for roots treated with As(V), whereas 61–70% As(III) for roots treated with As(III).

The As speciation in the solution was also influenced by the three factors, plant parts, As species and P status (Table 1). Excised pinnae did not cause significant change of As speciation in the solution during the 1-day experiment (Table 3). However, compared to the As (III) solution without plant, the

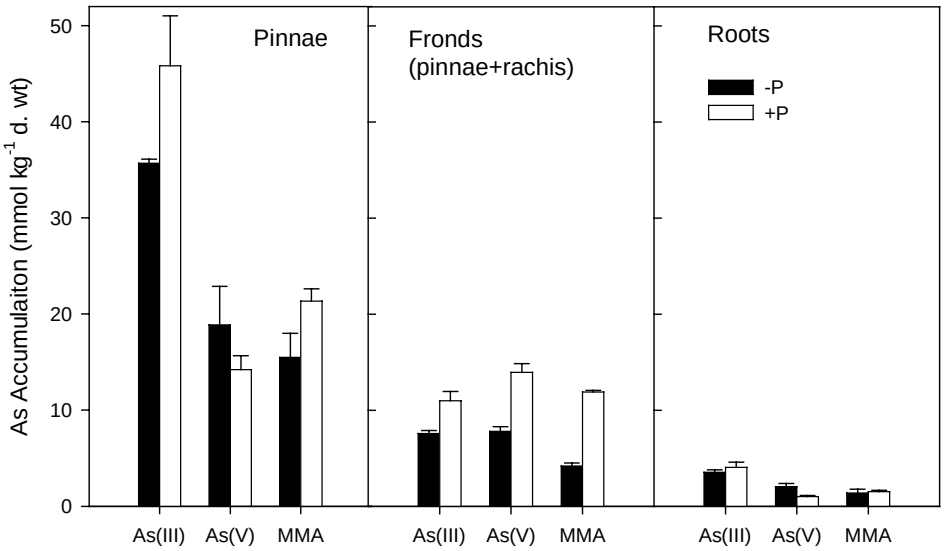

Fig. 1. Net As absorption by excised parts (pinnae, fronds and roots) of Chinese Brake fern after exposing to 0.5-strength Hoagland nutrient solution spiked with 667 μM of As(III), As(V) or MMA (with and without 500 μM P) for 1 day. The error bars are standard error of the means from three replicates.

Table 2
Effects of As species and P on As(III) concentrations (expressed as % of total As) in excised pinnae, fronds, and roots of Chinese Brake fern after exposing to 667 μM As ($P = 0$ μM or 500 μM) for 1 day

Treatment	Pinnae		Fronds		Roots	
	−P	+P	−P	+P	−P	+P
Control	78.1 (2.6)	92.6 (2.2)	90.1 (1.9)	90.4 (4.2)	–	–
As(III)	92.2 (0.3)	94.1 (1.5)	94.6 (2.9)	93.6 (2.8)	61.0 (3.8)	70.1 (5.9)
As(V)	85.7 (1.9)	94.2 (1.5)	44.6 (1.9)	92.9 (0.6)	24.0 (2.0)	34.3 (3.8)

Data in parentheses are standard error of three replicates.

presence of plant roots reduced As(III) concentrations by 20–28% for As(III) solution, whereas it increased As(III) concentrations by 3–17% for As(V) solution.

3.3. Thiol formation in excised plant parts

No significant amount of thiols was detected in excised roots except that a small amount of thiol was

Table 3
Effects of As species and P on As(III) concentrations (expressed as % of total As) in the hydroponic solution spiked with 667 μM of As after growing excised parts of Chinese Brake fern for 1 day ($P = 0$ μM or 500 μM)

Treatment	Pinna solution		Root solution	
	−P	+P	−P	+P
Control (without As)	94.0 (2.7)	97.0 (2.6)	–	–
As(III) (without plant)	100 (1.7)	101 (2.1)	99.8 (2.6)	100 (3.4)
As(III)	101 (4.5)	99.2 (4.2)	71.4 (4.6)	80.3 (5.3)
As(V)	4.60 (2.3)	4.40 (0.7)	3.10 (2.7)	17.0 (3.9)

Data in parentheses are standard error of three replicates.

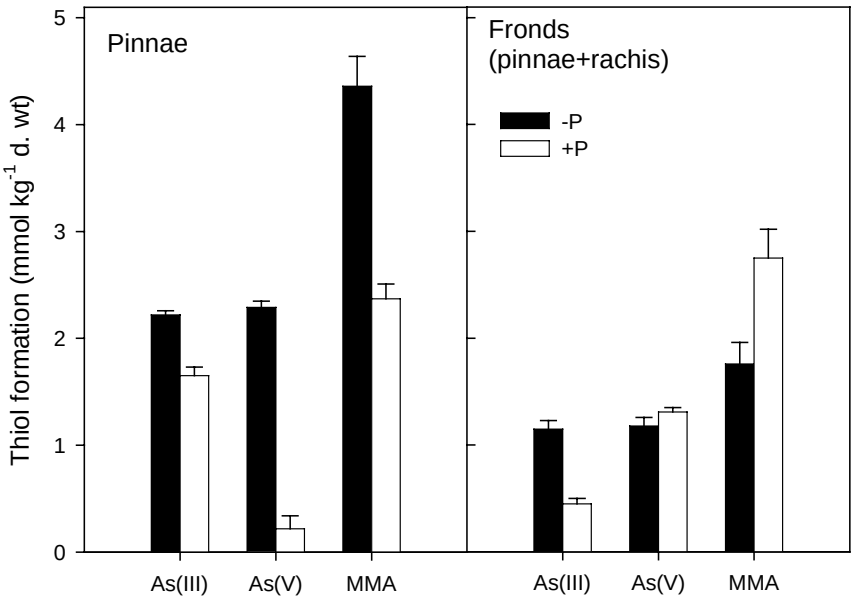

Fig. 2. Thiol formation by excised pinnae and fronds of Chinese Brake fern exposed in 0.5-strength Hoagland nutrient solution spiked with 667 μM of As(III), As(V) and MMA (with and without 500 μM P) for 1 day. The error bars are standard error of the means from three replicates.

detected when treated with As(III) (data not shown). Excised pinnae and fronds treated with As produced high amounts of thiols (Fig. 2), which was influenced by excised parts, As species, P status and their interactions (Table 1). In the absence of P, excised pinnae treated with As(III), As(V), and MMA resulted in 3.0-, 3.1-, and 5.8-fold more thiol production than in the control (0.75 ± 0.02 mmol kg^{-1} dry weight). Addition of P to solution reduced thiol concentrations in excised pinnae treated with As(III), As(V), and MMA by 26, 90 and 63%, and were only 2.0-, 0.3-, and 2.9-fold greater than in control (0.81 ± 0.05 mmol kg^{-1} dry weight).

Thiol concentrations in excised fronds were generally lower than those in the excised pinnae (Table 1 and Fig. 2), which were generally consistent with As accumulation in the plants (Fig. 1). The effect of P on thiol production varied with As species. Adding P decreased thiol production in fronds treated with As(III), but increased thiol production treated with As(V) and MMA (Fig. 2).

The relationship between thiol concentrations and total As, As(III) and As(V) concentrations in the plant could be best described by quadratic parabolic equations (Fig. 3), with the F-values of 16.8, 13.6, and 18.4, respectively (all $P < 0.001$). The coefficients of determination (R^2) for the regression equations of total As, As(V) and As(III) were 0.43 ($n = 48$), 0.45 ($n = 36$), and 0.53 ($n = 36$) (all $P < 0.001$).

4. Discussion

This experiment examined the effects of P and different As species on plant As absorption, As speciation, and thiol formation using excised parts of Chinese Brake fern during a 1-day period in a hydroponic system. It showed that excised pinnae, fronds and roots of Chinese Brake fern all effectively accumulated As(III), As(V), and MMA from solution with their capability in the order of pinnae > fronds ≫ roots (Table 1 and Fig. 1). This is consistent with the pattern of As distribution in Chinese Brake fern in the literature where 83% of As is distributed in the fronds (Ma et al., 2001) and 96% of total As in the aerial parts is found in the pinnae (Lombi et al., 2002). The efficiency of As absorption by the excised pinnae were demonstrated by its high accumulation

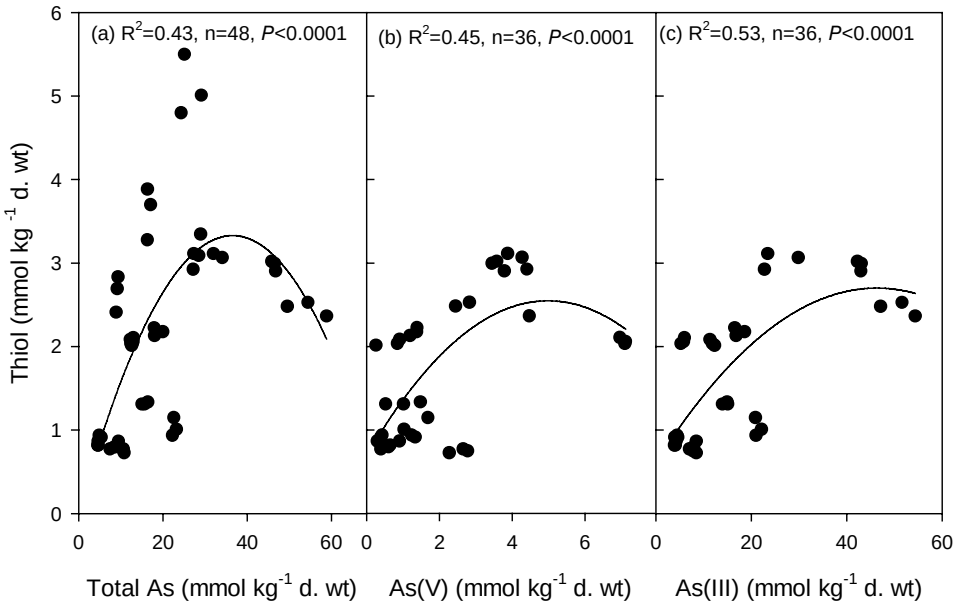

Fig. 3. Relationship between concentrations of thiol and concentrations of total As (a), As(V) (b) and As(III) (c) in Chinese Brake fern. The number of the samples in (a) included 12 samples from MMA treatment. The quadratic parabolic curves were drawn using Sigma Plot 7 Software. The regression fits are determined by coefficients of determination (R^2).

(1065–3433 mg kg^{-1}) after just 1-day period, which averaged to 44–143 mg kg^{-1} absorption rate on hourly basis. The rapid As accumulation by the excised plants may have relevance for phytoremediation of As contaminated water, i.e., excised plants can possibly be used to clean up As contaminated water by simply floating them in the water.

Among the three As species examined, the fern was most efficient in taking up As(III) regardless of the P status (Table 1 and Fig. 1). As discussed earlier, once taken up by Chinese Brake fern, As(V) is reduced to As(III), which is then sequestered in the vacuoles of the fronds (Lombi et al., 2002; Ma et al., 2001; Wang et al., 2002; Zhang et al., 2002). The plant's capability to reduce As(V) directly affects its ability to accumulate As as As(V) reduction is considered one of As detoxification mechanisms in Chinese Brake fern (Ma et al., 2001). Thus, compared to As(V) and MMA, it is easier for Chinese Brake fern to absorb As(III) since it can be directly sequestered into vacuoles without the reduction step. This is also consistent with As uptake in other plant species. For instance, in hydroponic culture, As uptake by two perennial coastal marsh grasses *Spartina patens* and *Spartina alterniflora* followed the trend of As(III) > As(V) ≅ MMA (Carbonell-Barrachina et al., 1998). Arsenic uptake by rice (*Oryza sativa*) in a long-term hydroponic culture was As(III) > MMA > As(V) (Marin et al., 1992).

Generally, addition of P increased plant absorption of As(III) and MMA possibly due to an improved plant P nutrition, which resulted in a significant increase of As absorption in the presence of P (Table 1). This may also suggest that both As(III) and MMA were not taken up by excised fern via the P transporter systems. On the other hand, addition of P significantly reduced plant absorption of As(V) except for the fronds, suggesting that As(V) enters the pinnae and roots via P transporting mechanisms.

In the presence of P, As(III) occurred as the dominant As species in excised pinnae and fronds, even in those plants treated with As(V). However, in excised roots [except those treated with As(III)], the opposite was observed (Table 2). In previous studies, analysis of As speciation in intact plants of Chinese Brake fern showed that 60–74% of the As in the fronds was present as As(III) compared to only 8% in the roots (Zhang et al., 2002). Similar results were reported for *Pityrogramma calomelanos*, another As

hyperaccumulating fern (Francesconi et al., 2002). The experimental data reported here suggested that As(V) reduction occurred mainly in the aboveground biomass, more precisely in the pinnae, not in the roots. Given that, in the roots, 30–39% As present as As(V) when exposed to As(III) and 24–34% As present as As(III) when exposed to As(V) suggested that both As(III) oxidation and As(V) reduction occurred in the roots (Table 2). Oxidation of As(III) has rarely been reported in the plants, but it has been reported in soil bacteria (Phillips and Taylor, 1976) and mineral leaching bacteria (Sehlin and Lindström, 1992). In general, two pathways are involved in production of different As species in plants (Marschner, 1995). One is oxidation/reduction of As species in the plant; another is direct uptake of different As species from the environment. The As speciation data in the hydroponic solution spiked with As(III) and As(V) with excised pinnae showed that there was no significant change in As speciation (Table 3), indicating that As reduction mainly occurred in the excised pinnae. In the presence of roots, however, in comparison with the control (As(III) solution without plant, 99.8–100% As(III)), As(III) concentrations increased by 3–17% in solution spiked with As(V) and decreased 20–29% in the solution spiked with As(III) (Table 3), suggesting an occurrence of As oxidation/reduction possibly by microbial activity in the solution or direct root exudation of As(V)/As(III) into the solution. The fact that more As(V) was present in the roots (24–34%) than the solution (3.1–17%), and more As(III) was present in the solution (71–80%) than the roots (61–70%) suggest that As oxidation occurred inside the roots (Tables 1 and 2).

Thiol content was significantly related to total As, As(V) and As(III) concentrations in Chinese Brake fern, indicating that As accumulation caused plant stress and thus resulting in thiol formation (Fig. 3). This was similar to the results described for the As tolerant plant *Holcus lanatus* by Hartley-Whitaker et al. (2001) who reported that content of total phytochelatins, thiol-rich polypeptides, generally increased with elevated external As(V) concentration. Thiols are considered precursors for formation of plant PCs (Grill et al., 1989). Among the three As species, MMA was the strongest inducer of thiol formation, followed by As(V) and As(III) (Table 1 and Fig. 2). Organic As (MMA) has long been considered less toxic than inorganic As species in human and animal (NRCC, 1978). However, studies in *S. patens* and radish (*Raphanus sativus* L.) as well as Chinese Brake fern found that the phytotoxicity of organic As, MMA and DMA, is greater than that of inorganic As, As(III) and As(V) (Carbonell-Barrachina et al., 1998, 1999; Tu and Ma, 2002). Such a result was supported by present experiment since MMA triggered much greater thiol formation than As(III) and As(V) regardless of the P status.

It is believed that P plays a key role in As detoxification since phosphate and arsenate are analogous. In this experiment, we found that addition of P to the hydroponic solution inhibited As(V) absorption by excised pinnae and roots (Fig. 1) but enhanced the reduction of As(V) (Tables 1 and 2). Arsenic(V) reduction to As(III) is considered one of the mechanisms for plant As detoxification since As(III) can easily complex with thiol-riched PCs (Cai and Ma, 2003). Hence it does not inhibit some of the P-related enzymes in Chinese Brake fern because As(III) is not chemically similar to phosphate (unpublished data); Thus, less As(V) uptake and high As(III) concentrations (i.e., more As(V) reduction), which were attributed to P, may constitute a part of the mechanism of As detoxification by Chinese Brake fern. This was further corroborated by the reduction of thiol level in the presence of P (Fig. 2), because a lower level of thiol indicated less As stress in the plants. As for the increased As absorption in excised fronds by addition of P, a different uptake mechanism may be used by the fronds since the absorption was through rachis immersed into the solution, possibly through xylem by transpiration. As a result, the high level of thiol in fronds treated with As(V) and MMA in the presence of P (Fig. 2) could also well be explained by their greater As accumulation since As concentration was positively related to thiol content (Fig. 3).

5. Conclusions

Excised plant parts were effectively used to characterize As absorption, speciation and thiol formation in Chinese Brake fern in this hydroponic experiment. The result showed that excised plants accumulated As in the order of pinnae > fronds ≫ roots. In the presence of P, the order of phytoavailability of As species

was As(III) > MMA > As(V) for excised pinnae and roots and As(V) > As(III) ≅ MMA for excised fronds. Arsenic reduction was rapidly carried out in the plant aerial parts and both As reduction and oxidation occurred in the roots, resulting in high As(III) concentrations in the fronds and high As(V) in the roots. Thiol formation was triggered by plant absorption of As species, especially MMA. Plant As detoxification, aided by P, was through suppression of As(V) absorption, enhancement of As(V) reduction and decrease of thiol formation. The results suggested that excised Chinese Brake fern could efficiently reduce As(V) into As(III) and synthesize thiols in the aerial parts, leading to As hyperaccumulation.

Acknowledgements

This research was supported in part by the National Science Foundation (Grant BES-0086768 and BES-0132114). The authors gratefully acknowledge the work provided by Mr. Tom Luongo for the GFAAS analysis of As and Dr. Nandita Singh and Dr. Mrittunjai Srivastava for proof-reading the manuscript. The thoughtful comments by two reviewers are highly appreciated.

References

Anderson, M.E., 1985. Tissue glutatione. In: Greenwald, R.A. (Ed.), Handbook of Methods for Oxygen Radical Research. CRC Press, Boca Raton, FL, pp. 317–323.

Asher, C.J., Reay, P.F., 1979. Arsenic uptake by barley *Hordeum-vulgare* cultivar zephyr seedlings. Aust. J. Plant Physiol. 6, 459–466.

Cai, Y., Ma, L., 2003. Metal tolerance, accumulation, and detoxification in plants with emphasis on arsenic in terrestrial plants. In: Cai, Y., Braids, O.C. (Eds.), Proceedings of the ACS Symposium Series 835 on Biogeochemistry of Environmentally Important Trace Elements. American Chemistry Society, pp. 95–114.

Carbonell-Barrachina, A.A., Aarabim, M.A., Delaune, R.D., Gambrell, R.P., Patrick Jr., W.H., 1998. The influence of arsenic chemical form and concentration on *Spartina patens* and *Spartina laterniflora* growth and tissue arsenic concentration. Plant Soil 198, 33–43.

Carbonell-Barrachina, A.A., Burlo, F., Lopez, E., Martinez-Sanchez, F., 1999. Arsenic toxicity and accumulation in radish as affected by arsenic chemical speciation. J. Environ. Sci. Heal. Part B 34, 661–679.

Clewer, A.G., Scarisbrick, D.H., 2001. Practical Statistics and Experimental Design for Plant and Crop Science. Wiley, New York.

Ellman, G.L., 1959. Tissue sulfhydryl groups. Arch. Biochem. Biophys. 82, 853–858.

Facanha, A.R., Okorokova-Facanha, A.L., 2002. Inhibition of phosphate uptake in corn roots by aluminum complexes. Plant Physiol. 129, 1763–1772.

Francesconi, K., Visoottiviseth, P., Sridokchan, W., Goessler, W., 2002. Arsenic species in an arsenic hyperaccumulating fern, *Pityrogramma calomelanos*: a potential phytoremediator of arsenic-contaminated soils. Sci. Total Environ. 284, 27–35.

Grill, E., Loffler, S., Winnacker, E.L., Zenk, M.H., 1989. Phytochelatins, the heavy-metal-binding peptides of plants, are synthesized from glutathione by a specific γ-glutamylcysteine dipeptidyl transpeptidase (phytochelatin synthase). Proc. Natl. Acad. Sci. U.S.A. 86, 6838–6842.

Hartley-Whitaker, J., Ainsworth, G., Vooijs, R., Bookum, W.T., Schat, H., Meharg, A.W., 2001. Phytochelatins are involved in differential arsenate tolerance in *Holcus lanatus*. Plant Physiol. 126, 299–306.

Hoagland, D.R., Arnon, D.I., 1938. The water culture method for prowling plants without soil. Calif. Agric. Exp. Stn. Bull. 347, 1–39.

Khattak, R., Page, A.L., Parker, D.R., Bakhar, D., 1991. Accumulation and interaction of arsenic, selenium, molybdenum and phosphorus in alfalfa. J. Environ. Qual. 20, 165–168.

Lombi, E., Zhao, F., Fuhrmann, M., Ma, L.Q., McGrath, S.P., 2002. Arsenic distribution and speciation in the fronds of the hyperaccumulator *Pteris vittata*. New Phytol. 156, 195–203.

Ma, L.Q., Komar, K.M., Tu, C., Zhang, W., Cai, Y., Kennelley, E.D., 2001. A fern that hyperaccumulates arsenic. Nature 409, 579.

Marin, A.R., Masscheleleyn, P.H., Patrick, W.H., 1992. The influence of chemical form and concentration of arsenic on rice growth and tissue arsenic concentration. Plant Soil 139, 175–183.

Marschner, H., 1995. Mineral Nutrition of Higher Plants, second ed. Academic Press, London.

Matschullat, J., 2000. Arsenic in the geosphere–a review. Sci. Total Environ. 249, 297–312.

Meharg, A.A., Hardey-Whitaker, J., 2002. Arsenic uptake and metabolism in arsenic resistant and nonresistant plant species. New Phytol. 154, 29–43.

Meng, X., Korfiatis, G.P., Jing, C., Christodoulatos, C., 2001. Redox transformations of arsenic and iron in water treatment sludge during aging and TCLP extraction. Environ. Sci. Technol. 35, 3476–3481.

Nissen, P., Bensun, A.A., 1982. Arsenic metablolism in fresh water and terrestial plants. Physiol. Plantarum 54, 446–450.

NRCC, 1978. Effects of Arsenic in the Canadian Environment. National Research Council Canada (NRCC), NRCC No. 15391, Ottawa, Ont.

Phillips, S.E., Taylor, M.L., 1976. Oxidation of arsenite to arsenate by *Alcaligenes faecalis*. Appl. Environ. Microbiol. 32, 392–399.

Ric De Vos, C.H., Vonk, M.J., Vooijs, R., Schat, H., 1992. Glutathione depletion due to copper-induced phytochelatin systhesis causes oxidative stress in *Silene cucubalus*. Plant Physiol. 98, 853–858.

Schmöger, M.E.V., Oven, M., Grill, E., 2000. Detoxification of arsenic by phytochelatins in plants. Plant Physiol. 122, 793–801.

Sehlin, H.M., Lindström, E.B., 1992. Oxidation and reduction of arsenic by *Sulfolobus scidocaldarius* strain BC. FEMS Microbiol. Lett. 93, 87–92.

Sneller, F.E.C., van Heerwaarden, L.M., Kraaijeveld-Smit, F.J.L., Bookum, W.M.T., Koevoets, P.L.M., Schat, H., Verkleij, J.A.C., 1999. Toxicity of arsenate in *Silene vulgaris*, accumulation and degradation of arsenate-induced phytochelatins. New Phytol. 144, 223–232.

Tu, C., Ma, L., 2002. Effects of arsenic concentrations and forms on arsenic uptake by the hyperaccumulator Ladder Brake. J. Environ. Qual. 31, 641–647.

Tu, C., Ma, L., 2003. Effects of arsenate and phosphate on their accumulation by an arsenic-hyperaccumulator *Pteris vittata* L. Plant Soil 249, 373–382.

Visoottiviseth, P., Francesconi, K., Sridokchan, W., 2002. The potential of Thai indigenous plant species for the phytoremediation of arsenic contaminated land. Environ. Pollut. 118, 453–461.

Waldrop, M.P., Sterling, T.M., Khan, R.A., Molin, W.T., 1996. Fate of prometryn in prometryn-tolerant and susceptible cotton cultivars. Pest. Biochem. Physiol. 56, 111–122.

Wang, J., Zhao, F.J., Meharg, A.A., Raab, A., Feldmann, J., McGrath, S.P., 2002. Mechanisms of arsenic hyperaccumulation in *Pteris vittata*. Uptake kinetics, interactions with phosphate, and arsenic speciation. Plant Physiol. 130, 1552–1561.

Zhang, G., Taylor, G.J., 1991. Effects of biological inhibitors on kinetics of aluminum uptake by excised roots and purified cell wall material of aluminum-tolerant and aluminum-sensitive cultivars of *Triticum aestivum* L. J. Plant Physiol. 138, 533–539.

Zhang, W., Cai, Y., Tu, C., Ma, L.Q., 2002. Arsenic speciation and distribution in an arsenic hyperaccumulating plant. Sci. Total Environ. 300, 167–177.

Zhao, F.J., Dunham, S.J., McGrath, S.P., 2002. Arsenic hyperaccumulation by different fern species. New Phytol. 156, 27–31.