Three new arsenic hyperaccumulating ferns

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Abstract

Phytoremediation, an emerging, plant-based technology for the removal of toxic contaminants from soil and water, has been receiving increased attention. The prerequisite for successful phytoremediation is the existence of hyperaccumulator plants. Designed to search for new arsenic (As) hyperaccumulators, an experiment was conducted under greenhouse conditions in a completely randomized design with four replications. This experiment identified \( Pteris biaurita \) L., \( P. quadriaurita \) Retz and \( P. ryukyuensis \) Tagawa as new hyperaccumulators of As and re-confirmed \( Pteris cretica \) as a hyperaccumulator. The average As concentration ranged from 1770 to 3650 mg kg\(^{-1}\) DW in the fronds and 182 to 507 mg kg\(^{-1}\) DW in the roots of \( P. cretica \), \( P. biaurita \) and \( P. ryukyuensis \) after having been grown in 100 mg As kg\(^{-1}\) soil. There was a greater percentage of As (III) as compared to As (V) in the fronds of these plants. Based on our study, \( P. ryukyuensis \) is the most promising candidate to phytoremediate As contaminated soils compared to the other three species. The nutrient requirements or distributions within the \( Pteris \) species were altered distinctly when the plants were exposed to As.

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1. Introduction

Arsenic (As) is one of the most toxic elements present in soils and water. Over the years, arsenic has been widely used in agriculture and industrial practices such as pesticides, fertilizers, wood preservatives, smelter wastes and coal combustion ash, which are of great environmental concern (Smith et al., 1998; Nriagu, 2002). Arsenic contamination affects biological activities as a teratogen, carcinogen and mutagen as well as having detrimental effects on the immune system (Squibb and Fowler, 1983). The evidence of health risks from As contamination is so compelling that in 2002 the Environmental Protection Agency lowered the maximum contaminant level of As in drinking water from 50 to 10 \( \mu \)g l\(^{-1}\), making remediation of As contaminated water an increasingly important and potentially expensive issue (Smith et al., 2002).

Phytorextraction, a promising new method that uses green plants to detoxify metals, is an alternative to the conventional means to remediate As contaminated sites (Salt et al., 1998). This technology is a relatively inexpensive form of ecological engineering that has proven effective in some cases (Raskin et al., 1994). Obvious prerequisites for successful phytorextraction are the existence of metal hyperaccumulators with the ability to accumulate large amounts of the metal contaminant in their aboveground tissues with a high biomass. The term metal hyperaccumulator was first used to describe a plant species that hyperaccumulates nickel (Ni). The
term was later broadened to characterize plants achieving metal concentrations \( >1000 \text{ mg kg}^{-1} \) \cite{Reeves and Baker, 2000}.

Many plants have been reported to accumulate more than \( 1000 \text{ mg kg}^{-1} \) arsenic in their tissues \cite{Porter and Peterson, 1975}, however, they cannot be classified as hyperaccumulators since arsenic accumulation in these plants occurs very slowly over an extended period of time. In addition, a large portion of the arsenic is sequestered in the roots. Most importantly, a lack of rapid growth, large biomass production and high uptake capacity render these plants unsuitable for phytoremediation.

Although the definition of arsenic hyperaccumulators is not clearly defined and can be considered arbitrary, the working definition is a plant that accumulates a minimum arsenic concentration of \( 1000 \text{ mg kg}^{-1} \) in the aboveground biomass, and has a higher concentration in the aboveground biomass than in both the roots and the soil \cite{Bondada and Ma, 2003; Meharg, 2003}.

The first known arsenic hyperaccumulating plant, \textit{Pteris vittata} L., also known as Chinese brake fern, was discovered by Komar et al. \cite{1998} from an arsenic-contaminated site that was contaminated from pressure-treating lumber using chromated-copper-arsenate (CCA). However, the hyperaccumulator was not well publicized until after 2001 when several other scientists made a similar discovery \cite{Ma et al., 2001a; Chen et al., 2002; Visoottiviseth et al., 2002}. Among the 14 plant species collected from the CCA site, this fern is the only one that hyperaccumulates arsenic, with arsenic concentrations in the fronds (aboveground biomass) being as high as \( 4360 \text{ mg kg}^{-1} \), as compared to \( 184 \text{ mg kg}^{-1} \) in the soil \cite{Komar et al., 1998}. In a subsequent screening study of 17 fern species, three cultivars of \textit{Pteris cretica} (i.e. \textit{albo-lineata}, \textit{mayii} and \textit{parkerii}), were classified as arsenic hyperaccumulators \cite{Ma et al., 2001b}. Arsenic concentrations in their fronds ranged from 1114 to 2046 \text{ mg kg}^{-1} after growing in an arsenic-contaminated soil containing \( 245 \text{ mg kg}^{-1} \) arsenic for 8 weeks. The other 14 species are not arsenic hyperaccumulators, all being non-\textit{Pteris} ferns and having arsenic concentrations less than \( 46.6 \text{ mg kg}^{-1} \) in the fronds.

In addition to \textit{P. vittata} and \textit{P. cretica}, several other arsenic hyperaccumulating plants have been reported recently including \textit{Pityrogramma calomelanos} \cite{Francesconi et al., 2001} and \textit{Pteris longifolia} and \textit{Pteris umbrosa} \cite{Meharg, 2003; Zhao et al., 2002}. Besides the three cultivars of \textit{P. cretica} identified by Ma et al. \cite{2001b}, four additional cultivars of \textit{P. cretica} have also been identified as arsenic hyperaccumulators, i.e. \textit{chilisi}, \textit{crista} and \textit{rowerii} \cite{Meharg, 2003} and \textit{wisnsetti} \cite{Zhao et al., 2002}. Except for \textit{P. calomelanos}, all known arsenic hyperaccumulator species are ferns and belong to the \textit{Pteris} genus. However, not all \textit{Pteris} species hyperaccumulate arsenic \cite{Meharg, 2003; Zhao et al., 2002}.

In this study, we hypothesized that screening ferns in the \textit{Pteris} genus could identify more arsenic hyperaccumulators. We tested the hypothesis by growing different \textit{Pteris} species under controlled greenhouse conditions. The biomass and nutrient [phosphorus (P), potassium (K), calcium (Ca) and magnesium (Mg)] accumulation in these ferns and their relations to As were also studied.

## 2. Materials and methods

### 2.1. Arsenic accumulation by \textit{Pteris} species grown from spores

Spores of \textit{P. cretica} L. (a known As hyperaccumulator), \textit{Pteris biaurita} L., \textit{P. quadrialurita} Retz. and \textit{P. ryukyuensis} Tagawa were collected from the natural habitat of each species. Spores were sprinkled onto a moist soil (50% sand, 25% peat and 25% garden soil) in a seed tray. The trays were covered with a plastic film to maintain moisture. After spore germination and the prothalli development, fertilizer was applied. Once sporelings grew to having two to three fronds, they were transplanted individually into 2-in. plastic pots containing just potting soil. After 1 month, these plants were transferred to pots containing 2.5 kg of soil.

The experiment was conducted in controlled environmental conditions with an 8-h light period at intensity of \( 350 \text{ \mu mol m}^{-2} \text{ s}^{-1}, 25^\circ\text{C/20}^\circ\text{C day/night temperature and 60–70% relative humidity. The study was set up as a completely randomized design in a 4 × 2 factorial. Four fern species: \textit{P. cretica}, \textit{P. biaurita}, \textit{P. quadrialurita} and \textit{P. ryukyuensis} (one plant in each pot) were grown in a clean soil (control) and a soil spiked with 100 mg As kg\(^{-1}\), added as Na\(_2\)HAsO\(_4\)-7H\(_2\)O. Each treatment was replicated four times. Forty-five days after transplanting, the plants were harvested and washed with deionized water. The plants were then separated into fronds (aboveground biomass) and roots (underground biomass). Plant parts were dried at 60 °C for 48 h and dry weights recorded.

### 2.2. Chemical analysis

Ground plant material (0.5 g) was digested with nitric acid and hydrogen peroxide on a temperature-controlled digestion block (Environmental Express, Mt. Pleasant, S.C.) using USEPA Method 3050A in
preparation for metals analysis, which was performed with either a transversely heated, Zeeman background correction equipped graphite furnace atomic absorption spectrophotometer (GFAAS for As; Perkin-Elmer SIMAA 6000, Norwalk, CT) or a flame atomic absorption spectrophotometer (for Ca, Mg and K; Varian, 220FS, Cupertino Ca.).

Phosphorus was determined by a modified molybdenum blue method (Carvalho et al., 1998). Briefly, the pH of the digestion solution was adjusted to around seven with NaOH and H₂SO₄. 10 ml of the solution was pipetted into a 20 ml glass test tube, to which 0.5 ml of L-cysteine (5% w/v in 0.6 M HCl) was added. The test tube was capped tightly and incubated for 5 min at 80 °C to allow complete reduction of arsenate into arsenite. The solution was cooled to room temperature and P was determined by the molybdenum blue method (Murphy and Riley, 1962).

Arsenic speciation was performed by extracting fresh plant samples ultrasonically in 10 ml of a methanol/water mixture (1/1 (v/v)) three times for a total of 6 h at 25 °C (Zhang et al., 2002). The three extracts were decanted into a 50 ml volumetric flask and diluted to 50 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). To check the reliability of this method, As speciation of *P. vittata* samples were previously analyzed by using both As cartridge-GFAAS method and HPLC-ICP-MS method (unpublished data). The sum of AsIII and AsV concentrations determined by HPLC-ICP-MS and the total As concentration determined by As cartridge-GFAAS agreed by 96±13%. A 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 the accuracy and precision of the As analysis.

2.3. Data analysis

All results were expressed as an average of four replications. Treatment effects were determined by analyses of variance using the general linear model procedure of the Statistical Analysis System (SAS Institute Inc., 1996). Duncan’s test at \( p \leq 0.05 \) was used for post hoc comparisons to separate treatments. Single correlation analyses were performed to investigate the relationships between arsenic in the plant parts of each fern species and macronutrient contents.

3. Results and discussion

3.1. Arsenic accumulation in fronds and root tissues

Arsenic accumulation was determined in the frond and root tissues of the four *Pteris* species. After 45 days of exposure to 100 mg kg⁻¹ As, all the plants were quite healthy and did not show any phytotoxicity symptoms except slight necrosis at the edge and tip of the fronds in
a few plants. This indicated that these fern species were relatively tolerant to arsenic. There was considerable variation in As accumulation among the four fern species (Fig. 1). Root As concentrations ranged from 182 to 507 mg kg\(^{-1}\) DW. Frond As concentrations varied from 1766 to 3647 mg kg\(^{-1}\) DW. The plant species exhibiting the highest root As concentration was *P. ryukyuensis* (Fig. 1). The fern species varied widely in their abilities to transport As to the fronds with the highest frond As concentration found in *P. ryukyuensis* (3647 mg kg\(^{-1}\)). Among the four species tested, *P. ryukyuensis* was the most efficient in As accumulation. Our findings are in agreement with Tu et al. (2002), who also reported more accumulation of As in fronds (6000 mg kg\(^{-1}\) DW) of *P. vittata*, a well known As hyperaccumulator when grown on As contaminated soil containing 98 mg kg\(^{-1}\) for 8 weeks (60 days).

### 3.2. Arsenic speciation in plant parts

As (III) and As (V) were the principal species found in the present study. This finding is in agreement with Tu et al. (2004) who also recorded the same As species in their study using *P. vittata*. The distribution of As species in these ferns is shown in Fig. 2. Plants generally contained a greater percentage of As (III) (79–94%) than As (V) in the fronds (Fig. 2).

### 3.3. Arsenic translocation and bioconcentration

One of the characteristics of hyperaccumulator plants is the ability to accumulate high concentrations of metal or metalloid in the aboveground biomass. The transfer factor has been used to describe a plant’s ability to transfer an element from roots to aboveground biomass, which is defined as concentration ratio of an element in the aboveground biomass to that in the roots. The frond/root ratios of As in the plants varied from 7.3 to 11.3 growing in the soil spiked with 100 mg kg\(^{-1}\) As (Fig. 3A). The bioaccumulation factor, on the other hand, measures the ability of a plant to concentrate the contaminant relative to the medium and is defined as the concentration ratio of an element in the plant to the soil medium. The bioconcentration factor ranged from 17.7 to 36.5 (Fig. 3B). The total As accumulation (fronds and roots) was the highest in *P. ryukyuensis* (Table 1). *P. biaurita* was the most efficient in translocating As from roots to fronds (Fig. 3A). Variation in the As accumulation was greatly influenced by the ability of the plants to translocate As. With respect to As accumulation, *P. ryukyuensis* was the most promising (Table 1).

### 3.4. Macronutrients in plant parts

Differences in concentrating nutrients by *Pteris* ferns could account for the differences between the species to remove As from the system. The results of this study indicate that *Pteris* species have different nutrient requirements and partitioning in the plant parts (Table 2). The results also indicated that the nutrient status and distribution varied with the amount of As present (Table 2). For instance, in the absence of metal or metalloid.
of As, *P. cretica* concentrated almost twice as much P, then *P. biaurita* or *P. quadriaurita*. However, when exposed to 100 mg kg\(^{-1}\) of arsenic, *P. ryukyuensis* was the species that accumulated more P than *P. biaurita* or *P. quadriaurita*.

The relative content of specific nutrients in the plant parts, however, may change depending on arsenic concentrations in the soil. For example, regardless of the soil As concentration, P concentrations in the fronds were greater than that in the roots for all *Pteris* species evaluated. Exposure of the plants to As (100 mg kg\(^{-1}\)) increased the frond P concentration in all the species of *Pteris* during this study except *P. cretica*. Increased P availability due to competitive adsorption (Gao and Mucci, 2001; Tu and Ma, 2003) and arsenic-induced physiological requirements of a plant (Carbonell et al., 1998) may be the reasons for the increased P uptake. *P. ryukyuensis* was the species

Table 1

<table>
<thead>
<tr>
<th>Fern species</th>
<th>Plant biomass (g/plant)</th>
<th>Plant biomass (g/plant)</th>
<th>As uptake (mg/plant)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Frond</td>
<td>Root</td>
<td>Frond</td>
</tr>
<tr>
<td><em>P. biaurita</em></td>
<td>1.99±0.42 A</td>
<td>1.60±0.44 A</td>
<td>4.08±0.75 B</td>
</tr>
<tr>
<td><em>P. quadriaurita</em></td>
<td>1.92±0.37 A</td>
<td>1.26±0.30 AB</td>
<td>5.42±0.90 AB</td>
</tr>
<tr>
<td><em>P. cretica</em></td>
<td>1.15±0.18 B</td>
<td>1.02±0.13 B</td>
<td>2.03±0.27 C</td>
</tr>
<tr>
<td><em>P. ryukyuensis</em></td>
<td>1.79±0.30 A</td>
<td>1.34±0.24 AB</td>
<td>6.53±0.94 A</td>
</tr>
</tbody>
</table>

Means in a column followed by the same letter are not significantly different at \(p \leq 0.05\).
Table 2
Concentrations of phosphorus (P), potassium (K), calcium (Ca) and magnesium (Mg) in fronds and roots of different *Pteris* species after growing in a soil with (100 mg kg\(^{-1}\) As) and without As for 45 days

<table>
<thead>
<tr>
<th>Species</th>
<th>P No As</th>
<th>P With As</th>
<th>K No As</th>
<th>K With As</th>
<th>Ca No As</th>
<th>Ca With As</th>
<th>Mg No As</th>
<th>Mg With As</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fronds</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. biaurita</em></td>
<td>2564 Ca</td>
<td>2925 Ba</td>
<td>10,785  Ba</td>
<td>23,416 Aa</td>
<td>11,792 Aa</td>
<td>6059 Bb</td>
<td>3864 Aa</td>
<td>2464 Ab</td>
</tr>
<tr>
<td><em>P. quadriaurita</em></td>
<td>2860 BCa</td>
<td>3193 Ba</td>
<td>21,289 Ba</td>
<td>21,356 Aa</td>
<td>12,324 Aa</td>
<td>8357 Aa</td>
<td>3732 Aa</td>
<td>2248 ABB</td>
</tr>
<tr>
<td><em>P. cretica</em></td>
<td>5242 Aa</td>
<td>3492 Bb</td>
<td>34,164 Aa</td>
<td>18,368 Aa</td>
<td>6321 Ba</td>
<td>5112 Ba</td>
<td>2513 Ba</td>
<td>1939 Ba</td>
</tr>
<tr>
<td>Roots</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. biaurita</em></td>
<td>2237 Ba</td>
<td>2017 Ba</td>
<td>10,118 Aa</td>
<td>10,622 Aa</td>
<td>13,880 ABA</td>
<td>13,023 Aa</td>
<td>2202 Bb</td>
<td>2420 Aa</td>
</tr>
<tr>
<td><em>P. quadriaurita</em></td>
<td>2265 Ba</td>
<td>2376 ABA</td>
<td>10,290 Aa</td>
<td>8325 Aa</td>
<td>15,256: Aa</td>
<td>10,916 Bb</td>
<td>2787 Aa</td>
<td>2136 Ba</td>
</tr>
<tr>
<td><em>P. cretica</em></td>
<td>3203 Aa</td>
<td>2143 ABB</td>
<td>8314 Aa</td>
<td>8480 Aa</td>
<td>11,124 Bb</td>
<td>9497 Ba</td>
<td>1593 Ca</td>
<td>1552 Ca</td>
</tr>
</tbody>
</table>

Means in a row followed by the same capital letter in a column are not significantly different at \( p \leq 0.05 \).

with the highest P concentration as a result of the mentioned processes.

The molar P/As ratio in plant tissue has been suggested as a good indicator of As phytotoxicity (Tu and Ma, 2005). We calculated the frond P to arsenic molar ratio by dividing the respective concentrations in the frond (mg kg\(^{-1}\)) for the molar weights. The addition of As to the soil reduced the P/As molar ratio from over 4000 to <5 in the fronds and from over 4600 to <30 in the roots (Table 3). The frond P/As molar ratio of approximately three (2.7 in *P. quadriaurita* and 2.9 in *P. ryukyuensis*) resulted in the highest As accumulation (Table 3).

*P. cretica*, growing in the soil with no arsenic exposure, was the most effective species to concentrate K in the fronds. This difference, however, disappeared when the plants were exposed to As. Also the *Pteris* species concentrated more K in the fronds than in the roots. Arsenic exposure also affected Ca and Mg partitioning in the plant organs. In the absence of As, *P. quadriaurita* and *P. ryukyuensis* had higher relative Ca and Mg concentrations in the roots. Interestingly, the concentrations of Ca in the fronds of the *Pteris* species were negatively correlated with arsenic in the fronds and roots of all species (Table 3).

Table 3
Phosphorus/arsenic (molar ratio) in fronds and roots of different *Pteris* species

<table>
<thead>
<tr>
<th>Fern species</th>
<th>P/As (molar ratio) with no arsenic</th>
<th>P/As (molar ratio) with arsenic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Frond: Root</td>
<td>Frond: Root</td>
</tr>
<tr>
<td><em>P. biaurita</em></td>
<td>2332: 1806</td>
<td>3.5: 26.7</td>
</tr>
<tr>
<td><em>P. quadriaurita</em></td>
<td>1560: 3287</td>
<td>2.7: 17.9</td>
</tr>
<tr>
<td><em>P. cretica</em></td>
<td>4623: 4601</td>
<td>4.8: 24.4</td>
</tr>
<tr>
<td><em>P. ryukyuensis</em></td>
<td>1569: 3988</td>
<td>2.9: 13.4</td>
</tr>
</tbody>
</table>

Means of a plant part followed by the same small letter are not significantly different at \( p \leq 0.05 \).

A similar trend was observed between frond Mg and frond and root Ca concentration; however, *P. ryukyuensis* was an exception. Root Ca and Mg concentration in *P. quadriaurita* and *P. ryukyuensis* fronds were negatively correlated with As in the frond and the roots (Table 4), while *P. biaurita* and *P. cretica* were not. Frond Ca concentration in *P. biaurita* and *P. quadriaurita* was reduced 40% to 50% when exposed to As. However, in the absence of As, *P. cretica* and *P. ryukyuensis* had significantly lower Ca concentrations as compared with *P. biaurita* and *P. quadriaurita* in the absence of As.

3.5. Arsenic hyperaccumulators

It is reported that an arsenic hyperaccumulator should have concentrations in excess of 1000 mg As kg\(^{-1}\) in its’ aboveground biomass (Bondada and Ma, 2003). In addition, its transfer factor and bioconcentration factor should be greater than one. Using these definitions, we have identified three new arsenic hyperaccumulators in the *Pteris* genus, namely, *P. biaurita*, *P. quadriaurita* and *P. ryukyuensis*, and re-confirmed *P. cretica* as an arsenic hyperaccumulator. These species are characterized by a high bioconcentration factor (>17.7) and high translocation factor (>7.3). Other arsenic hyperaccumulators in the *Pteris* genus includes *P. vittata* (Komar et al., 1998; Ma et al., 2001a,b), and *P. cretica*, *P. longifolia* and *P. umbrosa* (Ma et al., 2001c; Zhao et al., 2002; Meharg, 2003). However, not all the *Pteris* species are arsenic hyperaccumulators, for example, *P. stramina*, *P. tremula* and *P. semipinnata* (Meharg, 2003), and *P. ensiformis* (Srivastava et al., 2005).

It is interesting to note that the *Pteris* species and As hyperaccumulator *P. calomelanos* (Francesconi et
al., 2001) belong to the order Pteridales (Jones, 1987). That these species can withstand the high arsenic concentrations in the soil suggest that these plants may have a mechanism to detoxify accumulated As (Srivastava et al., 2005). The present study has shown that most of the As in the frond tissues is readily extracted into methanol and water, and this As is primarily present as inorganic As(III), i.e. arsenite.

Because of its status as a hyperaccumulator and its relatively hardy nature, *P. ryukyuensis* should be considered the most suitable species among the four fern species studied for phytoremediation purposes. Follow up work to compare the *P. ryukyuensis* with *P. vittata* under a variety of conditions may yield promising results. Further studies are required to explain the mechanisms for As detoxification in *Pteris* and other related species that hyperaccumulate As.

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