Arsenic distribution and speciation in the fronds of the hyperaccumulator *Pteris vittata*

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Summary

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• *Pteris vittata* is the first plant reported to be a hyperaccumulator of arsenic (As), and little is known about the mechanisms of As hyperaccumulation in this plant.
• Arsenic distribution at the whole plant (fronds) and cellular level was investigated using chemical analyses and energy dispersive X-ray microanalyses (EDXA). Speciation of As in the fronds was determined using X-ray absorption near edge spectroscopy (XANES) analyses.
• The majority of As was found in the pinnae (96% of total As). The concentration of As in pinnae decreased from the base to the apex of the fronds. Arsenic concentrations in spores and midribs were much lower than in the pinnae. EDXA analyses revealed that As was compartmentalized mainly in the upper and lower epidermal cells, probably in the vacuoles. The distribution pattern of potassium was similar to As, whereas other elements (Ca, Cl, K, Mg, P and S) were distributed differently.
• XANES analyses showed that approximately 75% of the As in fronds was present in the As(III) oxidation state and the remaining as As(V).

Key words: *Pteris vittata*, arsenic, hyperaccumulation, compartmentation, speciation.

Introduction

Arsenic is ubiquitous in the environment and its presence in soils is due to both natural and anthropogenic sources. In recent years, public concern regarding this element has increased due to new evidence that As may be toxic at lower concentrations than previously thought (Smith *et al.*, 1992; Chiou *et al.*, 1995).

A number of technologies to remediate As polluted soils are available (Lombi *et al.*, 2000). However, most of these technologies, which are engineering based, are generally expensive and may produce secondary wastes. Recently, environmentally-friendly, low-input approaches such as phytoremediation have been proposed for cleaning up soils contaminated with heavy metals and metalloids (McGrath *et al.*, 2002). A specific phytoremediation approach, called phytoextraction, makes use of hyperaccumulator plants which extract pollutants from the soil and accumulate them in harvestable parts of the plants. While studies have been conducted on the phytoextraction of heavy metals such as Cd, Ni and Zn,

very little is known about the phytoextraction of As. This is because the discovery of an As hyperaccumulator is very recent. Ma *et al.* (2001) reported for the first time that a fern, *Pteris vittata*, can accumulate extremely large concentrations (up to 23 000 mg kg^{-1}) of As in its above ground biomass. Several other fern species, including *Pityrogramma calomelanos* (Visoottiviseth *et al.*, 2002), and *Pteris cretica*, *Pteris longifolia* and *Pteris umbrosa* (Zhao *et al.*, 2002), have recently been reported to hyperaccumulate As to concentrations similar to *P. vittata*.

Inorganic arsenic species are generally highly toxic to plants. Arsenate acts as a phosphate analog and affects phosphate metabolism, whereas arsenite reacts with sulfydryl groups of enzymes and tissue proteins, leading to inhibition of cellular function and death (Meharg & Hartley-Whitaker, 2002). In As nonhyperaccumulating plants, resistance to environmental As has been shown to involve a decreased uptake of arsenate due to suppression of the high-affinity phosphate uptake system (Meharg & Macnair, 1991, 1992). Inside plant cells, arsenate may be detoxified through

reduction to arsenite, which is subsequently complexed with thiols, particularly phytochelatins (PCs) (Pickering *et al.*, 2000; Schmöger *et al.*, 2000; Hartley-Whitaker *et al.*, 2001). It is possible that As-PC complexes are sequestered in vacuoles, although direct evidence for this is still lacking (Meharg & Hartley-Whitaker, 2002).

Compared with many crop plant species, which typically suffer from phytotoxicity when As in shoots reaches 5–20 mg kg^{-1} d. wt (Kabata-Pendias & Pendias, 1992), As hyperaccumulating ferns posses a remarkable ability to tolerate exceedingly high concentrations (> 10 000 mg kg^{-1}) of As in the fronds. The mechanisms for internal detoxification of As in the hyperaccumulating ferns have not been elucidated. Ma *et al.* (2001) reported that As in fronds of *P. vittata* was present mainly (47–80%) as inorganic arsenite. Similarly, Francesconi *et al.* (2002) found that a large fraction of As in the fronds of *P. calomelanos* (up to 93%) was water extractable and present mainly as arsenite (up to 72%), the remaining As being arsenate with only traces of organoarsenic compounds. Because of the high affinity of arsenite to thiols, complexation with PCs may occur in these ferns. It is also likely that cellular/subcellular compartmentation plays an important role in the detoxification of As in these fern species. In a number of Zn and Ni hyperaccumulator plants, it has been shown that compartmentation at the cellular level is a key factor responsible for the detoxification of these metals (Küpper *et al.*, 1999, 2000, 2001; Frey *et al.*, 2000).

This paper aims to investigate the localization and speciation of As in fronds of *P. vittata*. This information is fundamental to understanding the mechanisms of As tolerance and hyperaccumulation in this species. In particular we focus on As localization in different parts of *P. vittata* fronds and its compartmentation at the cellular level using energy dispersive X-ray microanalyses (EDXA). Arsenic speciation in the fronds was determined using X-ray absorption near edge structure spectroscopy (XANES). This technique differs from the method used by Ma *et al.* (2001) and Francesconi *et al.* (2002), as it allows in-situ As speciation on plant samples to be determined without the need for extraction.

Materials and Methods

Plant material

Spores of *P. vittata* were germinated on a general purpose compost. At the 3–4 frond stage, sporelings were transferred to pots containing 0.5 kg of compost spiked with Na$_2$HAsO$_4$ to obtain a concentration of 300 mg As kg^{-1}. Plants were grown for 3 months, and at the end of this period samples were collected for EDXA and XANES analyses and to investigate As distribution in the fronds.

In order to obtain As distribution dot maps by EDXA, the As concentration in the plants had to be larger than that obtained by growing the plants in compost. This was achieved

by growing the fern hydroponically in the presence of As. Roots of seedlings developed in compost (3–4 frond stage) were washed carefully with water to remove adhering compost and transferred to 330-ml pots (one plant per pot) containing a modified Hoagland nutrient solution with $^1/_2$ strength major nutrients and full strength micronutrients (except that Fe was supplied as Fe-EDDHA at 100 μM). The nutrient solution was aerated continuously and renewed weekly. After 3 wk, Na$_2$HAsO$_4$ was added to the nutrient solution to give 500 μM As(V). Plants were grown for another 4 wk before collection of EDXA dot maps.

Plants grown either in the solid substrate or in hydroponics were kept inside a controlled environment growth chamber with the following conditions: 16 h light period with a light intensity of 350 m mole m^{-2} s^{-1}, 25°C/20°C day/night temperature, and 60–70% relative humidity.

Arsenic distribution in fronds

Two mature fronds of plants grown in compost were harvested to determine the As content of different parts of the frond. The midrib and pinnae (collected in pairs) of each frond were separated, oven-dried and their weight recorded. At the same time five pinnae were harvested and the spores separated from the laminae. Again the laminae and spores were oven dried and their weight recorded.

The different parts of the frond (pinnae, midribs) and of the pinnae (laminae, spores), were digested with ultrapure concentrated HNO$_3$ (2 ml) and 30% w/v H$_2$O$_2$ (2 ml) using a closed-vessel microwave digestion protocol (CEM Mars V, Matthews, NC, USA). In a subsequent prereduction step, 1 ml of concentrated HCl and 1 ml of a reducing solution (5% w/v KI and ascorbic acid) were added to 1 ml aliquot of the digest solution. After 16 h the samples were diluted with ultra-pure (*c.* 18 MΩ) water (ELGA Maxima, High Wycombe, UK) and analysed for As using a Perkin-Elmer 4100ZL atomic absorption spectrophotometer equipped with a Perkin-Elmer (Norwalk, CT, USA) FIAS 400 flow-injection hydride generation unit (HG-AA).

Energy dispersive X-ray microanalyses (EDXA)

This technique was used to investigate the distribution of As and other elements (Ca, Cl, K, Mg, P and S) at the cellular level in the bulk hydrated tissues of pinnae. The technique involved minimal sample preparation, which minimizes the possibility of element redistribution. The method used is described in detail by Küpper *et al.* (2000). Sections of eight pinnae from different plants grown in compost were excised and mounted in a stainless steel device. The specimens were then rapidly (within less than a minute after excision) frozen in liquid nitrogen and transferred to a fracturing chamber cooled to −170°C. A blade was used to cut through the cells. Subsequently, samples were evaporatively coated with

carbon. EDXA analysis was performed in a scanning electron microscope (SEM, Philips XL 40) on a cryostage (−160°C to −180°C), using an acceleration voltage of 30 kV and a working distance of 10 mm. Spectra from 0 to 20 KeV were collected at increments of 10 eV per channel with the electron beam focused on a rectangular area in the center of selected cells. For each tissue (upper and lower epidermis, palisade and spongy mesophyll) between 15 and 29 individual cells were recorded. These spectra were analyzed using the program Superquant (EDAX, San Francisco, CA, USA). A calibration between peak: background ratios for specific elements and their concentrations in the standard solutions was used to quantify the recorded data (Boekestein et al., 1984; Van Steveninck & Van Steveninck, 1991).

Two-dimensional distribution patterns of selected elements were recorded by scanning an area of the specimen repeatedly for up to 2 h and integrating the counts for the respective elements into dot-maps. As mentioned above, these analyses can be performed only when the As concentration is large enough to produce a peak/background signal greater than 2. This is because the spectrum-analysis software used does not allow for separation of the element-specific X-rays (net peak counts) from the background counts during the collection of the dotmap data. Therefore, plants grown hydroponically to induce large As accumulation were used to collect dot-maps.

The total concentration of As in the pinnae used for EDXA analyses was also determined by hydride generation – atomic absorption (HG-AA) analyses of microwave digest as described above.

X-ray absorption near edge structure analyses (XANES)

Fronds from the plants grown in As-spiked compost were cut, washed with deionized water, blotted dry, and frozen in liquid nitrogen. The sample was freeze-dried under vacuum for 3 d, and ground to a fine powder.

X-ray Absorption Spectroscopy (XAS) was done at beamline X-18B at the National Synchrotron Light Source (NSLS) at Brookhaven National Laboratory, Upton, NY, USA. Arsenic was analysed at the K-edge (11.86 keV). The channel cut Si (111) monochromator was detuned by about 20% to reduce the third harmonic. A 0.5-mm vertical slit was used at about 18 m from the source, resulting in an absolute energy resolution of about 3 eV. For standard samples, two 30-cm-long sealed ion chambers from Oxford Instruments (Nitney, UK) with Ar at atmospheric pressure were used to measure incident flux I_0 and the transmitted flux I_t through the sample. Arsenic (0) powder on tape was used for calibration and as reference between I_t and an I_{ref} ion chamber containing flowing Ar. At the samples, the beam was 0.5 mm in height and 15 mm across. The monochromator moved in steps of 0.5 eV in the XANES (X-ray Absorption Near Edge Spectroscopy) region. The edge energy was calibrated using the inflection of the first derivative of the metal reference. The As concentration in the plant samples was in the high mg kg^{-1} range and they were therefore measured in the fluorescence mode to enhance the signal to noise ratio. The sample was angled at 45° to the beam and a 13-element Ge detector (Canberra Industries, Meriden, CT, USA) was placed at 45° to the sample, 90° to the beam, to measure the fluorescent photons in integration mode. Multiple scans run at room temperature were averaged for each sample and standard. The powdered and freeze-dried plant material was packed into a plexiglass holder, measuring 7.5 mm thick. Kapton tape was placed on either side of the window to hold the powder. Standards consisted of fine grained As (0) powder, as well as As(III) and As(V) oxide compounds that were mixed with sucrose and ground to a fine powder. Concentrations of As in the standards were about 500 mg kg^{-1}. These were analyzed in the same configuration as the plant samples.

Results

Arsenic distribution in fronds

Plants grown for 3 months in compost spiked with 300 mg kg^{-1} of arsenate produced numerous fronds and were healthy. Mature fronds had more than 20 pairs of pinnae and had a d. wt of over 2 g per frond. The majority of the biomass produced was represented by pinnae (71%), the weight of midribs accounting for the remaining part (Table 1). The concentration of As was 24 times larger in the pinnae than in the midribs (6158 and 258 mg kg^{-1}, respectively). So although the midribs represented about 30% of the total

**Table 1** Dry weight and As concentrations in different plant parts

Part of frond/pinna	D. wt		As concentration	
	mg	% of total weight*	mg kg^{-1}	% of total weight
Pinnae	1539 (144)	71 (2)	6158 (1040)	96 (0.3)
Midrib	634 (9)	29 (2)	258 (23)	4 (0.3)
Lamina	27 (1.6)	78 (2)	6506 (321)	98 (1)
Spores	7 (0.6)	22 (2)	376 (83)	2 (1)

*Total weight of frond for pinnae and midrib, or of pinna for lamina and spores. SE are in parentheses.
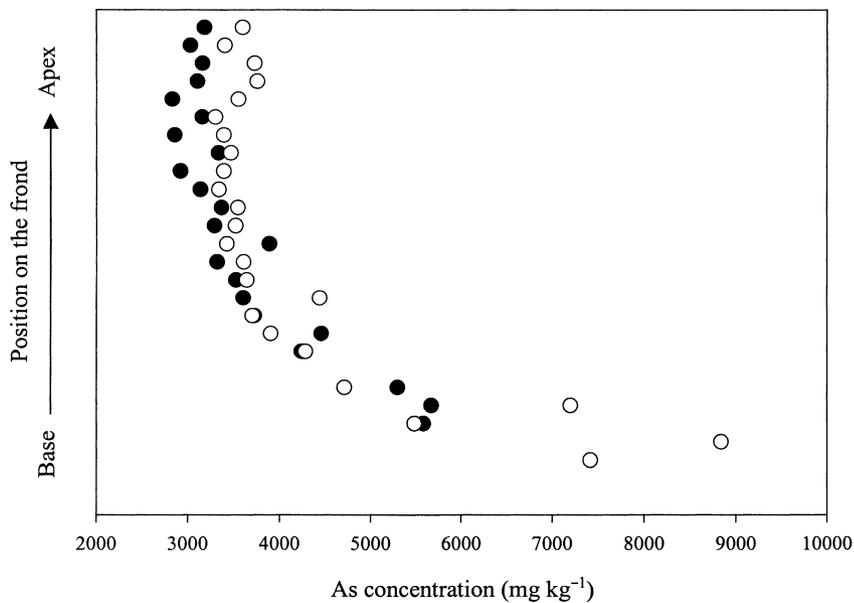
Fig. 1 Concentration of As in the pinnae along the midribs of two fronds. Open/closed symbols represent left and right pinnae.
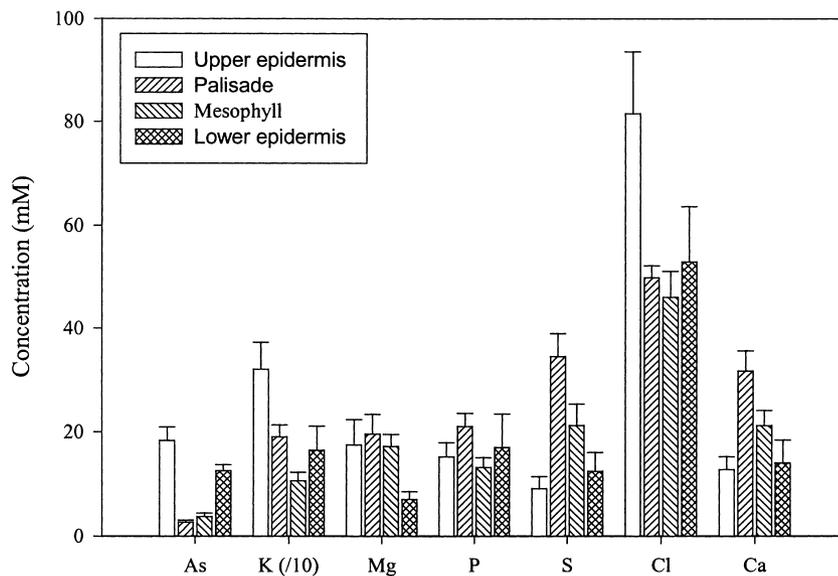
Fig. 2 Concentration of As and other selected elements in different cell types as determined by EDXA.

weight of the fronds, they accounted for only 4% of the As content of the above ground biomass.

The analysis of the different pinnae along each frond midrib showed that the concentration of As was highest in the basal pairs of pinnae (Fig. 1). The concentration of As increased from about 3000 mg kg^{-1} in the most apical pinnae to 6000–9000 mg kg^{-1} in the basal pinnae.

In mature pinnae, most of the weight (78%) was represented by the lamina but the weight of the spores was however, significant (Table 1). The concentration of As in the spores was similar to that in the midribs and therefore much lower than in the lamina. The great majority of As (98%) was contained in the lamina with only 2% in the reproductive tissue.

Since spores are likely to be dispersed in the environment it is advantageous that only a very small proportion of the As accumulated by the fern is contained in this part of the frond.

Energy dispersive X-ray microanalyses (EDXA)

The concentration of As in the pinnae used for EDXA analysis was 7200 ($\pm$ 300) mg As kg^{-1} d. wt. EDXA analysis showed that the distribution of different elements was not uniform between epidermis (upper and lower), palisade and spongy mesophyll (Fig. 2). Arsenic was significantly ($P < 0.001$) more abundant in the upper and lower epidermal cells (18 and 13 mM, respectively) than in the palisade and

spongy mesophyll (2.6 and 3.7 mM, respectively). EDXA also provides the determination of other elements, with atomic weight larger than Ne, present in large amounts in the leaves. Potassium and Cl were found to be in larger concentrations in the upper epidermis than in the other tissues of the pinnae. Magnesium and P were evenly distributed across the different tissues analysed, whereas S and Ca were more abundant in cells of the mesophyll and palisade than the epidermis. A significant positive correlation was found between the concentrations of As and K at cellular level in the upper epidermis (As = 4.4 + 0.043 K; $n = 25$, $R^2 = 0.75$, where As and K concentration are in mM).

In the plants grown in nutrient solution containing 500 μM arsenate, toxicity symptoms, in the form of necrotic areas, started to appear at the margins of the pinnae. EDXA analyses of pinnae sections revealed a high concentration of As in these damaged areas. As mentioned above, dot-maps of As can be collected only when the concentrations of these elements in the tissues are elevated. We analyzed the margins of pinnae that had high concentrations but did not show any evident toxicity symptoms (Fig. 3a). The dot-maps of As and K distribution (Fig. 3b,c) confirmed that these elements were localized preferentially in the upper and lower epidermis cells. Both elements appeared to be evenly distributed within the cells. The EDXA analyses of Ca distribution, in agreement with the data presented in Fig. 2, revealed that this element was more concentrated in the palisade and mesophyll than in the epidermis (Fig. 3d). In contrast with observations for other elements, Si was localized in the external cell walls of the upper and lower epidermis (Fig. 3e).

EDXA analysis of epidermis at high magnification further shows that As was localized mainly inside the cells, and not in the cell walls (Fig. 4). Because vacuoles occupy the majority of the cell volume in mature leaf cells, the distribution pattern shown in Fig. 4 suggests that As was stored mainly in the vacuoles.

X-ray absorption near edge spectroscopy analyses (XANES)

The K-edge XANES spectra for the As(III) and the As(V) oxide standards are shown in Fig. 5(a), along with the XANES spectrum for the plant sample. The plant edge is at an energy that is midway between the edges of the two standards, and the plant spectrum also contained a second peak that corresponds to the As(V) peak. Thus the plant contains a mixture of As(III) and As(V). A simple spreadsheet algorithm was used to proportion the standards spectra and compare the result with the plant spectrum, as shown in Fig. 5(b). The best match was a mixture of 75% As(III) and 25% As(V). At this ratio, both the edge position of the plant sample and the peak for As(V), coincided in energy with the model. The differences in the height of the peaks between the plant sample and the standards may be due to hydration of As species in the plant samples (Webb *et al.*, 2001).

Discussion

The majority of As accumulated by *P. vittata* is in the above ground biomass. Ma *et al.* (2001) reported that up to 93% of the total As accumulated was concentrated in the fronds. In this study we investigated the As distribution within the fronds and at cellular level in the pinnae. The concentration of As in the lamina of pinnae was found to be much greater than in midribs and spores. In particular, the pinnae at the base of the midribs had a greater concentration than those located in the central and apical part. This may be related to the development of the pinnae as those at the base are the oldest. The reproductive parts (spores) accumulated little As. Similarly, Zn and Cd were found to be largely excluded from flowers in the hyperaccumulator *A. halleri* (Küpper *et al.*, 2000).

At cellular level the As distribution in the pinnae of *P. vitatta* follows a pattern observed in the leaves of other hyperaccumulator plants. Similar to Zn in *Thlaspi caerulescens* (Küpper *et al.*, 1999) and Ni in *Alyssum lesbiacum*, *A. bertolonii* and *T. goesingense* (Küpper *et al.*, 2001), As was preferentially accumulated in the upper and lower epidermis cells in *P. vittata* (Figs 2 and 3). The As distribution was different from the pattern observed for other elements such as Mg, S and Ca. However, As and K in the upper epidermis cells were found to correlate positively. It is not clear whether such a correlation can be explained by the role that K$^+$ could have in counter-balance As anions in the vacuole. However, at vacuolar pH arsenite (pKa 9.2) should be undissociated and requires no cations (other than the undissociated protons) for charge balance, unless it forms negatively charged complexes.

X-ray microanalyses also revealed that As was localized in the central intracellular portion of cut cells. Since the majority of this area is occupied by the vacuole, this suggests that As was mainly contained in the vacuoles (Fig. 4). This is supported by the differences between the distribution of Si and As (Fig. 3). Both elements are mainly concentrated in the epidermal cells but Si appears to be localized in the external cell walls whereas As is more evenly distributed in the epidermal cells. This finding is in agreement with observed distributions of heavy metals in hyperaccumulating plants and indicates that compartmentalization of both metals and metalloids in cell vacuoles plays a key role in terms of metal/metalloid tolerance in these plants.

The XANES analyses showed that about 75% of the As contained in the fronds was in the As(III) oxidation state, and the remaining part was present as As(V). These results confirmed the findings of Ma *et al.* (2001) and Francesconi *et al.* (2002) who found similar proportions of arsenite and arsenate in *Pteris vittata* and *Pityrogramma calomelanos*, respectively. These authors used water/ methanol or water to extract frond samples and HPLC-ICPMS to separate As species in the extracts. This nonphysiological extraction may alter arsenic speciation in tissues. However, using a physiologically
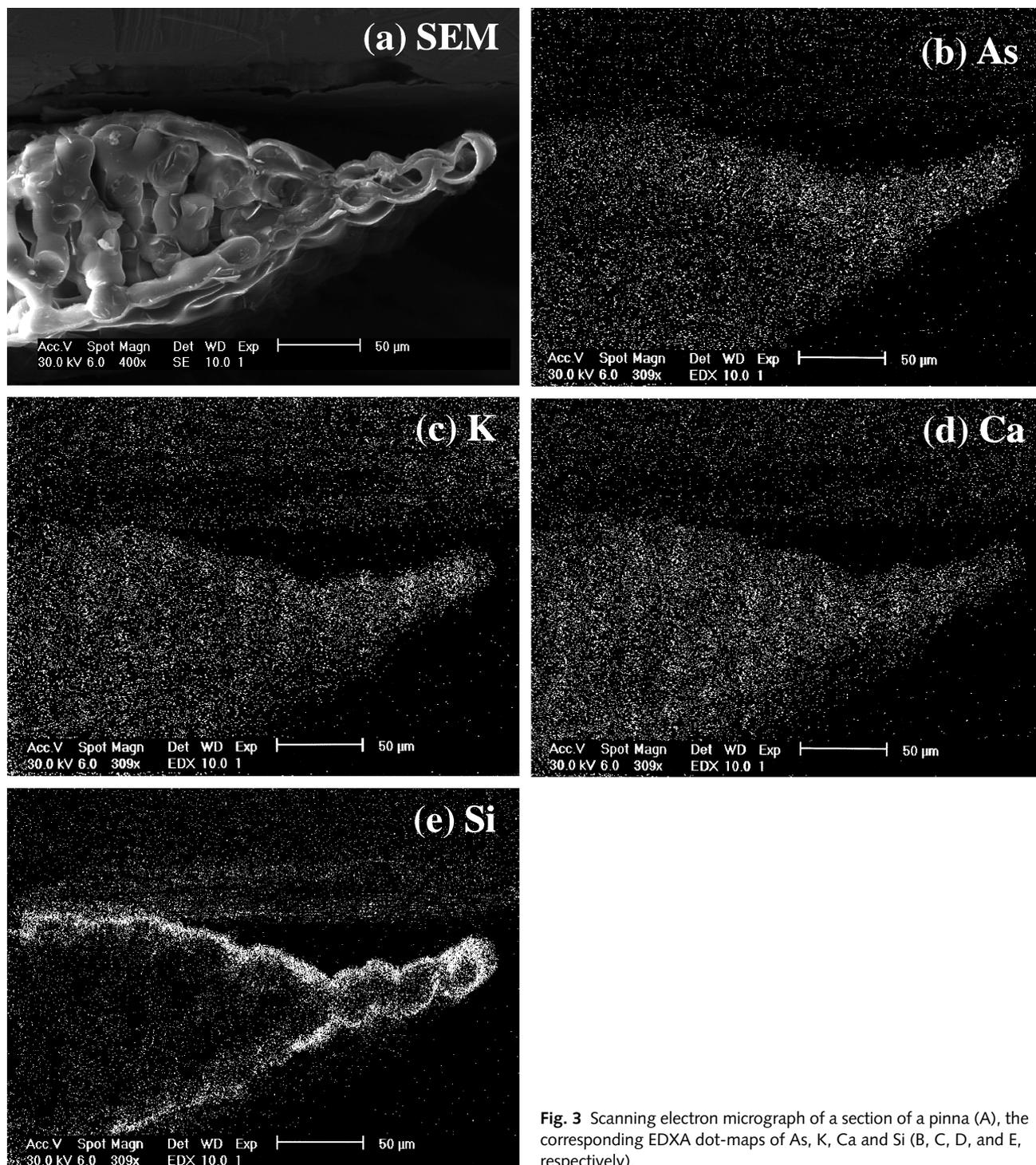
Fig. 3 Scanning electron micrograph of a section of a pinna (A), the corresponding EDXA dot-maps of As, K, Ca and Si (B, C, D, and E, respectively).

more relevant extraction (a phosphate buffer at pH 5.6), Wang *et al.* (2002) showed that > 85% of the As extracted from the fronds of *P. vittata* was in the form of arsenite, and the remaining mostly as arsenate. Francesconi *et al.* (2002) also reported that the largest percentage of arsenate was found in senescent fronds. In mature fronds, such as those used in our work, it is possible that some of the As was re-oxidized to As(V). This is consistent with C. Tu *et al.* (unpublished) who investigated As speciation in fronds of *P. vittata* growing in a soil spiked with 50 mg As kg^{-1} as sodium arsenate. They reported
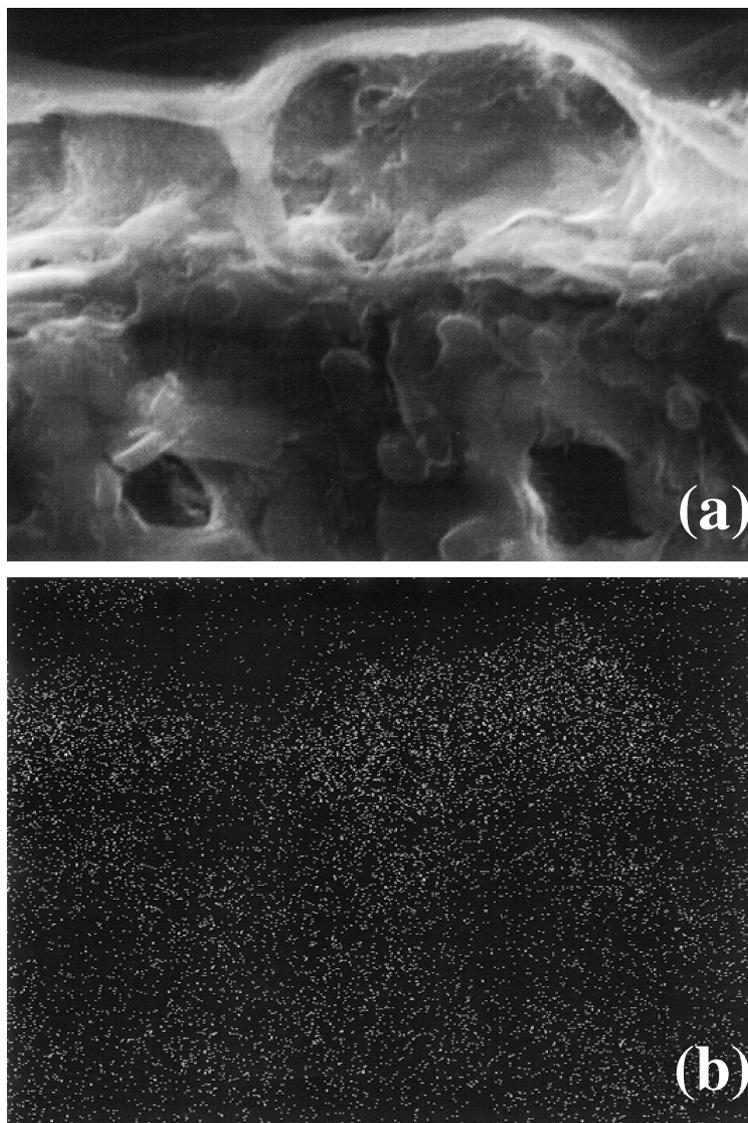
Fig. 4 Scanning electron micrograph of epidermal cells
(a), and the corresponding EDXA dot-map of As (b).

that essentially all As in young fronds was present as As(III), whereas 27–38% of the As in mature to old fronds was present as As(V), that is re-oxidation of As(III) occurs as fronds mature.

Reduction of arsenate to arsenite in plants has been reported by several other authors and appears to be related to mechanisms of tolerance due to the interference of arsenate with P mediated processes and metabolism (Delnomdedieu *et al.*, 1994; Mattusch *et al.*, 2000; Pickering *et al.*, 2000). However, arsenite is also toxic to plants due to reaction with sulfydryl groups of enzymes and proteins (Ullrich-Eberius *et al.*, 1989). Therefore, the compartmentation in the vacuoles, which we have observed using EDXA, may represent an efficient strategy to maintain a low As concentration in the cytoplasm. Another mechanism linked to As tolerance appears to be complexation of As(III) by phytochelatins (PCs). For instance, Pickering *et al.* (2000) reported that As(III) in

Brassica juncea is co-ordinated with three sulfur groups. Ha *et al.* (1999) showed that an *Arabidopsis thaliana* mutant that does not produce PCs was significantly more sensitive to As toxicity than the wild type. In consideration of this and other evidence, Meharg & Hartley-Whitaker (2002) proposed that As-PC complexes may be stored in the vacuoles where the acidic conditions are favorable for the stability of such complexes. The EDXA analyses did not reveal correlations between As and S and therefore it is unclear whether complexation of As by PCs and vacuolar storage of As-PC complexes are involved in As tolerance in *P. vittata*.

Acknowledgements
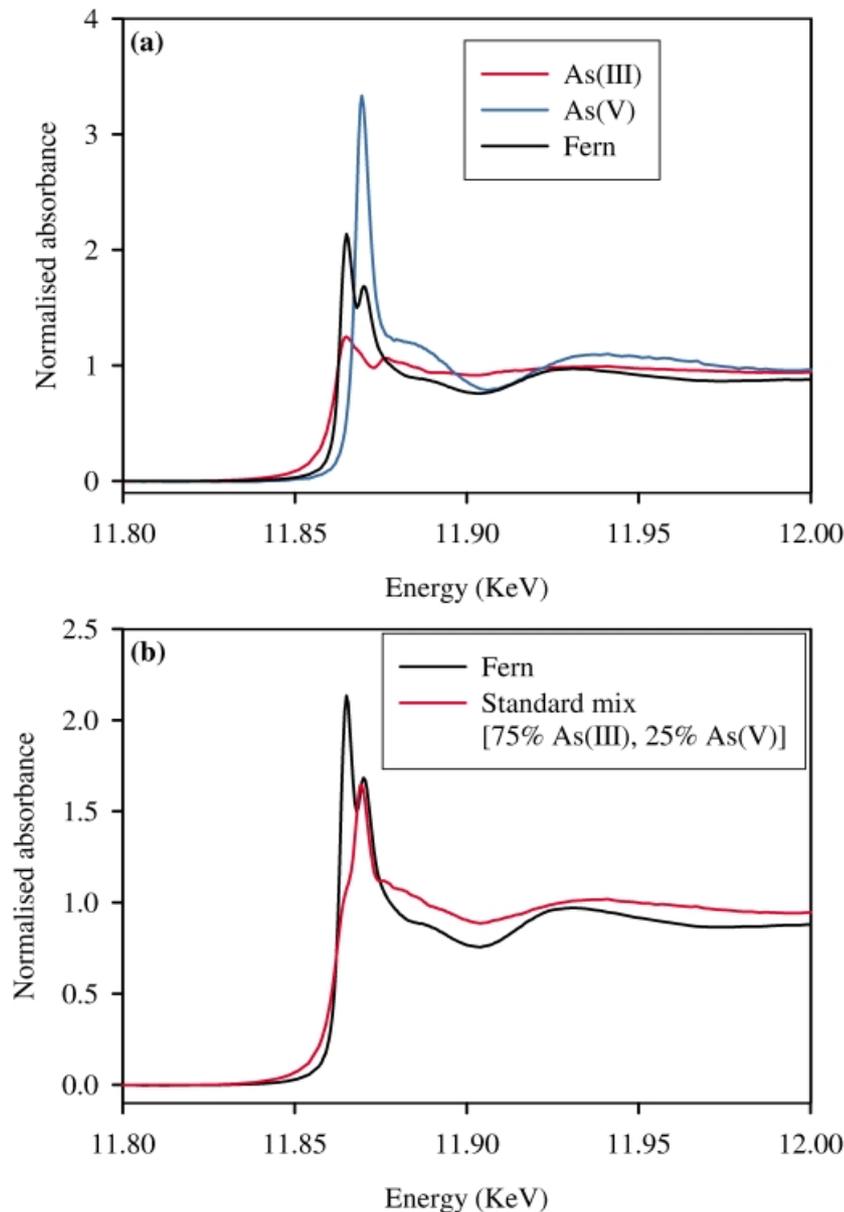
Fig. 5 XANES spectra of As(V) and As(III) standards and of freeze-dried fronds (a) and of a standard mixture of arsenite/arsenate (75/25 W W^{-1}) and freeze-dried fronds (b).

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