Journal of Bioscience and Bioengineering
VOL. 111 No. 3, 326–332, 2011

www.elsevier.com/locate/jbiosc

Effects of cultivation conditions on the uptake of arsenite and arsenic chemical species accumulated by *Pteris vittata* in hydroponics

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Received 17 August 2010; accepted 10 November 2010
Available online 24 December 2010

The physiological responses of the arsenic-hyperaccumulator, *Pteris vittata*, such as arsenic uptake and chemical transformation in the fern, have been investigated. However, a few questions remain regarding arsenic treatment in hydroponics. Incubation conditions such as aeration, arsenic concentration, and incubation period might affect those responses of *P. vittata* in hydroponics. Arsenite uptake was low under anaerobic conditions, as previously reported. However, in an arsenite uptake experiment, phosphorous (P) starvation-dependent uptake of arsenate was observed under aerobic conditions. Time course-dependent analysis of arsenite oxidation showed that arsenite was gradually oxidized to arsenate during incubation. Arsenite oxidation was not observed in any of the control conditions, such as exposure to a nutrient solution or to culture medium only, or with the use of dried root; arsenite oxidation was only observed when live root was used. This result suggests that sufficient aeration allows the rhizosphere system to oxidize arsenite and enables the fern to efficiently take up arsenite as arsenate. X-ray absorption near edge structure (XANES) analyses showed that long-duration exposure to arsenic using a hydroponic system led to the accumulation of arsenate as the dominant species in the root tips, but not in the whole roots, partly because up-regulation of arsenate uptake by P starvation of the fern was caused and retained by long-time incubation. Analysis of concentration-dependent arsenate uptake by *P. vittata* showed that the uptake switched from a high-affinity transport system to a low-affinity system at high arsenate concentrations, which partially explains the increased arsenate abundance in the whole root.

[**Key words:** *Pteris vittata*; Hydroponics; Arsenic species; Long time incubation; Arsenite oxidation]

Arsenic is a carcinogenic and mutagenic element, and is therefore detrimental to human health. It exists naturally in the soil and groundwater of many countries worldwide (1–4). In particular, arsenic contamination of groundwater poses a serious health risk in Asian countries such as Bangladesh, India, China, and Thailand. In many of these countries, as much as 90% of the drinking water comes from groundwater. Moreover, the arsenic concentration in groundwater, which has been measured at a maximum of 3 mg/L, is much higher than the guideline concentration of 10 μg/L, as determined by the World Health Organization (5–8). Ingestion of arsenic through drinking water or crops that are grown on arsenic-contaminated land is, therefore, of great concern for human health.

Recently, the arsenic hyperaccumulator *Pteris vittata* (Chinese brake fern) was found in arsenic-contaminated land in Florida, USA. This plant species accumulates large amounts of arsenic—up to 2% of its dry weight—with more than 90% of the accumulated arsenic in the aboveground tissue (9). Thus, *P. vittata* has attracted interest for phytoremediation (phytofiltration), which is a new technology for the treatment of arsenic-contaminated land and water. However, a few questions remain regarding arsenic treatment using this fern in hydroponics.

Arsenic reportedly occurs predominantly in the environment as the pentavalent arsenic form (As(V)), arsenate, which resembles phosphate in chemical form, and the tetravalent arsenic form (As(III)), arsenite, which is a reduced form of arsenate. Arsenate uptake by the fern was dependent on P starvation, and was thought to occur via the phosphate transport system (10), as was observed in other plants (11). On the other hand, arsenite uptake was not dependent on P starvation (10), and the uptake rate of the fern was slower than with arsenate. However, it was also reported that there was no significant difference between the amount of arsenic accumulation in the fern when exposed to arsenite and that when exposed to arsenate under a hydroponic system, and that *P. vittata* could efficiently remove arsenic from arsenic-contaminated groundwater (12,13), in which arsenite is more common due to anaerobic conditions (14). Therefore, it is assumed that arsenite oxidation occurs during arsenic uptake in hydroponics.

To date, *P. vittata* has also been extensively studied with respect to arsenic speciation in the fern, as this information helps us to understand the mechanism of arsenic detoxification, which is essential for its hyperaccumulation. Arsenic speciation analyses were conducted using various analytical methods, such as arsenic extraction followed by high-pressure liquid chromatography/inductively coupled plasma mass spectrometry (HPLC/ICP-MS) (15,16) or X-ray absorption spectroscopy techniques (17,18). Previously, non-destructive extended X-ray absorption fine structure (EXAFS) analysis

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doi:10.1016/j.jbiosc.2010.11.004

of *P. vittata* grown in soil showed that the fern predominantly accumulated arsenic in the pinnae as arsenite and a negligible amount of thiol-coordinated arsenic with an oxidation state of +3 (18). These results were confirmed by other analytical methods (15). On the other hand, it was reported that arsenate predominately existed in the roots (16,19,20), while another report found that arsenite was the most abundant arsenic species in the roots (21). It was previously observed that long-term incubation with a hydroponic system led to leaf senescence and a change in the arsenic species in the root tips (22). However, the reason for the change in arsenic species, arsenite and arsenate abundance in the fern roots is unknown.

Chemical transformations, both outside and inside of the roots, are very important for efficient arsenic removal by the hyperaccumulator, *P. vittata*. This is because arsenite transport is slower than arsenate transport by the fern, and therefore high arsenate abundance outside the roots provides an advantage for the efficient removal of arsenic from the external environment to the internal roots. Similarly, it has been suggested that arsenite is highly efficient for the xylem transport in the *P. vittata* (21) which is a key step for arsenic translocation into the shoots and for a high translocation factor ($[As]_{shoot}/[As]_{root}>1$), a characteristic of hyperaccumulators (23). In the present study, arsenite uptake under anaerobic and aerobic conditions was compared. The arsenic species were also investigated in two plant sites (the root tips and rachis) in two ferns of the same strain that were incubated for different periods. In addition, arsenite abundance in the fern root and the rate of uptake, as well as indicators of P starvation of the plant, were investigated with respect to incubation time. The results of these experiments provide useful information on the feasibility and efficient use of the Chinese brake fern for phytoremediation (phytofiltration).

MATERIALS AND METHODS

Plant materials The *P. vittata* L. with 5 to 6 fronds used in the present study were kindly donated by Fujita Co., Ltd. First, the ferns were planted in a mixture of sand and peat moss in a 9:1 volume ratio, and were allowed to grow for two weeks. Each fern was then transferred to a 350-ml vessel and incubated in 200 ml Hoagland medium (24) with shaking at 200 rpm. The medium was changed every 4 days. The vessels were wrapped with Al foil to prevent the roots from being exposed to light. Each fern was placed in a growth chamber with a 16 h light period, at 25°C/20°C and 70% relative humidity for the duration of the experiment.

Arsenite uptake under aerobic or anaerobic conditions Arsenic uptake under anaerobic or aerobic conditions has been investigated with respect to rice in paddy fields (23,25,26). In flooded soil, oxygen diffusion from the atmosphere into the soil decreases drastically, and the remaining free oxygen is consumed by microorganisms, which results in anaerobic conditions and arsenite predomination in the soil. In aerobic soil conditions, arsenite is oxidized to arsenate by inorganic compounds such as iron hydroxides/oxides, and, therefore, arsenate predominates (23). In the case of the fern used to treat arsenic-contaminated water, which is a terrestrial plant assumed to lack an oxygen supply to the roots via aerenchyma (23,27), aeration is conducted by the use of shaking vessels. Therefore, to create anaerobic conditions in the present experiments, N_2 was purged to remove O_2 from the medium to prevent arsenite oxidation. For the arsenic-uptake experiment, three independent ferns were chosen for hydroponic cultivation. The ferns were pre-cultured in Hoagland medium with various concentrations of phosphate (10 μM, 100 μM, and 1 mM) for two weeks to prepare ferns with different degrees of phosphorus (P)-starvation. After pre-cultivation, the ferns were incubated in a medium [5 mM 2-(N-morpholino) ethanesulfonic acid (MES) (pH6.0), 0.5 mM $CaCl_2$] that included arsenite at a final concentration of 15 μM. For anaerobic cultivation, the medium was degassed with N_2, which was also used for purging during the uptake experiment. In the arsenic-uptake experiments, the measurement was initiated when the arsenic was added to the medium with an immersed fern. Evaporation of medium during the uptake experiment was monitored, in order not to influence the arsenic concentration in the medium. The incubation conditions were as follows: temperature, 25°C; illumination, 22,000 lx; N_2 gas rate, 10 ml/min; aeration, reciprocal shaking at 120 rpm (Fig. 1A).

Arsenite oxidation experiment A three-month-old fern was pre-cultured for 2 weeks in a nutrient solution containing 100 μM Pi. The fern was then washed and transferred to 5 mM MES (pH6.0) buffer supplemented with 20 μM arsenite, and incubated for 80 h. Various samples, such as a normal fern root, a dried root, a culture solution incubated with a fern (culture solution), a culture solution that had been filtered through a 0.22-μm nylon membrane, a boiled culture solution, and a culture solution with 0.2 mM EDTA were incubated with 20 μM arsenite for 24 h.

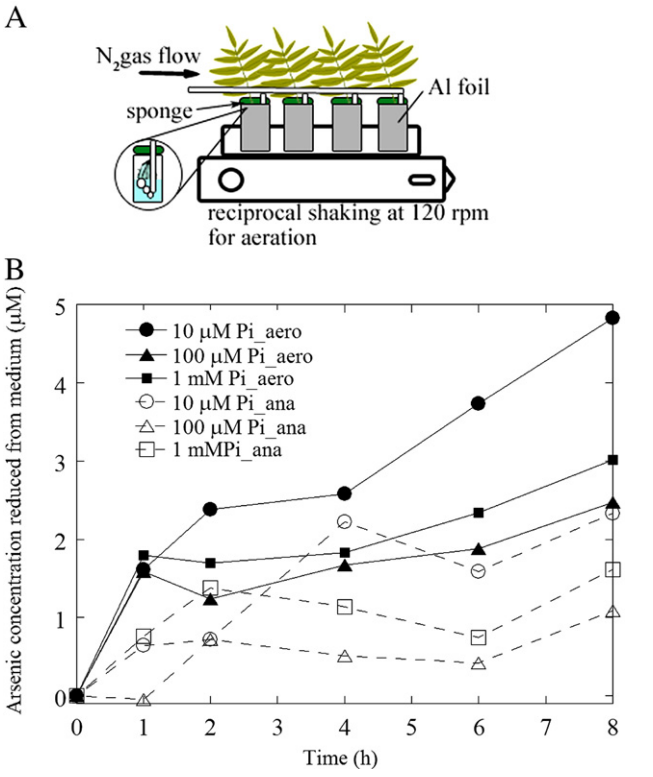

FIG. 1. (A) Diagram for the set-up of arsenite uptake experiment. The uptake experiment under anaerobic conditions was carried out by purging the medium with N_2 gas to prevent arsenite oxidation to arsenate during the uptake experiment. The fern was pre-incubated with 10 μM, 100 μM, or 1 mM phosphate in the nutrient solution to create different degrees of P-starved ferns. Data represents one of two determinations. (B) Arsenite uptake by *P. vittata* under aerobic conditions (10 μM Pi, filled triangle; 100 μM, Pi filled circle; 1 mM Pi, filled squares) and anaerobic conditions (10 μM Pi, open triangle; 100 μM Pi, open circle; 1 mM Pi, open squares). Incubation conditions were as follows: temperature, 25°C; illumination, 22,000 lx; N_2 gas rate, 10 ml/min; aeration, reciprocal shaking at 120 rpm, as described.

Inductively coupled plasma mass spectrometry (ICP-MS) Prior to arsenic analysis, the culture medium and plant samples were treated as follows. For the culture medium, 200 μl of medium was added to 25 ml of milliQ-water (18.3 mV cm^{-1} resistivity). The diluted medium was then filtered through a 0.45-μm (pore size) nylon membrane filter. The plant samples were dried at 60°C for two days. The dried plant samples were digested in concentrated HNO_3 (10 ml) at 130°C for 20 min. H_2O_2 was then added at a final concentration of 1 mM, and the samples were digested until the plant debris was eliminated. The extracts were diluted with milliQ-water. Each of the diluted samples contained an internal standard, which was yttrium at a final concentration of 10 ppb in 3% HNO_3 (final concentration). The total arsenic concentration was measured using an ICP-MS ELAN900 (Perkin-Elmer, Shelton, CT).

X-ray absorption spectroscopy (XAS) measurement The chemical state of arsenic in the living plant samples was determined using X-ray absorption near edge spectra (XANES) and EXAFS at the As K absorption edge. The XAS experiments were performed with the BL-12C in the Photon Factory (PF), High Energy Accelerator Research Organization (KEK), Tsukuba, Japan (Proposal No. 2007U005). The experimental conditions used in this analysis were as follows: an incident beam monochromized by a Si(111) double crystal was focused to ca. 1 mm × 1 mm using a bent cylindrical mirror, and its intensity was monitored using a N_2 gas flow ionization chamber. XAFS spectra at the As K absorption edge were measured in the fluorescence yield mode, using a multi-element solid-state detector to count the fluorescent X-rays emitted from the samples.

XAS measurements were obtained from a single point of the midrib, the root tips, which were newly synthesized and white in color, and the root tops, 1 cm below the boundary of roots and shoot. Samples of the ferns were prepared as described above. The ferns were hydroponically cultivated in Hoagland medium supplemented with 300 μM arsenate and no phosphate for either two weeks or two months. The roots were then rinsed with 1 L of milliQ-water and blotted dry three times. At least 3 fine living roots were combined to be irradiated with X-rays, and were placed on the center of an acrylic sample folder without being cut from the ferns. Arsenic accumulation in *P. vittata* was confirmed by measurement of the arsenic concentration in the pinnae of the fronds using ICP-MS. As a negative control, one fern was hydroponically cultivated