

V. 6. PIXE Study on Absorption of Arsenate and Arsenite by Arsenic Hyperaccumulating Fern (*Pteris vittata*)

Yamazaki H.¹, Ishii K.², Matsuyama S.², Kikuchi Y.², Takahashi Y.², Terakawa Y.², Kawamura Y.², Yamanaka K.², Watanabe M.², Tuboi S.², Satoh T.³, and Inoue C.³

¹Cyclotron and Radioisotope Center, Tohoku University

²Graduate School of Engineering, Tohoku University

³Graduate School of Environmental Studies, Tohoku University

Introduction

Arsenic contamination of soils and groundwater from various sources such as mines and urban wastes and wood preservatives is of serious environmental problem. A number of technologies for cleaning up arsenic-contaminated soils have been proposed¹⁻³⁾ Recently, environmentally friendly and low-input phytoremediation of several kinds of phytoextraction, rhizofiltration, phytovolatilization and phytostimulation has been proposed for remediation of soils contaminated with heavy metals and metalloids⁴⁾. Phytoextraction using an arsenic hyper-accumulator, *Pteris vittata* L., has generated increasing interest worldwide due to its both environmentally sound and cost effectiveness⁵⁾. Although many studies have been conducted about the arsenic hyper-accumulator fern⁶⁻⁸⁾, the entry system of arsenic to *Pteris vittata* is not well understood and the mechanism of arsenic accumulation by this plant is not clear at this time. To develop practical application of the arsenic accumulator fern, it is necessary to explicate the accumulation mechanism based on elemental translocation using an in-vivo analysis of representative parts of the fern cultivated under various conditions of different arsenic oxidation states and different. This study shows the *in-vivo* uptake of arsenate (As(V)) and arsenite (As(III)) by a hydroponic culture of *Pteris vittata* using both an in-air submilli-PIXE for different parts of the fern and an in-air micro-PIXE

Material and Methods

P. vittata L., a perennial and pinnate fern which grows worldwide in a dried area with long sunshine duration, was used throughout the experiment. In our experiments, fern

seedlings were used three months after spore germination. In this stage of growth, a rootstock of fern was in several fronds which have not opened completely. The roots were washed carefully in tap water to remove soil particles. The seedlings were then transferred to hydroponic water containing nutrients in concentrations of 8.10 mg/dm³ KNO₃, 9.50 mg/dm³ Ca(NO₃)₂·4H₂O, 5.00 mg/dm³ MgSO₄·7H₂O, 1.15 mg/dm³ NH₄H₂PO₄, 15 µg/dm³ NaFe-EDTA, 3.0 µg/dm³ H₃BO₃, 1.8 µg/dm³ MnCl₂·4H₂O, 0.2 µg/dm³ ZnSO₄·7H₂O, 0.05 µg/dm³ CuSO₄·5H₂O and 0.02 µg/dm³ Na₂MoO₄·2H₂O. The nutrient solution was aerated continuously. The seedlings were grown on the hydroponic solution for different periods more than one month in a growth chamber with a 16 hours light period, 25°C/20°C of day/night temperature and 70% relative humidity.

After a desired period of cultivation on hydroponic solutions, the roots were washed well in tap water. The fern frond of selected growth stage was placed in 100 ml conical beaker filled with 100 ml uptake solution of the same nutrient concentration as culture solution. Then arsenic in the form either As(V) as sodium arsenate (Na₂HAsO₄) or As(III) as sodium arsenite (NaAsO₂) was added to the uptake solution to be the final concentration of 23 to 50 mg-As per liter. In order to compare the effect of phosphate, NH₄H₂PO₄-free nutrient solution was also used for several arsenic-uptake experiments. The treated ferns were incubated in the growth chamber at the same operating condition as above for one day. After 24 hours incubation, total weight of the beaker was measured to determine the amount of transpiration by the fern. Then the roots were washed and the fern sample was placed in 100 ml of fresh solution with the same concentrations of nutrients and arsenic as above. This cycle was repeated for more than 5 days. Arsenic concentrations of the uptake solutions were determined by ICP-MS (Hewlett Packard, HP-4500).

Two kinds of in-air PIXE measurements of several tens mm² and several hundreds µm² sized areas were performed by using the in-air submilli-PIXE camera and the in-air micro-PIXE camera at Tohoku University, Japan⁹⁻¹¹). A part of lamina of *P. vittata* was plucked at three different growth stages; an early active stage at which all leaves have just opened, although the leaves are not opened completely at the beginning stage of growth, a middle active stage at which the area of an apex has served as maximum, and the mature growth stage at which all pinnas have grown most. In the in-air submilli-PIXE analysis, 3 MeV proton beams of beam current ~800 pA and <0.5 mm beam spot were scanned on a surface area of 10×20 mm² of a selected fern frond which was fixed to a target frame just after the beam exit window of 12.5 µm thick Kapton film. The plant sample was kept

alive by dipping the stalk part into a hydroponic solution. The distance from the beam exit window and a sample is around 5 mm. The X-ray energy and the beam position were simultaneously measured in order to obtain spatial distribution of elements. For elemental mapping on cells of different plant tissues by the in-air micro-PIXE camera, a sliced fern section with moisture was mounted between two polycarbonate films of 5 μm thickness which were glued to sample holder, and $50 \times 50 \sim 400 \times 300 \mu\text{m}^2$ sized areas were analyzed by a proton beam with a diameter of typically $1.5 \times 1.5 \mu\text{m}^2$ and energy of 3 MeV at beam currents of approximately 100 pA. In both submilli- and micro-PIXE analyses, X-rays from targets were measured with two Si(Li) detectors; No.1 detector (7.5 μm thick Be window, 10 mm^2 active area) with a low geometric efficiency is well suited for detection of an element of low atomic number, and No.2 detector (12.5 μm thick Be window, 60 mm^2 active area) with a 100- μm Mylar absorber allows detection of X rays $> 4 \text{ keV}$ and the removal of recoil protons. The list mode data acquisition system can sort the data for a selected element / energy region and generate an elemental image even while the data are accumulated. This resulted in a decrease of the dead time of signal processing. Quantitative PIXE analysis was performed using the GeoPIXEII software¹²⁾.

Results and Discussion

Many plants living on a soil contaminated by harmful elements usually maintain the vital activity in such a way that the absorption of harmful elements is blocked on the roots and the epigeal leaves remain free from harmful effects. On the other hand, *P. vittata* fern accumulate arsenic in a very high concentration harmful to the vital activity of the epigeal fronds without blocking arsenic on the roots, indicating a detoxication mechanism of arsenic in the lamina part of ferns¹³⁾. Because of the similarity in chemical forms between orthophosphate and arsenate, the transportation route of phosphorus can be utilized for absorbing arsenate by the fern.

Figure 1 shows a time course of arsenic concentration in hydroponic culture solution with addition of 23 mg/dm^3 arsenic. In this experiment, ferns of the beginning growth stage were treated for two weeks using hydroponics of 1/10 or 1/100 of the ordinary phosphate concentration (1 mM) in the hydroponic culture solution. The ferns under different phosphorus deficiency conditions and the fern under ordinary phosphorus concentration were moved to phosphorus-free hydroponic solutions with the same concentration of arsenic. Arsenate in similar chemical form to orthophosphate was more

rapidly transferred into the plants under heavier phosphorus deficiency. The genetic expression of phosphorus transportation is reported for the roots of *Capsella bursapastoris* and *Lycopersicon esculentum* corresponding to a phosphate deficiency condition in cultivation. It is considered that a transportation route of phosphorus in *P. vittata* participates in absorption of arsenate. On the other hand, uptake of arsenite for initial 15 min was very quick regardless of phosphate concentrations, indicating different absorption path of arsenite from phosphorus transportation in the fern. After two hours, arsenite was oxidized to arsenate, which was confirmed using liquid chromatography, and the arsenic uptake rate changed greatly with the phosphorus deficiency states of ferns. It is inferred that arsenite influx into the roots of *P. vittata* is much greater than arsenate influx. In addition to this, the oxidation state of arsenic in the fern at early active stage of growth was determined using X-ray absorption near edge structure (XANES) analyzer (Rigaku, R-XAS Looper) with the X-ray tube worked at 23 kV-80 mA and the analyzing crystal of Si (620). These measurements indicated that arsenate ions in hydroponic solution directly entered from the roots of *P. vittata* and were quickly translocated to the epigeal fronds. Moreover, this transported arsenate was reduced to trivalent state and accumulated in the lamina part of the fern.

Three kinds of fern samples of which the growth stage differs were moved to a culture solution included by arsenic 50 mg/dm^3 , the absorption experiment of arsenic was conducted for five days, and the element distribution on each terminal pinna was measured by the submilli-PIXE camera. Figure 2-1 shows the elemental mapping for a fern apex in early stage of growth under the arsenite treatment, and Fig. 2-2 shows the results under the arsenate treatment. A measurement domain was $10 \times 20 \text{ mm}^2$, and elemental concentrations were displayed as a function of the position in the rectangular portion of a 4 mm width on the leaf. The concentrations were derived from the relevant X-ray yields integrated on the rectangle segments on a leaf based on the assumption of the leaf matrix of $100 \text{ }\mu\text{m}$ thickness in composition of 80% H_2O and 20% $\text{C}_4\text{H}_6\text{O}_3$ with the density of 1 g/cm^3 13).

Since 1 mM orthophosphate was contained in the culture solutions, a slow uptake rate of arsenate resulted in low arsenic concentration profile on the apex of fern exposed to arsenate contamination. However, there was no difference in the elemental distribution for two ferns on culture solutions containing different chemical forms of arsenic. That is, potassium and calcium were almost uniformly distributed over whole stenophyllous apex,

except for disordered leaf matrix of the venation in the middle of the apex. Arsenic, irrespective of the oxidation states, was transported to the leaf edge distant from the vascular bundle acting as a conducting tissue of elements. Although the results are not shown due to the lack of space, the distribution of K and As did not change when the fern progressed to more mature stage. However, calcium was accumulated in some parts of the vein and some spots on an apex at high concentration.

Figure 3 shows the concentrations of K, Ca and As analyzed at the measurement domains of $10 \times 20 \text{ mm}^2$ on the apexes in three growth steps; the early active stage of growth, the middle one and the mature stage. The five-day uptake of arsenate increased remarkably, when orthophosphate was not contained in the culture solution, and arsenite was absorbed by the fern without the influence of orthophosphate. Although the result was not shown here, arsenic of pentavalent was extremely distributed in the edge of apexes under the phosphorous deficiency state. This finding indicates that a growth function of fern leaf changes under the condition of a phosphorous deficiency and it influences the arsenic transportation to the growth part. Although K concentration was uniformly high in the leaves at the early active and the middle active stages of growth, it decreased 1/7 to 1/5 in the mature stage of growth. Moreover, very high-concentration spots over $10,000 \mu\text{g/g}$ were detected for Ca on the vein portion and the arsenic-accumulated edge position of the leaf in the mature stage of growth.

Although a plant of active metabolism of growth requires much potassium to prepare the metabolic pathway of cells, this element is not needed so much in the mature stage of the growth to which metabolic activity decreases. Calcium is also largely required for the maintenance of a structure and a function of cell walls at the intense growth phase of cell division. In a low metabolic activity of mature plant, calcium ions combine with carbonate or oxalate ions and then are condensed in a vacuolar part. Especially when harmful organic acid exists superfluously, calcium is taken in high concentration by a plant mostly due to the antidotal effect. The knowledge about elements needed for keeping the metabolism activity of plants put an interpretation on change of elemental concentrations in the fern leaf shown in Fig. 3. That is, arsenic is taken into cells in the fern leaf of an active metabolism requiring a lot of potassium, and it is transported mainly to a perimeter part of low metabolism. In the grown-up leaf at which cell growth stopped, calcium is conveyed and utilized for detoxification of absorbed arsenic and conservation of a cell wall, but an excessive transported calcium is segregated in a part of venation and pinna of a fern.

Hydroponics of the fern in early stages of growth was carried out for one month on culture solutions which contained arsenate in the concentration of 25mg per liter and the nutritional elements except phosphorous. The in-air micro-PIXE analysis was carried out for a sliced vascular bundle of fern lamina in $50 \times 50 \mu\text{m}^2$ area (Fig. 5) and for a sliced mesophyll of fern pinna in $300 \times 400 \mu\text{m}^2$ area (Fig. 6). The distribution of essential elements such as K and Ca revealed that the vascular bundle of the fern was formed in many small cells of only a size of 10 to 20 μm . Arsenic was also distributed over the vascular bundle cells along with Mn, Fe, Cu and Zn added as a nutrient to the culture solution. These results suggest that arsenic, like other ingredients, is originally conveyed with water and passes through a conductive tissue of the fern pinna. The elemental mapping of a sliced mesophyll showed that arsenic and potassium were highly distributed over the front part of a pinna in the sun light and on the contrary sulfur and calcium were distributed over the back side of the leaf at rather high concentrations. It is probable that a high concentration of potassium is required to adjust the metabolism of cells in the mesophyll of pinna which actively uptakes arsenic unnecessary for vital activity of a leaf. On the other hand, it is considered that the high concentration of arsenic results in change to the function of a cell wall, and then calcium diffuses out from those cells. In the conductive tissue in a vascular bundle and the mesophyll cells of a pinna of the fern, arsenic is not condensed into a certain specific tissue, but is uniformly distributed over the cells in each tissue. Therefore, the specific organization which detoxifies arsenic does not exist in *P. vittata* fern, but arsenic is accumulated in the vacuole which accounts for a big volume fraction in a plant cell and then arsenic distribution becomes uniform in the tissue of fern fronds.

Conclusion

The centimeter-scale area mapping of elements using the in-air submilli-PIXE camera reveals elemental distribution of living fronds of *P. vittata* fern, and the micrometer-scale area mapping using the in-air micro-PIXE camera indicates the translocation of elements in living tissue of the fern. Hence, the in-air PIXE analysis is an effective measure for undertaking phytoremediation research. Since *Pteris vittata* absorbs a lot of arsenic into the fronds, it is most effective in arsenic phytoextraction to mow the fronds of well growth stage on a polluted soil. The perennial fern is repeatedly applicable to the decontamination of an arsenic polluted land and is effective irrespective of the

valence state of arsenic. However, the rate of absorption of arsenate (pentavalent) from a polluted medium with sufficient concentration of phosphorus becomes slow compared with the rate of absorption of the arsenite (trivalent). The translocation of essential elements for plants like potassium and calcium occur in the fern fronds which absorbed a lot of arsenic, and then the vital activity of the fern is maintained. The tissue morphology of the fern fronds is also kept in satisfactory condition, and arsenic is distributed in the lamina cells of *P. vittata* ferns at all growth stages.

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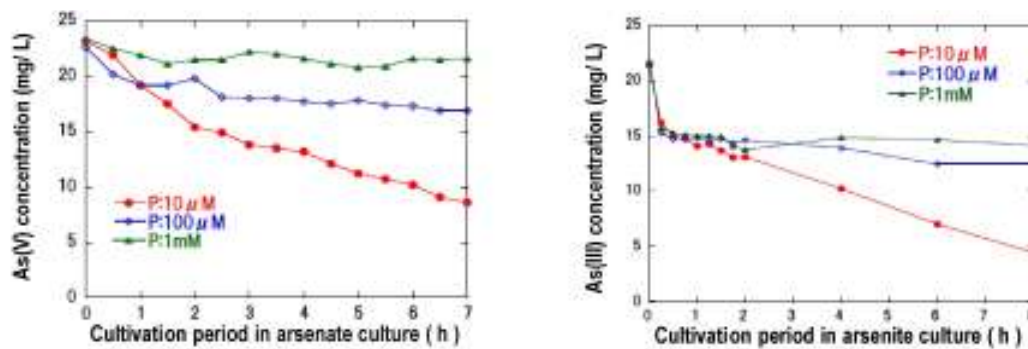


Figure 1. Influence of phosphate deficiency in cultivation on arsenic absorption rates by the fern.

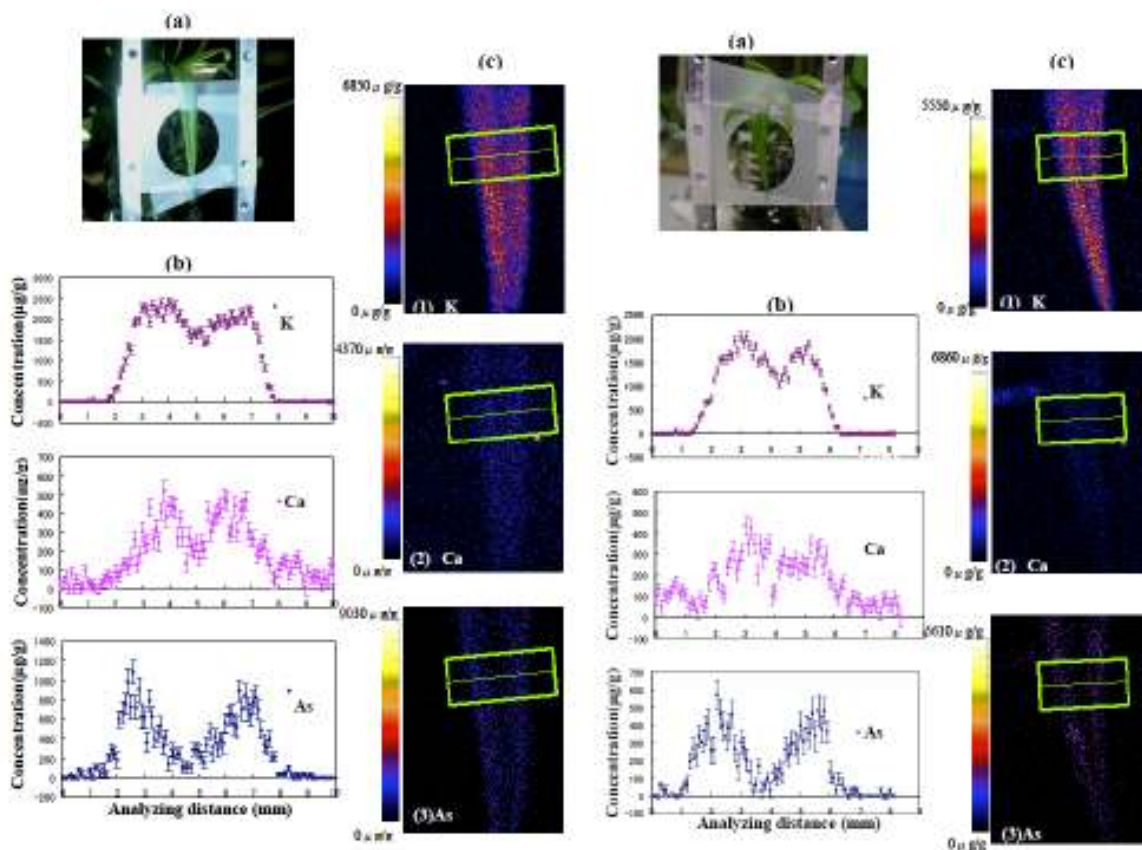


Figure 2-1 (left). Photograph (a), elemental concentration profiles (b) and maps (c) of an apex of *P. vittata* in an early active growth stage for five-day uptake of As(III) using hydroponic culture solution with 1mM phosphate.

Figure 2-2 (right). Photograph (a), elemental concentration profiles (b) and maps (c) of an apex of *P. vittata* in an early active growth stage for five-day uptake of As(V) using hydroponic culture solution with 1 mM phosphate.

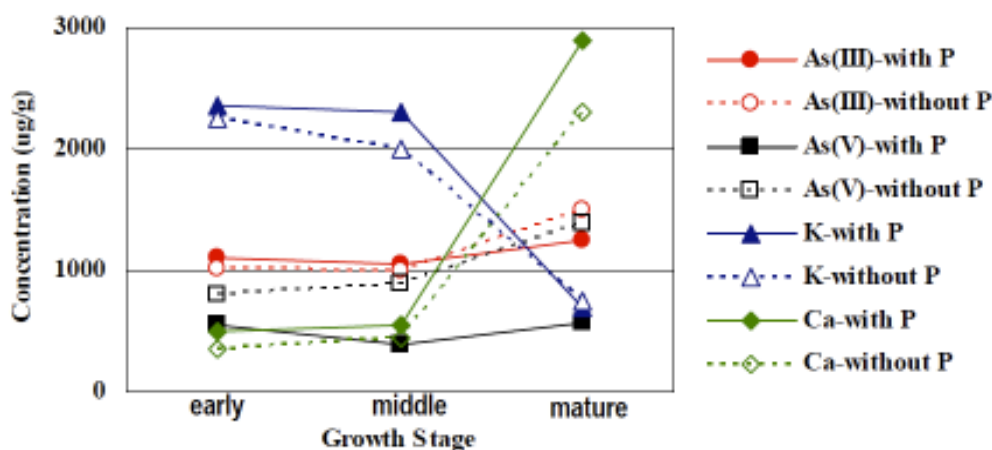


Figure3. Change in concentrations of K, Ca and As^{III} or ^V at three different growth stages of apexes of *P. vittata* on hydroponic solutions with or without addition of 1 mM phosphorus nutrient.

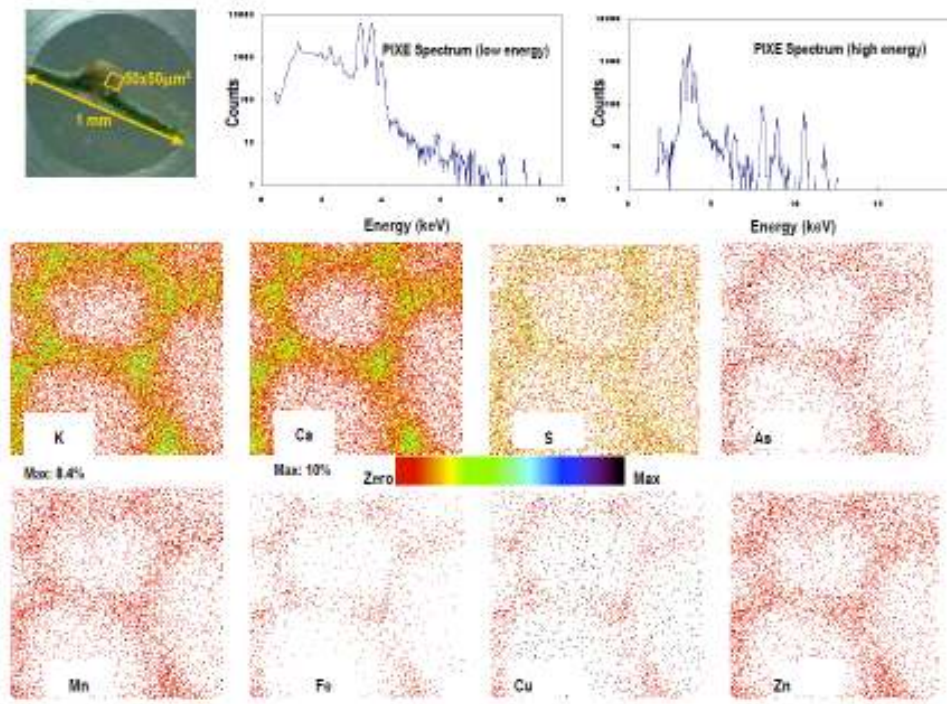


Figure 4. Photograph, PIXE spectra and elemental maps for a sliced vascular bundle of an apex of *P. vittata* under arsenate treatment.

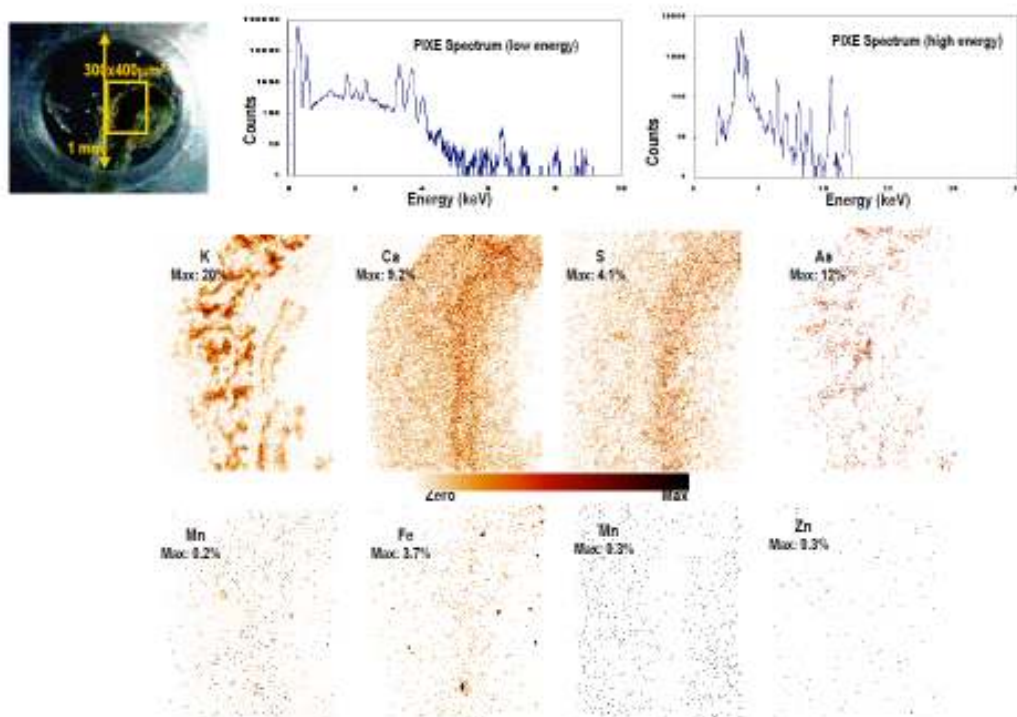


Figure 5. Photograph, PIXE spectra and elemental maps for a sliced mesophyll of an apex of *P. vittata* under arsenate treatment.