Localisation of trace metals in hyper-accumulating plants using μ-PIXE

R. Siegele [a], A.G. Kachenko [b], Y.D. Wang [c], M. Ionescu [a], N.P. Bhatia [a], and D.D. Cohen [a]

[a] *Australian Nuclear Science and Technology Organisation (ANSTO), PMB1, Menai, NSW 2234, Australia*
[b] *Faculty of Agriculture, Food and Natural Resources, The University of Sydney, NSW 2006, Australia*
[c] *School of Botany, The University of Melbourne, VIC 3010, Australia*

Abstract. PIXE is a very sensitive technique that can fast and reliably measure a wide range of elements simultaneously with high sensitivity. Using a focused microbeam elemental distributions can be mapped with high spatial resolution. We demonstrate high resolution mapping of metals in plant leafs at 5 μm resolution and its application in detecting sites of metal accumulation in metal-accumulating plant tissues. The importance of biological sample preparation is discussed by direct comparison of freeze-substitution and freeze-drying techniques routinely used in biological sciences. The advantages and limitations of quantitative elemental imaging using these techniques are also discussed.

Keywords: localization, metal hyperaccumulation, nuclear microprobe, PIXE.

INTRODUCTION

PIXE is a powerful analytical technique used in many research areas. Combined with a microbeam it becomes a powerful tool to map the distribution of elements across a specimen.

At ANSTO we have used μ-PIXE extensively to map trace elements in plant and animal tissues. More recently we have applied μ-PIXE to study the localisation of trace metals in metal indicator and hyperaccumulating plant species. A plant is said to be a metal hyper-accumulator if it concentrates trace metals above a minimum threshold concentration in above ground tissues. This threshold varies according to the metal involved for example: more than 1000 mg/kg dry weight (DW) for cobalt, copper, lead or nickel or more than 10,000 mg/kg DW for zinc or manganese [1-2]. In contrast, metal uptake in indicator plants to aboveground tissues is relatively unregulated, thus internal concentrations are a passive reflection of external levels [3].

To understand the mechanisms that allow these metal-accumulating plants to tolerate high concentrations of normally toxic metals, the spatial localisation of the metal accumulation has to be known. The objective of this study was to demonstrate the use of μ-PIXE in mapping the cellular and sub cellular localisation of trace metals in metal accumulating plant tissues using freeze-drying and freeze-substitution sample preparation techniques.

EXPERIMENTAL

Plant samples were analysed using the ANSTO High Energy Heavy Ion Microprobe (HIMP) [4]. Ion beams with an ME/q^2 of up to 100 can be focused at the HIMP with spot sizes down to 3 µm, providing sufficient current to use it for various Ion Beam Analytical techniques. The samples were analysed using a 3 MeV proton beam with a typical spot size of between 3 and 5 µm. At this spot size beam currents between 0.1 and 0.5 nA can be achieved, which is sufficient for PIXE analyses.

A high purity Ge detector was used with a 100 mm^2 active area, located 33 mm from the sample. A 100 µm Mylar foil was used to reduce low energy X-rays and thus pile-up in the µ-PIXE spectrum. This setup allowed the detection of the accumulated trace metals such as Ni and Cu with high sensitivity.

SAMPLE PREPARATION AND RESULTS

In the microanalysis of biological tissues sample preparation is one of the most important steps, which is also the case for µ-PIXE. Plant samples need to be dried and thin sectioned for µ-PIXE analysis. In the case of plant material exposed to metals it has to be ensured that this process does not result in the redistribution of the metal and that the cell ultrastructure is preserved. Because of the high spatial resolution of µ-PIXE even small movements have to be avoided.

In previous experiments, we have employed a simple technique that involved the hand sectioning of the samples followed immediately by snap freezing of the sections in liquid nitrogen. The sections were subsequently freeze dried [5]. However, with this technique at the best sections of 50 µm can be achieved. This limits the lateral resolution in µ-PIXE, because of the possible overlap of the distribution of a particular element from different sample depth. As a result cellular resolution is difficult to achieve because overlapping cell layers are mapped in this case.

In order to prepare thinner (<50 µm) sections we employed a freeze-substitution technique using dry tetrahydrofuran (THF) as a solvent. Pålsgård et al. [6] described this technique and found it suitable for biological sample preparation. Using this sample preparation technique, sections of ca. 10 µm or thinner can be prepared with a microtome.

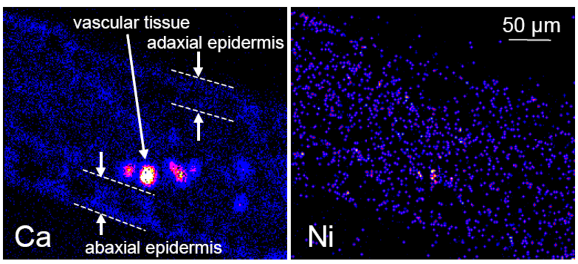

FIGURE 1. Elemental maps for Ca (left) and Ni (right) of the *Hybanthus floribundus* subsp. *floribundus* leaf section prepared by freeze substitution in THF

A leaf section of Ni-hyperaccumulating *Hybanthus floribundus* subsp. *floribundus* was prepared by this technique and analysed with µ-PIXE. Elemental maps shown in Figure 1 were extracted using GeoPIXE [7]. The Ca concentration map demonstrates that in the epidermis layer cellular resolution can be achieved and that most of the Ca is located in the cell walls. However, in the central region of the leaf, individual cells can not be resolved because the cells in this region are much smaller and a number of cell layers overlap. This can also be seen in the optical micrograph (not presented) where the cell structure is not visible in this part of the leaf.

In contrast K showed a homogeneous distribution across the analysed section (not presented), with the average K concentration across the section much lower than the K concentration measured by ICP-AES. The K concentration from ICP-AES was 2.0% DW, while the average concentration across the leaf calculated from µ-PIXE was 0.4% DW. Similarly, the Ni concentration was much lower in freeze-substituted sections (0.1%) than measured using ICP-AES (0.8%) DW. These results suggest that some of the K and Ni were washed out of the sample during the freeze-substitution process.

These findings are consistent with recently published results by D. Budka et al. [8] who found a substantial loss of Ni in Ni-hyperaccumulating *Berkheya coddii* prepared by this technique. The authors reported Ni loss of up to 90% in leaf samples treated with THF. Like Ni, K is considered a readily mobile element and in our study freeze-substitution with THF resulted in significantly lower K concentrations than ICP-AES results.

The Ni image showed some indication of the cell structure in the epidermal layers with Ni enriched in the cell walls. However, the Ni concentration was too low to quantify the distribution pattern. In order to achieve better statistics the µ-PIXE maps were used to calculate quantitative elemental profiles across the central regions of the leaf section (Figure 2). Both the Ni and Ca profiles showed a peak at the leaf surfaces and correspond to the adaxial and abaxial epidermis. Higher Ni and Ca concentrations were also observed in the central part of the leaf around the vascular tissue.

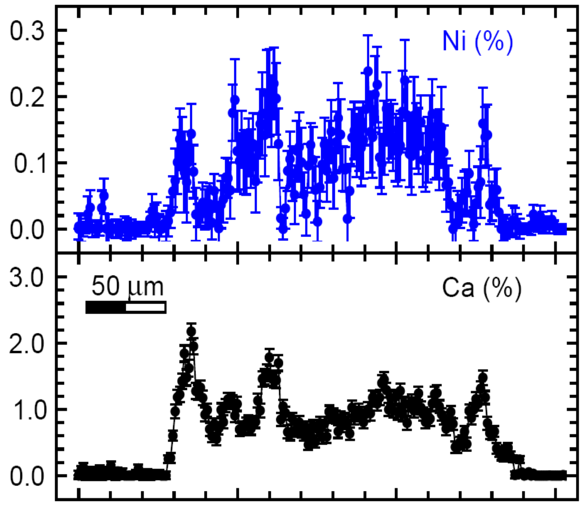

FIGURE 2. Quantitative elemental profiles of Ni and Ca across the freeze-substituted leaf section shown in Figure 1.

To compare these results a set of samples were hand sectioned, cryo-fixed and freeze-dried following the procedure described by Bhatia et al. [5]. Figure 3 shows elemental maps from the samples prepared in this way.

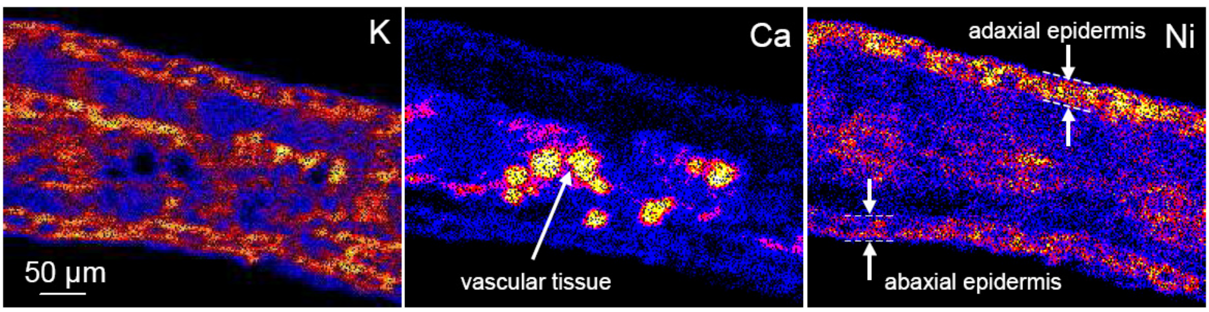

FIGURE 3. Elemental maps of K (left), Ca (centre) and Ni (right) taken on a hand sectioned cryo-fixed freeze-dried *Hybanthus floribundus* subsp. *floribundus* leaf.

These maps clearly showed a variable distribution of metals across the leaf section. Notably, across all three maps, the adaxial and abaxial epidermis was clearly defined. Moreover, in the K and Ca maps the cell walls were visible in the epidermis, although not with the same clarity as in Figure 1. The Ni map also suggested enrichment of Ni in the cell walls.

However, compared with the images of the freeze-substituted sample, the Ni image of the freeze-dried sample clearly illustrated the anatomical structure and corresponding localization pattern. In particular Ni concentrations were highest in the adaxial and abaxial epidermal layers, but also in the vascular bundles that dominate the central region of the leaf.

The K map showed a similar distribution with high K concentrations in the epidermal layers of the leaf surface. In addition, it showed a second layer with high K concentration, that was likely vascular bundles.

The Ca map showed that the Ca is concentrated in the epidermal cell walls, however, this is less apparent, because of the overlap of a number of layers smearing out the image. Furthermore, the Ca concentration in the vascular bundles was approximately 5-10 fold higher than in the epidermal tissues.

Quantitative elemental profiles for Ni and K (Figure 4) taken across the central region of the freeze-dried section clearly showed the higher concentration of both elements in the epidermal tissues and vascular bundles supporting the results of the elemental maps in Figure 3.

A leaf section of *Haumaniastrum robertii*, a Cu indicating plant was also analysed using μ-PIXE after it had been prepared by hand-sectioning and freeze-drying. This species is well known as a "copper flower" and it is endemic to soils with high Cu content [9]. In this study, it was found to accumulate small quantities of Cu in its leaf tissues (less than 0.1% DW), indicating this species does not hyperaccumulate Cu.

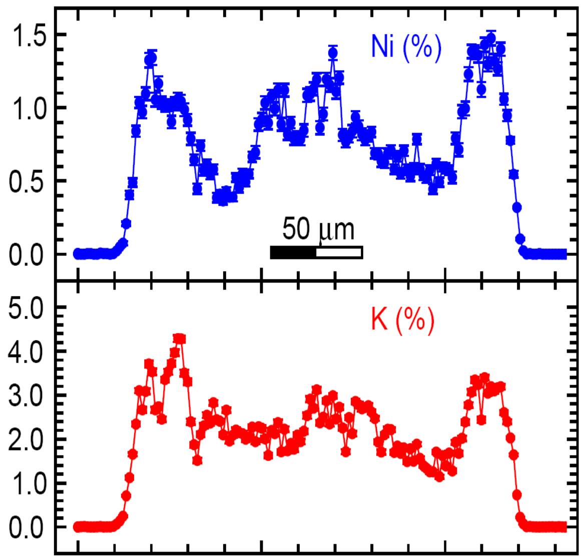

FIGURE 4. Quantitative elemental profiles of Ni and K across the freeze-dried leaf section shown in Fig. 3.

Elemental maps extracted from μ-PIXE are shown in Figure 5. Individual cells are visible in the K map, however, the image is blurred because PIXE is integrating over the probing range of the beam. Individual cells were not observed in the Cu map.

The K map illustrated a high K concentration in the epidermis layer and a second layer of high K concentration just below the elongated cells of the palisade layer.

The Cu concentration measured in the leaf was very low and within the noise throughout most parts of the leaf, except for a clear band parallel to the lower surface, between the palisade and spongy layer (Figure 5). These areas of high Cu content are also illustrated in the K map as areas with increased K content. In addition, the Cu map clearly showed a positive correlation between Cu and K content.

Figure 6 depicts quantitative elemental profiles across the central portion of the leaf. The Ca profile showed lower concentrations of Ca in the epidermal tissues as compared to *H. floribundus* subsp. *floribundus* epidermal tissues. Moreover, in this species the epidermis contained much less Ca than the remaining leaf tissues. The Ca concentration in the epidermis was between 0.1 and 0.2%, as compared to 0.6 and 0.8% in the central region of the leaf.

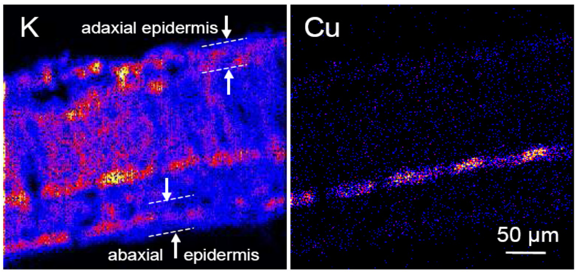

FIGURE 5. Elemental maps for K (left) and Cu (right) taken of a hand sectioned freeze-dried *Haumaniastrum robertii* leaf.

The K profile showed K concentrations of approximately 1% in the adaxial epidermis as compared to 0.7% in the palisade layer below. A clear peak in the K concentration was visible in the centre of the leaf, which coincides with the peak Cu concentration observed, indicating a link between the accumulation of Cu and K. The whole leaf section was analysed with µ-PIXE, but this band with the high K and Cu content extended only through part of the leaf section.

Since the optical micrographs (not presented) showed no clear plant structure in the areas where Cu concentrations were high, it can only be speculated which plant regions accumulate the Cu. We suggest that it is a vascular bundle running across the leaf section that contained elevated concentrations of Cu. Vascular bundles are typically found below the palisade layer.

The fact the band visible in the K and Cu does not extent through the whole leaf section can be explained by the fact that the vascular bundle was cut off when the leaf was sectioned. Since no structure was visible in the optical micrograph, this suggests that the vascular bundle is at some depth below the cut tissue surface.

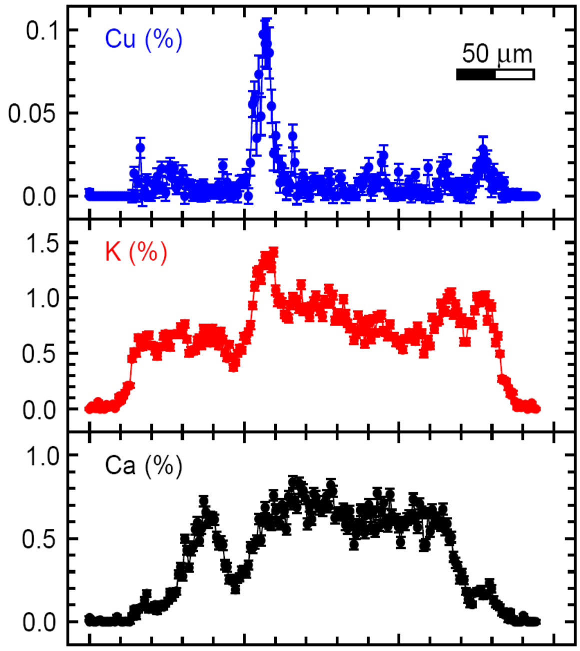

FIGURE 6. Quantitative elemental profile for Cu, K and Ca across the freeze-dried leaf section shown in Fig. 5.

CONCLUSIONS

We have demonstrated that µ-PIXE can be used to localise the areas of metal accumulation in leaf tissues.

The simultaneous mapping of various elements can be used to explore the physiological mechanisms that allow these plants to accumulate and in some cases hyperaccumulate metals.

In sections prepared using freeze-substitution (THF), we showed that cellular and to a lesser extent sub-cellular resolution can be achieved. However, this procedure resulted in the loss of metals and possible redistribution. Conversely freeze-dried leaf sections preserved cellular metal concentrations, however only cellular resolution was achieved.

ACKNOWLEDGMENTS

The authors thank Kevin Ansary for his help in running the accelerator and the Tandem Accelerator Operations Team for their efforts. A.G. Kachenko, B. Singh, Y.D. Wang and A.M.J.

Baker acknowledge funding by grants from the Australian Institute of Nuclear Science and Engineering (AINSE). A.G. Kachenko acknowledges the financial assistance provided by the Australian Government through an Australian Postgraduate Award scholarship.

REFERENCES

1. A.J.M. Baker, S.P. McGrath, R.D.Reeves and J.A.C. Smith, "Metal Hyperaccumulator Plants: A Review of the Ecology and Physiology of a Biological Resource for Phytoremediation of Metal-Polluted Soils," in *Phytoremediation of Contaminated Soil and Water,* edited by N. Terry and G. Bañuelos, Boca Raton, FL, USA: CRC Press Inc, 2000, pp. 85-107.
2. A.J.M. Baker,R.R. Brooks, *Biorecovery* **1** (1989) 81-126.
3. A.J.M. Baker, *J. Plant. Nutr.* **3** (1981) 643-654
4. R. Siegele, D.D. Cohen, N. Dytlewski, *Nucl. Instr. and Meth.* **B158** (1999) *31-38.*
5. N.P. Bhatia, K.B. Walsh, I. Orlic, R. Siegele, N. Ashwath, A.J.M. Baker. *Fun. Plant Biol.* **31** (2004) 1061-1074.
6. E. Pålsgård, U. Lindh, G.M. Roomans, Microsc. *Res. Tech.* **28** (1994) 254-258.
7. C.G Ryan, *Nucl. Instr. and Meth.* **B181** (2001) 170–179.
8. D. Budka, J. Mesjasz-Przybyłowicz, G. Tylko, W.J. Przybyłowicz, *Nucl. Instr. and Meth.* **B231** (2005) 338-344.
9. R. R. Brooks, *Plant Soil* **48** (1977) 541-544.