

Localisation of trace metals in metal-accumulating plants using μ -PIXE[†]

R. Siegele,^{1*} A. G. Kachenko,² N. P. Bhatia,¹ Y. D. Wang,³ M. Ionescu,¹ B. Singh,²
A. J. M. Baker³ and D. D. Cohen¹

¹ Australian Nuclear Science and Technology Organisation (ANSTO), PMB1, Menai, NSW 2234, Australia

² Faculty of Agriculture, Food and Natural Resources, The University of Sydney, NSW 2006, Australia

³ School of Botany, The University of Melbourne, VIC 3010, Australia

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Particle induced x-ray emission (PIXE) is a very sensitive technique that can quickly 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 leaves at 5 μ m 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. Copyright © 2008 John Wiley & Sons, Ltd.

INTRODUCTION

Particle induced x-ray emission (PIXE) is a powerful analytical technique that is widely used in many research areas. When combined with a microbeam the distribution of elements across a specimen can be mapped, which leads to a wide range of new applications.

μ -PIXE has been extensively used in environmental and biological research to map trace elements in plant and animal tissues. μ -PIXE has been used to study the localisation of trace metals in metal indicator and hyperaccumulating plant species. A plant is said to be a metal hyperaccumulator, 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 above-ground 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.

*Correspondence to: R. Siegele, Australian Nuclear Science and Technology Organisation (ANSTO), PMB1, Menai, NSW 2234, Australia. E-mail: rns@ansto.gov.au

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EXPERIMENTAL

Plant samples were analysed using the ANSTO High Energy Heavy Ion Microprobe (HIMP).⁴ Ion beams with an ME/q² of up to 100 can be focused at the HIMP with spot sizes down to 3 μ m, 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 between 3 and 5 μ m. 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 mm² active area, located 33 mm from the sample. A 100 μ m 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. Typically, plant samples are 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. On account 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 the best sections of 50 μ m can be achieved. This limits the lateral resolution in μ -PIXE, because of the possible overlap of the distribution of

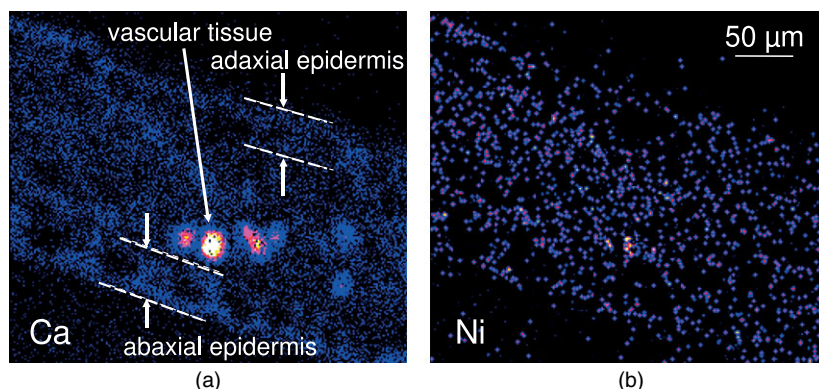


Figure 1. Elemental maps for Ca (a) and Ni (b) of the *Hybanthus floribundus* subsp *floribundus* leaf section prepared by freeze-substitution in THF.

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. Further, this technique may smear cellular contents during the cutting action that is restricted to a small fraction of cells on the cut surface, but is unlikely to cause redistribution between cells.

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.

A leaf section of Ni-hyperaccumulating *Hybanthus floribundus* subsp *floribundus* was prepared by this technique and analysed with µ-PIXE. Elemental maps shown in Fig. 1 were obtained 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 cannot 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 invisible 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 inductively coupled plasma-atomic emission spectroscopy (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 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 (Fig. 2). Both the Ni and Ca profiles showed a peak at the leaf surfaces corresponding 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.

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 Fig. 3 shows elemental maps from a sample prepared in this way, which was approximately 50 µm thick.

These maps clearly showed a variable distribution of the elements 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 Fig. 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

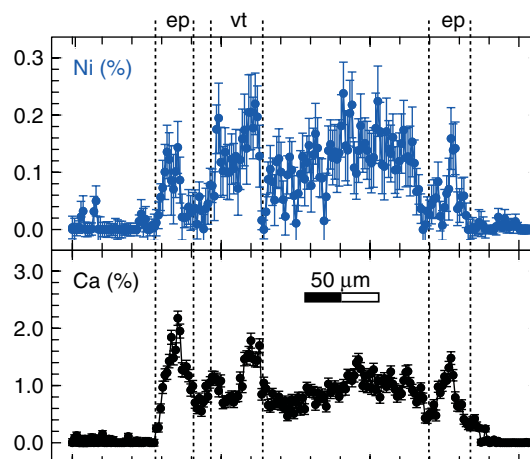


Figure 2. Quantitative elemental profiles of Ni and Ca across the freeze-substituted leaf section shown in Fig. 1. (ep – epidermis, vt – vascular tissue).

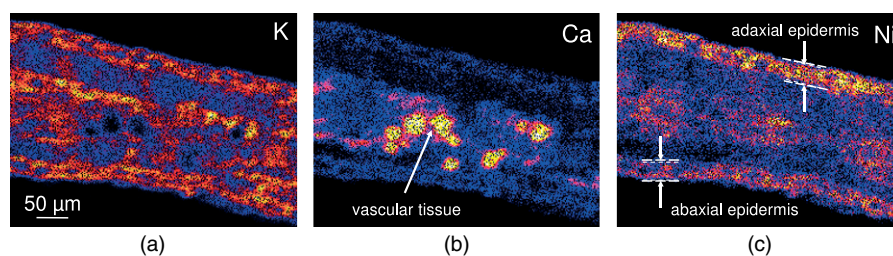


Figure 3. Elemental maps of K (a), Ca (b) and Ni (c) taken on a hand-sectioned cryo-fixed freeze-dried *Hybanthus floribundus* subsp *floribundus* leaf.

clearly illustrated the anatomical structure and corresponding localisation pattern. In particular, Ni concentration was at par (0.78% DW) with bulk tissue analysis and was highest not only 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 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 (Fig. 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 Fig. 3.

A leaf section of *Haumaniastrum robertii*, a Cu indicating plant was also analysed using μ -PIXE after it had been prepared by cryo-fixing followed by freeze-drying and hand-sectioning. 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 that this species does

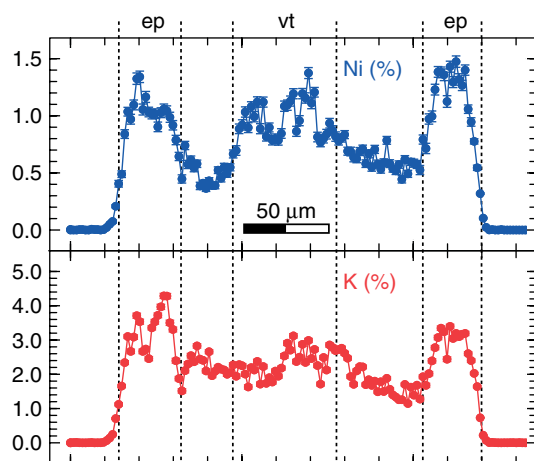


Figure 4. Quantitative elemental profiles of Ni and K across the freeze-dried leaf section shown in Fig. 3. (ep – epidermis, vt – vascular tissue).

not hyperaccumulate Cu.

Elemental maps extracted from μ -PIXE are shown in Fig. 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 and the cell structure of this sample was not as well preserved as in the previous samples. The damage of the sample was most likely due to the hand-sectioning of the fragile freeze-dried leaf sample. Individual cells were not observed in the Cu map.

The K map illustrated a high K concentration in the abaxial and adaxial epidermis and a thin layer in the lower part of the leaf 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 (Fig. 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, in this band with high Cu 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.

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 that the band visible in the K and Cu does not extend 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

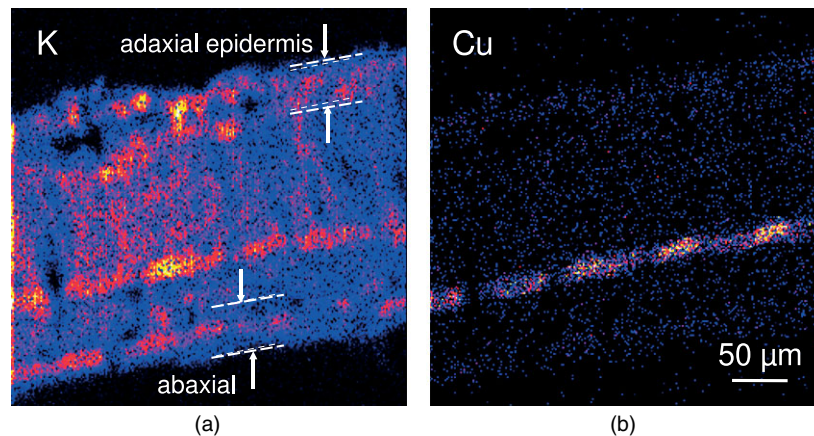


Figure 5. Elemental maps for K (a) and Cu (b) taken of a hand-sectioned freeze-dried *Haumaniastrum robertii* leaf.

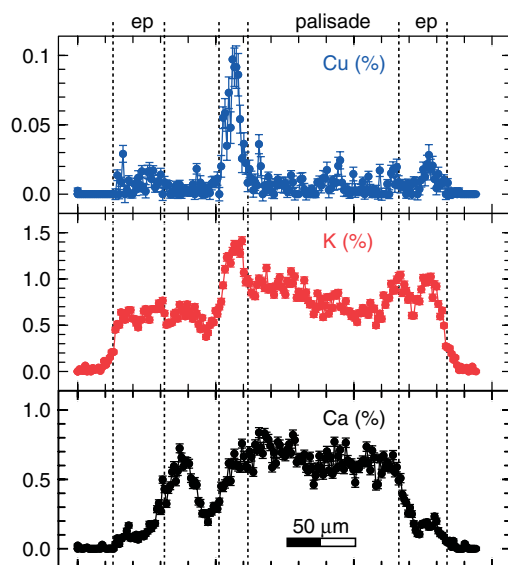


Figure 6. Quantitative elemental profile for Cu, K and Ca across the freeze-dried leaf section shown in Fig. 5. (ep – epidermis).

micrograph, this suggests that the vascular bundle is at some depth below the cut tissue surface.

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.

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