# Tools for the Discovery of Hyperaccumulator Plant Species and Understanding Their Ecophysiology

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**Abstract**

Globally the discovery of hyperaccumulator plants has been hindered by systematic screening of plant species, and is highly biased towards Ni hyperaccumulators. This is mainly due to the existence of a reagent paper test that is only specific to nickel (based on dimethylglyoxime) such that more than 400 of the approximately 500 known hyperaccumulators species are for Ni. New technical advances now permit massive screening of herbarium specimens using non-destructive, portable X-Ray Fluorescence Spectroscopy (XRF), an approach that has already led to the discovery of numerous hyperaccumulator species new to science. The elemental distribution in selected hyperaccumulator plant tissues can then be further studied using techniques such as desktop or synchrotron micro-XRF, nuclear microprobe (PIXE), scanning/transmission electron microscopy with energy-dispersive spectroscopy (SEM/TEM-EDS), secondary ion mass spectrometry (SIMS) or laser ablation inductively-coupled plasma mass spectrometry (LA-ICP-MS). The use of histochemical dyes combined with light microscopy further aids in the identification of anatomical and structural features of the studied plant tissues.

## Introduction

### Hyperaccumulator Plant Discovery

The high proportion of identified Ni hyperaccumulator species in comparison to hyperaccumulators that accumulate other elements is primarily due to the fact that ultra- mafic soils are by far the most widespread metalliferous anomaly worldwide (Echevarria 2017). Furthermore, the availability of an easily deployed, field-testing method for Ni using dimethylglyoxime (DMG)-treated paper has helped identification of Ni hyperaccumulator plants substantially. After initial field screening using a spot test (e.g. DMG-paper, see Fig. 1 for an example), confirmation of hyperaccumulator status has traditionally been achieved using Atomic Absorption Spectrophotometry (AAS) (e.g. Kelly et al. 1975; Jaffre et al. 1979b; Kersten et al. 1979) and Inductively Coupled Plasma- Atomic Emission Spectroscopy (ICP-AES) (e.g. Reeves et al. 1996, 2007; Fernando et al. 2009; van der Ent and Reeves 2015) after acid digestion of dried leaf material samples. Although the analysis with AAS or ICP-AES itself takes only minutes, the sample preparation is time and resource consuming (e.g. 100 samples take 2–3 days to digest). Table-top XRF (X-ray fluorescence) was also used for this purpose in the past (e.g. Mesjasz-Przybylowicz et al. 1994). XRF analysis can be done on intact specimens, but for more reliable results homogenisation and preparation of pellets is recommended. Recent technological advances in more sensitive and compact X-ray fluorescence (XRF) instruments enable non-destructive elemental screening of a vast numbers of samples, including herbarium specimens, in a relatively short period of time. This chapter introduces the use of portable XRF as an efficient tool for hyperaccumulator species discovery in herbaria, and shortly summarizes which micro-analytical techniques can be used to

### Handheld X-Ray Fluorescence Technology for Herbarium Screening

In order for hyperaccumulator species to be dis- covered, systematic screening of plant species must be conducted. The use of handheld XRF instruments is a time- and cost-effective method. It has the ability to measure a range of different elements simultaneously within 30–60 s in dry samples. The latest generation of such XRF instruments (equipped with an Ag anode 6–50 kV, 0–200 μA) have detection limits in the range of ~100–300 μg g-1 for most transition elements (i.e. Co, Ni and Zn). The XRF instrument works by subjecting the sample to a beam of focused, high-energy X-rays generated from an X-ray tube in the device. The spectrum of excited fluorescent X-rays is then analyzed to determine the presence of different elements and to calculate their relative concentrations in the sample. Recently, handheld XRF systems have been validated for the measurement of elements in plant samples, although this has involved powder- ing and pelletisation of sample material before measurement (McLaren et al. 2012; Reidinger et al. 2012; Guerra et al. 2014). However, XRF can be employed for non-destructive analysis of plant material, and with the advent of portable instruments utilizing the latest type of fast (Silicon drift detectors or SDDs) detectors, has made it possible to perform measurements of tens of thousands of samples in a relatively short time span (see Fig. 2 for the setup of XRF scanning of herbarium specimens). As such, elements such as Ni or Zn in herbarium specimens can be

The herbarium specimen sheet is placed on a titanium metal sheet that serves as a X-ray radiation block, with the portable XRF device fitted with a backscatter shield placed. The XRF device is connected to a computer to read out the acquired data measured at a rate of ~300 specimens per day (translating to ~6000 specimens per month). Hence, this makes it feasible to scan the entire phylogenetic lineages, as well as specimens of the same species from many different collections or localities. Additionally, XRF screening may be combined with the digitisation process of herbarium specimens, an effort already underway in many global herbaria. From such initial mass screening, species may be selected for further investigation to elucidate elemental distribution at the tissue and cellular level, after dedicated field collection of samples. The results of XRF measurements of herbarium specimens may vary somewhat within a specimen, depending on the exact region

of the leaf surface measured (e.g. mid-vein or lamina), and it is advisable to measure multiple spots within a specimen (see Fig. 3). Another consideration when undertaking XRF measurements is that some herbarium specimens were treated with $HgCl_2$ for long-term insect protection and there- fore readings for Hg can be extremely high for such samples (i.e. >500 μg $g^{-1}$). Furthermore, surficial contamination with soil particles must also be considered (easily gauged from Fe and Cr concentrations), as this also raises the apparent concentrations of Ni, Co and Mn. In order to obtain quantitative data, the XRF system should be properly calibrated (Markowicz 2008). In the example provided in the case study below, a set of parallel foliar samples were analyzed by ICP-AES to obtain element-specific correlation coefficients for calibrating the XRF concentration values obtained by a handheld instrument.

**The Case of New Caledonia**

New Caledonia is located in the southwest Pacific, approximately 1500 km east of Australia, and consists of one main island (Grande Terre) and a group of smaller islands with a total area of approximately 19,000 km2 (Pillon et al. 2010). It is a biodiversity hotspot (Myers et al. 2000; Mittermeier et al. 2004), harbouring over 3371 vascular plant species of which nearly 75% are endemic (Morat et al. 2012). In addition, there is 16% endemism at the generic level for vascular plant species, and New Caledonia has three endemic families, namely the Amborellaceae, Oncothecaceae, and Phellinaceae (Morat et al. 2001; Heads 2010). The highly distinctive plant diversity of New Caledonia results in part from its geological his- tory (Pelletier 2006), its isolation (Morat 1993; Murienne 2009; Morat et al. 2012), and the climatic stability (Murienne 2009), although the latter is yet to be proven (Pillon 2012). The high species richness and endemicity also raise questions on their origins, with both vicariance (e.g. Ladiges et al. 2003; Barker et al. 2007), and long-distance dispersal purported as possible mechanisms for speciation (Setoguchi et al. 1998; Swenson et al. 2001; Bartish et al. 2005; Pillon et al. 2014). The geological evidence suggests that the species diversity is dominated by neo-endemism and disharmonic colonization, hence not of ancient relictual Gondwanan origins (Murienne et al. 2005; Grandcolas et al. 2008). Pillon et al. (2010) showed that plant families with low levels of endemism but high effective dispersal capacities were under-represented on the island of New Caledonia. Therefore, they concluded that dispersal was not a significant factor in sorting the species in the flora, but instead that ultramafic soils may have been a major force for speciation, as previously emphasized by Jaffre (1993).

More than 70% of the 2492 endemic plant species in New Caledonia grow on ultramafic soils, of which over 1200 species are ultramafic soil obligates, representing ~50% of the endemic flora of the island (Isnard et al. 2016). New Caledonia has extensive outcrops of ultramafic soils (totalling approximately 5600 km2) that are strongly weathered (i.e. laterization) (L'Huillier and Edighoffer 1996). The parent ultramafic rocks contain 0.16–0.4% Ni, but the portion of Ni increases during soil weathering up to complete laterization, mainly in saprolitic horizons (Echevarria 2017). The deficiency of essential plant nutrients (e.g. P, K, Ca) in ultramafic soils and the bioavailability of potentially toxic elements (i.e. Co, Mn, Ni) may cause stresses for plants to survive (Alexander and DuShey 2011). This so-called 'serpentine syndrome' in general is more pronounced on soils developed on serpentinite, i.e. Magnesic Cambisols (Isnard et al. 2016; Echevarria 2017).

**Herbarium XRF Scanning in New Caledonia**

In New Caledonia, currently 65 Ni and 11 Mn hyperaccumulator plant species are known (van der Ent et al. 2015a). Jaffre et al. (1976) discovered that the New Caledonian endemic tree, *Pycnandra acuminata* (formerly *Sebertia*) has a latex with more than 25 wt% dry weight Ni. XRF herbarium screening was undertaken with a portable XRF instrument at the Herbarium of the Institute for Research and Development (IRD) in New Caledonia on over 7500 dried herbarium specimens. The selection of herbarium specimens to scan was based on families that were already known to contain numerous hyperaccumulator species (Jaffre et al. 1976, 1979a,

2013; Swenson and Munzinger 2010) (e.g. Cunoniaceae, Phyllanthaceae, Salicaceae, Sapotaceae and Violaceae), as well as a systematic screening of 1–4 specimens (depending on availability) of species known to occur on ultramafic soils in New Caledonia (3996 specimens covering 1087 species). The measurements led to the recording of numerous new hyperaccumulators: restricting to high range records only (i.e. Ni >5000 μg $g^{-1}$, Mn >20 000 μg $g^{-1}$, Co >1000 μg $g^{-1}$, and Zn 10 000 μg $g^{-1}$), there were 87 taxa for nickel (including 22 new records), 68 taxa for manganese (including 59 new records), 8 taxa for cobalt (none previously recorded), and 4 taxa for zinc (none previously recorded). This demonstrates that XRF screening of herbarium specimens has the potential to discover vast numbers of new hyperaccumulator species, even in well-studied floras such that of New Caledonia.

**Knowledge Gaps: Priority Regions for Exploration and Discovery**

Currently it is estimated that hyperaccumulation occurs in 0.2% of Angiosperms and 1–2% of the known global ultramafic flora (Baker 1981; Baker and Brooks 1989; van der Ent et al. 2015b). Systematic herbarium specimen XRF scanning, combined with auxiliary collection data, can provide insights into phylogenetic patterns of hyperaccumulation, and has the potential to complement and add insights to bio- geographical and phylogenetic studies. This approach also facilitates the selection of plant species targeted for more detailed investigations, using the methods described below. Rapid plunge cryo-fixation: fast plunging of small samples into a liquid cryogen bath (usually propane, ethane or isopentane) cooled by liquid nitrogen. Liquid nitrogen should not be used as primary cryogen due to the Leidenfrost phenomenon (i.e. the formation of an insulating gas layer around the warm object that results in a slow freezing speed). Metal mirror fixation: quickly pressing small excised samples against a solid block of Cu (sometimes covered by Au) cooled by liquid nitrogen. This method is possible in the field. High pressure freezing: requires specialized equipment, with several restrictions regarding the method of preparation and the specimen size. This method is mainly recommended for further cryo-ultramicrotomy. The recommended starting point for investigations is collecting samples from plants growing in their natural habitat. Collecting plant tissue samples in the field is, however, challenging, but two strategies have been successfully used: (i) collecting whole plants with soil in pots from the metalliferous outcrops and trans- port to a suitably equipped laboratory (Koosaletse-Mswela et al. 2015), or (ii) freezing tissue samples in the field using metal-mirror freezing and transport to laboratory in a cryoshipper at liquid nitrogen temperature (Mesjasz-Przybylowicz et al. 2016; van der Ent et al. 2017). It is important that cryo-fixation is done as soon as possible (i.e. within seconds after

**Sample Preparation for Micro- Analytical Technique Investigations**

The preparation of plant tissue samples for physiological investigations is critical to achieve preservation of the intrinsic elemental distribution. Proper preservation of plant tissues for micro-analytical investigations principally involves using cryo-techniques–various forms of cryo-fixation followed by analysis of frozen hydrated or freeze-dried plant tissue material. There are three main approaches to cryo-fixation: excision) to minimise artefacts associated with sample deterioration. Air drying, or chemical fixation followed by resin embedding (including freeze-substitution) should be avoided for micro-analytical investigations because of the very substantial risks for elemental re-distribution. Once cryo-fixed, the samples can be analyzed in either frozen-hydrated state (e.g. Tylko et al. 2007a, b; Wang et al. 2013) or in freeze- dried state, employing a very slow, temperature controlled freeze-drying regime. Samples may also be sectioned for analysis of specific tissues and cells. This approach is advisable because the penetration depth of the X-ray is greater than the thickness of a cell layer, and where there is more than one overlapping layer it becomes impossible to ascertain from which layer the fluorescence signal is originating (Donner et al. 2012). A cryomicrotome can easily produce sections ~30 μm thick which typically corresponds to a single cell layer. When using a cryo-ultramicrotome, semi-thin sections of the order of 1–2.5 μm in thickness and thinner, can be obtained. Cryo-

sections need to be kept at cryo- genic temperature (-130C or colder) until micro-analytical examination, or they can be freeze-dried.

**Elemental Localization Within Plant Tissues**

A range of approaches can be utilized for assessing elemental distribution within plant tissues. Below, a number of techniques are discussed, including those that illuminate the sample with photons (desktop micro-XRF, synchrotron XFM), irradiate the sample with electrons (SEM/TEM-EDS) or ions (PIXE and SIMS). The elemental detection is based upon either the emitted fluorescent X-rays (desktop micro-XRF, synchrotron XFM, SEM/TEM-EDS and PIXE) or by mass spectrometry (SIMS and LA-ICP-MS). In addition, we also describe the use of light microscopy and histochemistry, as well as autoradiography. The various techniques differ in their sensitivity (i.e. the limits of detection), lateral resolution, and sample preparation requirements. Although discussed in detail below, desktop micro-XRF, synchrotron XFM, SEM-EDS, PIXE, and LA-ICP-MS potentially allow analysis of (frozen) hydrated samples, thereby reducing the likelihood of experimental artefacts. Synchrotron (XFM) and microprobe (PIXE) facilities are often not easily accessible for many users, which is in contrast to recently, the development of Environmental SEM has facilitated the analysis of hydrated/fresh samples. Although it is possible to obtain high resolution when imaging with SEM, elemental information is limited to a resolution of ca. 1–5 μm due to the interaction of the electrons with the sample, resulting in a tear drop-shaped penetration of the electrons into the sample. Figure 4 shows SEM-EDS elemental maps of a petiole cross-section.

**SEM-EDS**

In SEM-EDS, electrons are used to excite core electrons of the elements of interest within the plant tissue sample, with the subsequent measurement of emitted fluorescent X-rays. Of the techniques considered here, SEM-EDS has relatively] poor detection limits, in the order of 1000–5000 μg g 1 for most transition elements. 'Traditional' SEM analysis is conducted in a vacuum, requiring samples that are dehydrated, although samples can be analyzed in frozen state where a cryogenic stage is available. More recently, the development of Environmental SEM has facilitated the analysis of hydrated/ fresh samples. Although it is possible to obtain high resolution when imaging with SEM, elemental information is limited to a resolution of ca. 1–5 μm due to the interaction of the electron with the sample, resulting in a tear drop-shaped penetration of the electrons into the sample. Figure 4 shows SEM-EDS elemental maps of a petiole cross-section.

**Desktop Micro-XRF**

Desktop micro-XRF instruments uses a focused beam of high-energy X-rays as the excitation source. These types of instruments provide high spatial resolution with spot sizes as small as 25 μm and detection limits in the order of 5–50 μg g 1 for most transition elements. It relies on the same principle as a handheld XRF, but has the ability to raster-scan a sample, thereby producing elemental distribution maps. Figure 5 shows a micro-XRF image of a hyperaccumulator plant leaf with the distribution of different elements marked by different colours. The sample preparation for desktop micro-XRF analysis is minimal (samples of living leaves or other plant parts can be analyzed directly), however, it is preferable that the leaf samples are dehydrated to increase relative elemental concentration during analysis, and to reduce scattering due to water content.

**Synchrotron Micro-XRF Microscopy**

Synchrotron light is emitted by electrons when affected by a magnetic field resulting in photons that range from infrared and ultraviolet to X-ray wavelengths (Watson and Perlman 1978). The electromagnetic radiation is delivered to 'end stations' in individual beamlines. At 'end stations' the X-rays illuminate samples in a spectrometer, and fluorescence spectra may be recorded by different types of detectors (Miller and Dumas

2006; Sarret et al. 2013; Vijayan et al. 2015). Techniques that are possible using synchrotron XRF microscopy (XFM) beamlines include X-ray fluorescence microscopy (XFM), X-ray fluorescence microcomputed tomography (XRF-μCT); X-ray absorption Spectroscopy (XAS), and differential phase contrast imaging of low-Z ultrastructure (DPC), amongst others. The XFM beamline at the Australian Synchrotron achieves a spatial resolution of ~1 μm, with an energy range of 4–25 keV operating on an in-vacuum undulator source (Paterson et al. 2011). XFM beamlines have the ability to generate fine-scale elemental maps with spatial resolutions and quantification within plant tissues, cells, and organelles (McRae et al. 2009).

Fluorescence emitted from the irradiated spots is detected as a function of the location on the specimen, when the specimen is moved relative to the stationary X-ray beam. Synchrotron XRF is a highly sensitive technique and hydrated samples can be analyzed in vivo, and it has the capacity to perform simultaneous, multi-elemental analysis (Sarret et al. 2013). Figure 6 shows the analytical measurement conditions at the XFM beamline of the Australian Synchrotron with the Maia detector system, with Fig. 7 showing the elemental maps of a mid-vein portion of a Ni hyperaccumulator leaf. New-generation fast detector systems such as the Maia detector system, which comprises of an array of 384 detectors can process photon rates exceeding 107 s 1, For samples fully penetrated by the proton beam, the thickness can be also determined using the scanning transmission ion microscopy (STIM) method (Pallon et al. 2009). Numerous studies of hyperaccumulators have been conducted mainly at the three laboratories–iThemba LABS in South Africa, MIC in Slovenia and ANSTO in Australia. The first two laboratories offer facilities suitable for analysis of frozen-hydrated samples and have reported results of elemental mapping of hyperaccumulator plants (Tylko et al. 2007a, b;] Wang et al. 2013; Vavpetic et al. 2013). However, the majority of studies were conducted on freeze-dried samples. The first PIXE elemental mapping of hyperaccumulator plants was undertaken in 1993 (Mesjasz-Przybylowicz et al. 1993, 1994). Overviews of numerous micro-PIXE studies related to hyperaccumulation of Ni, Co, As, Mn, Zn and Cd can be found e.g. in Przybylowicz et al. (1997) and Mesjasz-Przybyłowicz and Przybyłowicz (2011). Studies in which micro- PIXE was employed extended from elemental mapping of organs, tissues and plant cells, to the role of mycorrhizas in hyperaccumulating plants and the relations between these plants and herbivorous insects feeding on them (Mesjasz-Przybyłowicz and Przybyłowicz 2011).

**NanoSIMS**

Secondary ion mass spectrometry (SIMS) uses ions as the incident beam that collides with the sample surface and causes atoms and molecules from the upper layers of the sample to be ejected into the vacuum (De Rue et al. 2006; Hoppe et al. 2013). Ionized particles, referred to as secondary ions, are extracted to a mass spectrometer for analysis (which also enables isotopic identification). Given that the sputtering depth is 5–20 nm (Hoppe et al. 2013), NanoSIMS is a surface- sensitive technique, allowing for a spatial resolution as low as <50 nm. The primary beam is typically O- or Cs+, with a negatively charged primary beam (O-) tending to favour the production of positively charged secondary ions; a positively charged primary beam (Cs+) tends to favour the production of negatively charged secondary ions. Thus, for metals (such as Ni), an O- beam is generally preferable, whereas for metalloids (such as As) a Cs+ beam is generally preferable. For some elements, such as Zn, Cd, and Mn, a poor secondary ion yield makes analyses more difficult (Zhao et al. 2014). NanoSIMS analyses are conducted within an ultra-high vacuum, and hence plant samples must first be processed and dehydrated prior to examination (Moore et al. 2011). In the hyperaccumulator *Alyssum lesbiacum*, Smart et al. (2007) used NanoSIMS to examine leaf sections, with Ni found to accumulate in the peripheral regions of large unicellular trichomes and in the epidermal cell layer. In a similar manner, Smart et al. (2010) examined the distribution of Ni and other elements in highly vacuolated leaf tissue of *A. lesbiacum*.

**LA-ICP-MS**

Laser ablation inductively-coupled plasma mass spectrometry (LA-ICP-MS) uses a focused laser beam to ablate the surface, with ablated particles then transported to the mass spectrometer. Elemental and isotopic analyses are both possible, as for NanoSIMS. However, the spatial resolution of LA-ICP-MS is 2–3 orders of magnitude worse than for NanoSIMS, being 10–50 μm. However, in contrast to NanoSIMS, it is possible to analyze hydrated samples using LA-ICP-MS, in which analyses are performed at atmospheric pressure and room temperature. Moradi et al. (2010b) examined Ni in cross sections of fresh (hydrated) roots of the hyperaccumulator *Berkheya coddii*, with Ni concentrations found to be higher in the cortex compared to the stele. Using LA-ICP-MS, Tian et al. (2011) examined Cd distribution in *Sedum alfredii* using fresh (entire) leaves and freeze-dried sections taken from both leaves and stems. These authors reported that there was preferential accumulation of Cd within parenchyma cells, especially in stems. Wu et al. (2009) used LA-ICP-MS to examine elemental distribution in fresh (hydrated) leaves of *Elsholtzia splendens*.

**Autoradiography and MRI**

The use of radioactive isotopes in autoradiography is a powerful technique for the visualisation of specific radiotracers within intact living plants. It offers high resolution and the ability for time- resolved studies, but its application is limited primarily due to occupational health and safety concerns. The Cd distribution has been elucidated in *N. caerulescens* and *Arabidopsis halleri* using the $^{109}Cd$ radiotracer (Cosio et al. 2005; Huguet et al. 2012). An alternative approach that does not involve radioactive isotopes, but has many of the same advantages, is the use of Magnetic Resonance Imaging (MRI), which has been successfully demonstrated for in situ Ni distribution in the rhizosphere of *Berkheya coddii* (Moradi et al. 2010a).

**Histochemical Methods Localisation and Light Microscopy**

Knowledge of anatomical features is crucial for accurately interpreting the elemental distribution in the hyperaccumulator plant tissues being studied. For bright-field light microscopy samples are usually fixed in 1–3% glutaraldehyde ($C_5H_8O_2$) buffered in cacodylate or phosphate-buffered saline (PBS). Fixation is often followed by dehydration in a graded ethanol (C2H6O) series, with or without propylene oxide (C3H6O), followed by embedding in a low-viscosity resin [such as Spurr's resin (Spurr 1969)]. Sections 0.5–2 μm thick can then be cut, stained with dyes, and imaged. If subcellular information is needed on the cells in which elemental distribution is being investigated, ultrathin sections of gold interference colour (80–90 nm) can be cut from the same resin blocks and mounted onto grids, stained with 2% uranyl acetate (UO2(CH3COO)2) and lead citrate ($(C_6H_5O_7)_2Pb_3$) (Reynolds 1963), and then examined by TEM. It is better, however, to use tissue samples that are rapidly frozen and then freeze substituted and treated with a secondary fixative.

**Use of Chelating Dyes and Light Microscopy**

Chelating dyes have also been used to stain metal (loids) in plant tissues. Dimethylglyoxime ($C_4H_8N_2O_2$) forms a crimson complex with $Ni^{2+}$ and has been used as a histochemical stain for the localization of Ni within tissues of hyperaccumulator plants (Mizuno et al. 2003; Bhatia et al. 2004). However, artefacts are evident, as demonstrated by Bhatia et al. (2004) who found re-distribution of Ni. Similarly, Zincon ($C_{20}H_{15}N_4NaO_6S$), which forms a blue complex with $Zn^{2+}$, has been used for staining Zn in *N. caerulescens* (Macnair and Smirnoff 1999; Kozhevnikova et al. 2016). Autometallography is another histological technique in which target ions (such as $Cu^{2+}$, $Cd^{2+}$, $Pb^{2+}$, $Zn^{2+}$) are precipitated with sulphides upon which metallic Ag is subsequently deposited by use of a reductant (Danscher 1984). The Ag clusters can then be imaged by light microscopy or SEM/TEM. The main disadvantage is that the sulphide reaction is non-specific and precipitates a range of transition elements, although at neutral pH the selective formation of ZnS nanocrystals is favoured

(McRae et al. 2009). Selectivity may also be achieved using immunohistochemical methods via monoclonal anti-bodies to specifically bind chelated target ions, as demonstrated for $Cd^{2+}$ in plant tissue (Gao et al. 2015).

**Use of Fluorophores and Confocal Microscopy**

Fluorescence microscopy is sometimes employed for further interpretation of plant structure that may be pertinent for interpreting elemental distribution patterns in specific tissues. These include, for example, the identification of apoplastic barriers such as Casparian bands in roots stained with the fluorophore berberine ($C_{20}H_{18}NO_4$) (Brundrett et al. 1988). Ion-selective fluorophores have been used to image the distribution of Ni and Zn in hyperaccumulator plants. Zinpyr-1 ($C_{46}H_{36}Cl_2N_6O_5$) was used to image $Zn^{2+}$ in *N. caerulescens* (Kozhevnikova et al. 2014, 2016). The high sensitivity of this fluorophore also enabled imaging of $Zn^{2+}$ in non-hyperaccumulator plants, such as in the roots of *Arabidopsis thaliana* (Sinclair et al. 2007). Leadmium Green was used to map $Zn^{2+}$ and $Cd^{2+}$ in the hyperaccumulators *Sedum alfredii* and *Picris divaricata* (Lu et al. 2008; Hu et al. 2012). Newport Green ($C_{43}H_{30}Cl_2N_4O_8$) has been applied for imaging Ni2+ in cells of *A. murale* (Agrawal et al. 2013) and *A. lesbiacum* (Ingle et al. 2008). Although fluorophores have a number of merits, including high selectivity, the ability to be used in live tissues in time-resolved visualization, and an inherently high resolution via visible light microscopy, there are also limitations related to unknown penetration and binding to target metal(loid) ions. Fluorophores will only bind to free ions that are not already strongly ligated; for example, $Al^{3+}$ binds strongly to the cell wall but the fluorophore morin ($C_{15}H_{10}O_7$) does not bind to this wall-bound Al, and this method was thus unable to identify this major compartment of Al (Eticha et al. 2005). Another concern relates to the application method of fluorophores to the sample: loading vibratome sectioned samples in a solution with the fluorophore will ostensibly result in removal of any soluble metal(loid) ions present. Delivery of cell permeable fluorophores into a nutrient solution, followed by uptake in the plant, could circumvent this issue, but avoiding re-distribution during sectioning remains difficult. Attempts have also been made at fixing mobile target ions ($Cd^{2+}$ or $Zn^{2+}$) by *in situ* precipitation as metal sulphides or with strong chelators before microtoming and microscopy (Hu et al. 2009, 2012; Gao et al. 2015). There is a need for independently validating the use of fluorophores for mapping metal(loid) ions in hyperaccumulator plants, for instance by mapping of fluorophore labelled tissues with other micro-analytical techniques, such as XFM or PIXE.

**Conclusions and Outlook**

The use of a portable XRF-instruments enables mass screening of herbarium collections that will likely lead to the discovery of substantial numbers of hyperaccumulator plant species. The data obtained from systematic screening of entire phylogenetic lineages can lead to better insights in the evolution of hyperaccumulator plants. The major limitation for researchers in using XFM and PIXE in their experiments is the restrictive nature of access to synchrotron and accelerator facilities. There is, therefore, a need to bridge the gap between what is currently possible in the laboratory environment and the capability of large analytical facilities. Technological developments, including very high flux X-ray sources (Hemberg et al. 2003), and even table-top accelerators (Graves et al. 2014), will not replace synchrotron-based XFM, but will permit combining their individual strengths, for example, by whole organism mapping at the local laboratory followed by investigation of target cells at a major synchrotron or accelerator facility.

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**References**

Agrawal B, Czymmek KJ, Sparks DL, Bais HP (2013) Transient influx of nickel in root mitochondria modulates organic acid and reactive oxygen species strategies in the response of plants to heavy metals. J Plant Nutr 3:643–654

Baker AJM, Brooks RR (1989) Terrestrial higher plants which hyperaccumulate metallic elements—a review of their distribution, ecology, and phytochemistry. Biorecovery 1:81–126

Barker NP, Weston PH, Rutschmann F, Sauquet H (2007) Molecular dating of the 'Gondwanan' plant family Proteaceae is only partially congruent with the timing of the break-up of Gondwana. J Biogeogr 34:2012–2027

Bartish IV, Swenson U, Munzinger J, Anderberg A (2005) Phylogenetic relationships among New Caledonian Sapotaceae (Ericales): molecular evidence for generic polyphyly and repeated dispersal. Am J Bot 92:667–673

Bhatia NP, Walsh KB, Orlic I, Siegele R, Ashwath N, Baker AJM (2004) Studies on spatial distribution of nickel in leaves and stems of the metal hyperaccumulator *Stackhousia tryonii* Bailey using nuclear microprobe (micro-PIXE) and EDXS techniques. Funct Plant Biol 31:1061–1074

Brundrett MC, Enstone DE, Peterson CA (1988) A berberine-aniline blue fluorescent staining procedure for suberin, lignin, and callose in plant tissues. Protoplasma 146:133–142

Cosio C, DeSantis L, Frey B, Diallo S, Keller C (2005) Distribution of cadmium in leaves of *Thlaspi caerulescens*. J Exp Bot 56:765–775

Danscher G (1984) Autometallography: a new technique for light and electron microscopic visualization of metals in biological tissues (gold, silver, metal sulphides and metal selenides). Histochemistry 81:331–335

De´ Rue C, Gibouin D, Demarty M, Verdus M-C, Lefebvre F, Thellier M, Ripoll C (2006) Dynamic- SIMS imaging and quantification of inorganic ions in Frozen-hydrated plant samples. Microsc Res Tech 69:53–63

Donner E, Punshon T, Guerinot M, Lombi E (2012) Functional characterisation of metal(loid) processes in planta through the integration of synchrotron techniques and plant molecular biology. Anal Bioanal Chem 402:3287–3298

Echevarria G (2017) Genesis and behaviour of ultramafic soils and consequences for nickel biogeochemistry. In: van der Ent A, Echevarria G, Morel JL (eds) Agromining: farming for metals. Springer

Eticha D, Stass A, Horst J (2005) Localization of aluminium in the maize root apex: can morin detect cell wall-bound aluminium? J Exp Bot 56:1351–1357

Fernando DR, Guymer G, Reeves RD, Woodrow IE, Baker AJM, Batianoff GN (2009) Foliar Mn accumulation in eastern Australian herbarium specimens: prospecting for 'new' Mn hyperaccumulators and potential applications in taxonomy. Ann Bot 103:931–939

Gao W, Nan T, Tan G, Zhao H, Tan W, Meng F, Li Z, Li QX, Wang B (2015) Cellular and subcellular immunohistochemical localization and quantification of cadmium ions in wheat (*Triticum aestivum*). PLoS One 10:e0123779–e0123716. doi:10.1371/journal. pone.0123779

Grandcolas P, Murienne J, Robillard T, Desutter- Grandcolas L, Jourdan H, Guilbert E, Deharveng L (2008) New Caledonia: a very old Darwinian island? Philos Trans R Soc Lond B 363:3309–3317

Graves WS, Bessuille J, Brown P, Carbajo S, Dolgashev V, Hong KH, Ihloff E, Khaykovich B, Lin H, Murari K, Nanni EA, Resta G, Tantawi S, Zapata LE, Ka¨rtner FX, Moncton DE (2014) Compact X-ray source based on burst-mode inverse Compton scattering at 100 kHz. Phys Rev ST Accel Beams 17:120701

Guerra MBB, de Almeida E, Carvalho GGA, Souza PF, Nunes LC, Junior DS, Krug FJ (2014) Comparison of analytical performance of benchtop and handheld energy dispersive X-ray fluorescence systems for the direct analysis of plant materials. J Anal At Spectrom 29:1667–1674

Heads M (2010) The endemic plant families and the palms of New Caledonia: a biogeographical analysis. J Biogeogr 37:1239–1250

Hemberg O, Otendal M, Hertz HM (2003) Liquid-metal- jet anode electron-impact X-ray source. Appl Phys Lett 83:1483. doi:10.1063/1.1602157

Hoppe P, Cohen S, Meibom A (2013) NanoSIMS: technical aspects and applications in cosmochemistry and biological geochemistry. Geostand Geoanal Res 37:111–154

Hu P-J, Qiu R-L, Senthilkumar P, Jiang D, Chen Z-W, Tang Y-T, Liu F-J (2009) Tolerance, accumulation and distribution of zinc and cadmium in hyperaccumulator *Potentilla griffithii*. Environ Exp Bot 66:317–325

Hu P-J, Gan Y-Y, Tang Y-T, Zhang Q-F, Jiang D, Yao N, Qiu R-L (2012) Cellular tolerance, accumulation and distribution of cadmium in leaves of hyperaccumulator *Picris divaricata*. Pedosphere 22:497–507

Huguet S, Bert V, Laboudigue A, Barthes V, Isaure M-P, Llorens I, Schat H, Sarret G (2012) Cd speciation and localization in the hyperaccumulator *Arabidopsis halleri*. Environ Exp Bot 82:54–65

Ingle RA, Fricker MD, Smith JAC (2008) Evidence for nickel/proton antiport activity at the tonoplast of the hyperaccumulator plant *Alyssum lesbiacum*. Plant Biol 10:746–753

Isnard S, L'Huillier L, Rigault F, Jaffre´ T (2016) How did the ultramafic soils shape the flora of the New Caledonian hotspot? Plant Soil 403–2:53–76

Jaffre´ T (1993) The relationship between ecological diversity and floristic diversity in New Caledonia. Biodivers Lett 1:82–87

Jaffre´ T, Brooks RR, Lee J, Reeves RD (1976) *Sebertia acuminata*: a hyperaccumulator of nickel from New Caledonia. Science 193:579–580

Jaffre´ T, Brooks RR, Trow JM (1979a) Hyperaccumulation of nickel by *Geissois* species. Plant Soil 51:157–161

Jaffre´ T, Kersten W, Brooks RR, Reeves RD (1979b) Nickel uptake by Flacourtiaceae of New Caledonia. Proc R Soc Lond B Biol Sci 205:385–394

Jaffre´ T, Pillon Y, Thomine S, Merlot S (2013) The metal hyperaccumulators from New Caledonia can broaden our understanding of nickel accumulation in plants. Front Plant Sci 4:279. doi:10.3389/fpls.2013.00279

Johansson SAE, Campbell JL, Malmqvist KG (eds) (1995) Particle-Induced X-Ray Emission Spectrometry (PIXE). Wiley, Chichester

Kelly PC, Brooks RR, Dilli S, Jaffre´ T (1975) Preliminary observations on the ecology and plant chemistry of some nickel-accumulating plants from New Caledonia. Proc R Soc Lond B Biol Sci 189:69–80

Kersten W, Brooks RR, Reeves RD, Jaffre´ T (1979) Nickel uptake by New Caledonian species of Phyllanthus. Taxon 28:529–534

Kirkham R, Dunn PA, Kuczewski AJ, Siddons DP, Dodanwela R, Moorhead GF, Ryan CG, De Geronimo G, Beuttenmuller R, Pinelli D, Pfeffer M, Davey P, Jensen M, Paterson DJ, de Jonge MD, Howard DL, Küsel M, McKinlay J (2010) The Maia spectroscopy detector system: engineering for integrated pulse capture, Low-Latency Scanning and Real-Time Processing. AIP Conf Proc 1234:240–243

Koosaletse-Mswela P, Przybyłowicz WJ, Cloete KJ, Barnabas AD, Torto N, Mesjasz-Przybyłowicz J (2015) Quantitative mapping of elemental distribution in leaves of the metallophytes *Helichrysum candolleanum*, *Blepharis aspera*, and *Blepharis diversispina* from Selkirk Cu-Ni mine, Botswana. Nucl Instr Meth Phys Res B 363:188–193

Kozhevnikova AD, Seregin IV, Erlikh NT, Shevyreva TA, Andreev IM, Verweij R, Schat H (2014) Histidine-mediated xylem loading of zinc is a species-wide character in *Noccaea caerulescens*. New Phytol 203:508–519

Kozhevnikova AD, Seregin IV, Gosti F, Schat H (2016) Zinc accumulation and distribution over tissues in Noccaea caerulescens in nature and in hydroponics: a comparison. Plant Soil 411(1):5–16

L'Huillier L, Edighoffer S (1996) Extractability of nickel and its concentration in cultivated plants in Ni rich ultramafic soils of New Caledonia. Plant Soil 186:255–264

Ladiges PY, Udovicic F, Nelson G (2003) Australian biogeographic connections and the phylogeny of large genera in the plant family Myrtaceae. J Biogeogr 30:989–998

Lu LL, Tian SK, Yang XE, Wang XC, Brown P, Li TQ, He ZL (2008) Enhanced root-to-shoot translocation of cadmium in the hyperaccumulating ecotype of *Sedum alfredii*. J Exp Bot 59:3203–3213

Macnair MR, Smirnoff N (1999) Use of Zincon to study uptake and accumulation of zinc by zinc tolerant and hyperaccumulating plants. Commun Soil Sci Plant Anal 30:1127–1136

Mando PA, Przybyłowicz WJ (2009) Particle-Induced X-ray Emission (PIXE). In: Meyers RA (ed) Encyclopedia of Analytical Chemistry. Wiley, Chichester

Markowicz AA (2008) Quantification and correction procedures. In: Potts PJ, West M (eds) Portable X-ray fluorescence spectrometry capabilities for in situ analysis. Royal Society of Chemistry, Cambridge, Cambridge

McLaren TI, Guppy CN, Tighe MK (2012) Rapid and nondestructive plant nutrient analysis using portable X-ray fluorescence. Soil Sci Soc Am J 76:1446–1453

McRae R, Bagchi P, Sumalekshmy S, Fahrni CJ (2009) In situ imaging of metals in cells and tissues. Chem Rev 109:4780–4827

Mesjasz-Przybyłowicz J, Przybyłowicz WJ (2002) Micro-PIXE in plant sciences: present status and perspectives. Nucl Instr Meth Phys Res B 189(1):470–481

Mesjasz-Przybyłowicz J, Przybyłowicz WJ (2011) PIXE and metal hyperaccumulation: from soil to plants and insects. X-Ray Spectrom 40(3):181–185

Mesjasz-Przybyłowicz J, Balkwill K, Przybylowicz WJ, Annegarn HJ (1994) Proton microprobe and X-ray fluorescence investigations of nickel distribution in serpentine flora from South Africa. Nucl Instr Meth Phys Res B 89:208–212

Mesjasz-Przybylowicz J, Przybylowicz W, Barnabas A, van der Ent A (2016) Extreme nickel hyperaccumulation in the vascular tracts of the tree *Phyllanthus balgooyi* from Borneo. New Phytol 209:1513–1526

Miller LM, Dumas P (2006) Chemical imaging of biological tissue with synchrotron infrared light. Biochim Biophys Acta 1758:846–857

Mittermeier RA, Robles Gil P, Hoffmann M, Pilgrim J, Brooks T, Mittermeier CG, Lamoreux J, da Fonseca GAB (2004) Hotspots revisited: Earth's biologically richest and most endangered terrestrial ecoregions. CEMEX, Mexico City

Mizuno N, Nosaka S, Mizuno T, Horie K, Obata H (2003) Distribution of Ni and Zn in the leaves of *Thlaspi japonicum* growing on ultramafic soil. Soil Sci Plant Nutr 49:93–97

Moore KL, Schroder M, Wu Z, Martin BGH, Hawes CR, McGrath SP, Hawkesford MJ, Feng Ma J, Zhao FJ, Grovenor CRM (2011) High-resolution secondary ion mass spectrometry reveals the contrasting subcellular distribution of arsenic and silicon in rice roots. Plant Physiol 156:913–924

Moradi AB, Oswald SE, Nordmeyer-Massner JA, Pruessmann KP, Robinson BH, Schulin R (2010a) Analysis of nickel concentration profiles around the roots of the hyperaccumulator plant *Berkheya coddii* using MRI and numerical simulations. Plant Soil 328:291–302

Moradi AB, Swoboda S, Robinson B, Prohaska T, Kaestner A, Oswald SE, Wenzel WW, Schulin R (2010b) Mapping of nickel in root cross-sections of the hyperaccumulator plant *Berkheya coddii* using laser ablation ICP-MS. Environ Exp Bot 69:24–31

Morat P (1993) Our knowledge of the flora of New Caledonia: endemism and diversity in relation to vegetation types and substrates. Biodivers Lett 1:72–81

Morat P, Jaffre´ T, Veillon J-M (2001) The flora of New Caledonia's calcareous substrates. Adansonia 23:109–127

Morat P, Jaffre´ T, Tronchet F, Munzinger J, Pillon Y, Veillon J-M, Chalopin M (2012) The taxonomic database « FLORICAL » and characteristics of the indigenous flora of New Caledonia. Adansonia se´r 3(34):177–219

Murienne J (2009) Testing biodiversity hypotheses in New Caledonia using phylogenetics. J Biogeogr 36:1433–1434

Murienne J, Grandcolas P, Piulachs MD, Belle´s X, D'Haese C, Legendre F, Pellens R, Guilbert E (2005) Evolution on a shaky piece of Gondwana: is local endemism recent in New Caledonia? Cladistics 21:2–7

Myers N, Mittermeier RA, Mittermeier CG, da Fonseca GAB, Kent J (2000) Biodiversity hotspots from conservation priorities. Nature 403:853–858

Pallon J, Ryan CG, Arteaga MN, Elfman M, Kristiansson P, Nilsson EJC, Nilsson C (2009) STIM evaluation in GeoPIXE to complement the quantitative dynamic analysis. Nucl Instr Meth Phys Res B 267:2080–2084

Paterson D, de Jonge MD, Howard DL, Lewis W, McKinlay J, Starritt A, Kusel M, Ryan CG, Kirkham R, Moorhead G, Siddons DP (2011) The X-ray fluorescence microscopy beamline at the Australian Synchrotron. AIP Conf Proc 1365 (1):219–222

Pelletier B (2006) Geology of the New Caledonia region and its implications for the study of the New Caledonian biodiversity. In: Payri C, Richer de Forges B (eds) Compendium of marine species from New Caledonia. Centre IRD de Noume´a, Noume´a

Pillon Y (2012) Time and tempo of diversification in the flora of New Caledonia. Bot J Linn Soc 170:288–298 Pillon Y, Munzinger J, Amir H, Lebrun M (2010) Ultra- mafic soils and species sorting in the flora of New Caledonia. J Ecol 98:1108–1116

Pillon Y, Hopkins HCF, Rigault F, Jaffre T, Stacy EA (2014) Cryptic adaptive radiation in tropical forest trees in New Caledonia. New Phytol 202:521–530

Przybylowicz WJ, Mesjasz-Przybylowicz J, Prozesky VM, Pineda CA (1997) Botanical applications in nuclear microscopy. Nucl Instr Meth Phys Res B 130:335–345

Reeves RD, Baker AJM, Borhidi A, Berazaın R (1996) Nickel-accumulating plants from the ancient serpentine soils of Cuba. New Phytol 133:217–224

Reeves RD, Baker AJM, Becquer T, Echevarria G, Miranda ZJG (2007) The flora and biogeochemistry of the ultramafic soils of Goia´s state, Brazil. Plant Soil 293:107–119

Reidinger S, Ramsey M, Hartley S (2012) Rapid and accurate analyzes of silicon and phosphorus in plants using a portable X-ray fluorescence spectrometer. New Phytol 195:699–706

Ryan CG, Siddons DP, Kirkham R, Dunn PA, Kuczewski A, Moorhead G, De Geronimo G, Paterson DJ, de Jonge MD, Hough RM, Lintern MJ, Howard DL, Kappen P, Cleverley J (2010) The new Maia detector system: methods for high definition trace element imaging of natural material. AIP Conf Proc 1221:9–17

Ryan CG, Siddons DP, Kirkham R, Li ZY, Jonge MD, Paterson DJ, Kuczewski A, Howard DL, Dunn PA, Falkenberg G, Boesenberg U, Geronimo GD, Fisher LA, Halfpenny A, Lintern MJ, Lombi E, Dyl KA, Jensen M, Moorhead GF, Cleverley JS, Hough RM, Godel B, Barnes SJ, James SA, Spiers KM, Alfeld M, Wellenreuther G, Vukmanovic Z, Borg S (2014) Maia X-ray fluorescence imaging: capturing detail in complex natural samples. J Phys Conf Ser 499(1):012002

Sarret G, Pilon Smits EAH, Castillo Michel H, Isaure MP, Zhao FJ, Tappero R (2013) Use of synchrotron-based techniques to elucidate metal uptake and metabolism in plants. Adv Agron 119:1–82

Setoguchi H, Osawa TA, Pintaud JC, Jaffre T, Veillon JM (1998) Phylogenetic relationships within Araucariaceae based on rbcL gene sequences. Am J Bot 85:1507–1516

Sinclair SA, Sherson SM, Jarvis R, Camakaris J, Cobbett CS (2007) The use of the zinc-fluorophore, Zinpyr-1, in the study of zinc homeostasis in *Arabidopsis* roots. New Phytol 174:39–45

Smart KE, Kilburn MR, Salter CJ, Smith JAC, Grovenor CRM (2007) NanoSIMS and EPMA analysis of nickel localisation in leaves of the hyperaccumulator plant Alyssum lesbiacum. Int J Mass Spectrom 260:107–114

Smart KE, Smith JAC, Kilburn MR, Martin BGH, Hawes C, Grovenor CRM (2010) High-resolution elemental localization in vacuolate plant cells by nano- scale secondary ion mass spectrometry. Plant J 63(5):870–879

Spurr AR (1969) A low-viscosity epoxy-resin embedding medium for electron microscopy. J Ultrastruct Res 26:31–43

Swenson U, Munzinger J (2010) Taxonomic revision of *Pycnandra* subgenus *Trouettia* (Sapotaceae), with six new species from New Caledonia. Austral Syst Bot 23:333–370

Swenson U, Backlund A, McLoughlin S, Hill RS (2001) Nothofagus biogeography revisited with special emphasis on the enigmatic distribution of subgenus *Brassospora* in New Caledonia. Cladistics 17:28–47 Tian S, Lu L, Labavitch J, Yang X, He Z, Hu H,

Sarangi R, Newville M, Commisso J, Brown P (2011) Cellular sequestration of cadmium in the hyperaccumulator plant species *Sedum alfredii*. Plant Physiol 157:1914–1925

Tylko G, Mesjasz-Przybyłowicz J, Przybyłowicz WJ (2007a) X-ray microanalysis of biological material in the frozen-hydrated state by PIXE. Microsc Res Tech 70:55–68

Tylko G, Mesjasz-Przybylowicz J, Przybylowicz WJ (2007b) In-vacuum micro-PIXE analysis of biological specimens in frozen-hydrated state. Nucl Instr Meth Phys Res B 260:141–148

van der Ent A, Reeves RD (2015) Foliar metal accumulation in plants from copper-rich ultramafic outcrops: case studies from Malaysia and Brazil. Plant Soil 389:401–418

van der Ent A, Jaffre´ T, L'Huillier L, Gibson N, Reeves RD (2015a) The flora of ultramafic soils in the Australia–Pacific region: state of knowledge and research priorities. Aust J Bot 63:173–190

van der Ent A, Rajakaruna N, Boyd R, Echevarria G, Repin R, Williams D (2015b) Global research on ultramafic (serpentine) ecosystems (8th International Conference on Serpentine Ecology in Sabah, Malaysia). Aust J Bot 63:1–6

van der Ent A, Callahan DL, Noller BN, Mesjasz-Przybylowicz J, Przybylowicz WJ, Barnabas A, Harris HH (2017) Nickel biopathways in tropical nickel hyperaccumulating trees from Sabah (Malaysia). Sci Rep 7:41861. doi:10.1038/srep41861

Vavpetic P, Pelicon P, Vogel-Mikus K, Grlj N, Pongrac P,Jeromel L, Ogrinc N, Regvar M (2013) Micro-PIXE on thin plant tissue samples in frozen hydrated state: A novel addition to JSI nuclear microprobe. Nucl Instr Meth Phys Res B 306:140–143

Vijayan P, Willick IR, Lahlali R, Karunakaran C, Tanino KK (2015) Synchrotron radiation sheds fresh light on plant research: the use of powerful techniques to probe structure and composition of plants. Plant Cell Physiol 56:1252–1263

Wang YD, Mesjasz-Przybylowicz J, Tylko G, Barnabas AD, Przybylowicz WJ (2013) Micro-PIXE analyzes of frozen-hydrated semi-thick biological sections. Nucl Instr Meth Phys Res B 306:134–139

Watson RE, Perlman ML (1978) Seeing with a new light: synchrotron radiation. Science 199:1295–1302

Wu B, Zoriy M, Chen Y, Becker JS (2009) Imaging of nutrient elements in the leaves of *Elsholtzia splendens* by laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS). Talanta 78:132–137

Zhao F-J, Moore KL, Lombi E, Zhu Y-G (2014) Imaging element distribution and speciation in plant cells. Trends Plant Sci 19:183–192

## FIGURES AND TABLES

**Fig. 1** Field spot test based on the Ni-specific colorimetric reagent dimethylglyoxime demonstrating the reaction with Ni from the fresh leaf of the Ni hyperaccumulator species *Psychotria gabriellae* (Rubiaceae) in the field in New Caledonia. The test is fast and sensitive and has aided in the identification of numerous Ni hyperaccumulators globally.

**Fig. 2** The typical setup during XRF scanning of herbarium specimens, here conducted at the Forest Research Centre herbarium in Sabah, Malaysia. The herbarium specimen sheet is placed on a titanium metal sheet that serves as a X-ray radiation block, with the portable XRF device fitted with a backscatter shield placed. The XRF device is connected to a computer to read out the acquired data.

**Fig. 3** Examples of herbarium specimens from the IRD herbarium in New Caledonia. The panels illustrate some of the challenges encountered with measuring such specimens, including small leaves, clumped leaves and uneven plant parts.

**Fig. 4** Examples of elemental maps obtained with SEM-EDS analysis. The top left panel shows the typical SEM image, with the other panels showing the elemental maps of Ca, P and K respectively. The sample is a freeze-dried petiole cross-section of the Ni hyperaccumulator *Rinorea bengalensis* (Violaceae).

**Fig. 5** Colour-composite elemental maps of a hyperaccumulator (*Rinorea javanica*, Violaceae) leaf. The data was obtained with a Bruker microXRF instrument that was fitted with a 30-Watt X-ray source coupled to a polycapillary X-ray optics permitting down to 25 μm spot size.

**Fig. 6** The analytical measurement conditions of the XFM beamline at the Australian Synchrotron. The system consists of the Maia detector and motion stages with the samples mounted on Perspex frame (between sheets of Kapton thin film). In this experiment, a nitrogen 'cryo-stream' is used to keep the hydrated sample frozen.

**Fig. 7** Examples of elemental maps obtained with XFM analysis. The sample is a freeze-dried portion of a Ni hyperaccumulator leaf (*Actephila alanbakeri*, Phyllanthaceae). The top left panel shows the Compton map (which represents scattered X-rays and provides information on the sample structure and density), and the other panels show the elemental maps of Ca, Ni and Mn respectively.

**Fig. 8** Live plants (cowpea, *Vigna unguiculata*) to be analyzed with the Maia detector system at the XFM beamline of the Australian Synchrotron. The leaves were scanned (4 μm pixels, area of 25 mm 5 mm) repeatedly for up to 48 h after exposure to 30 μM Mn.

**FIGURE 1**

**FIGURE 2**

**FIGURE 3**


L. Barrabé
1161
Herbier IRD de Nouvelle-Calédo
Myrtaceae Juss.
Cloezia aquarum (Guillaumin) J. W. Dawson
Dét. : L. Barrabé
Nouvelle-Calédonie
Province Sud, Creek Pernod
Herbier de Nouve

**FIGURE 4**

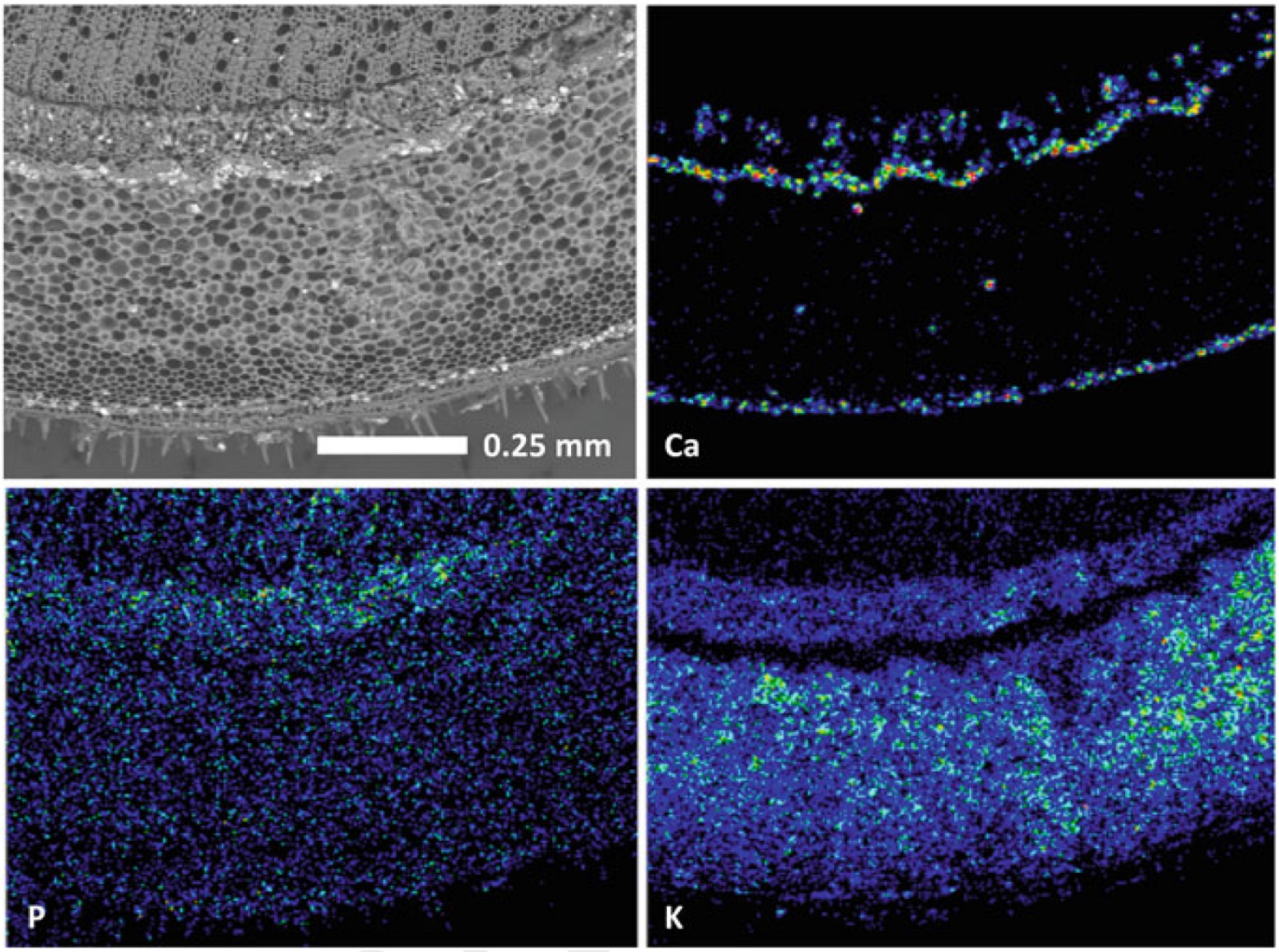

**FIGURE 5**

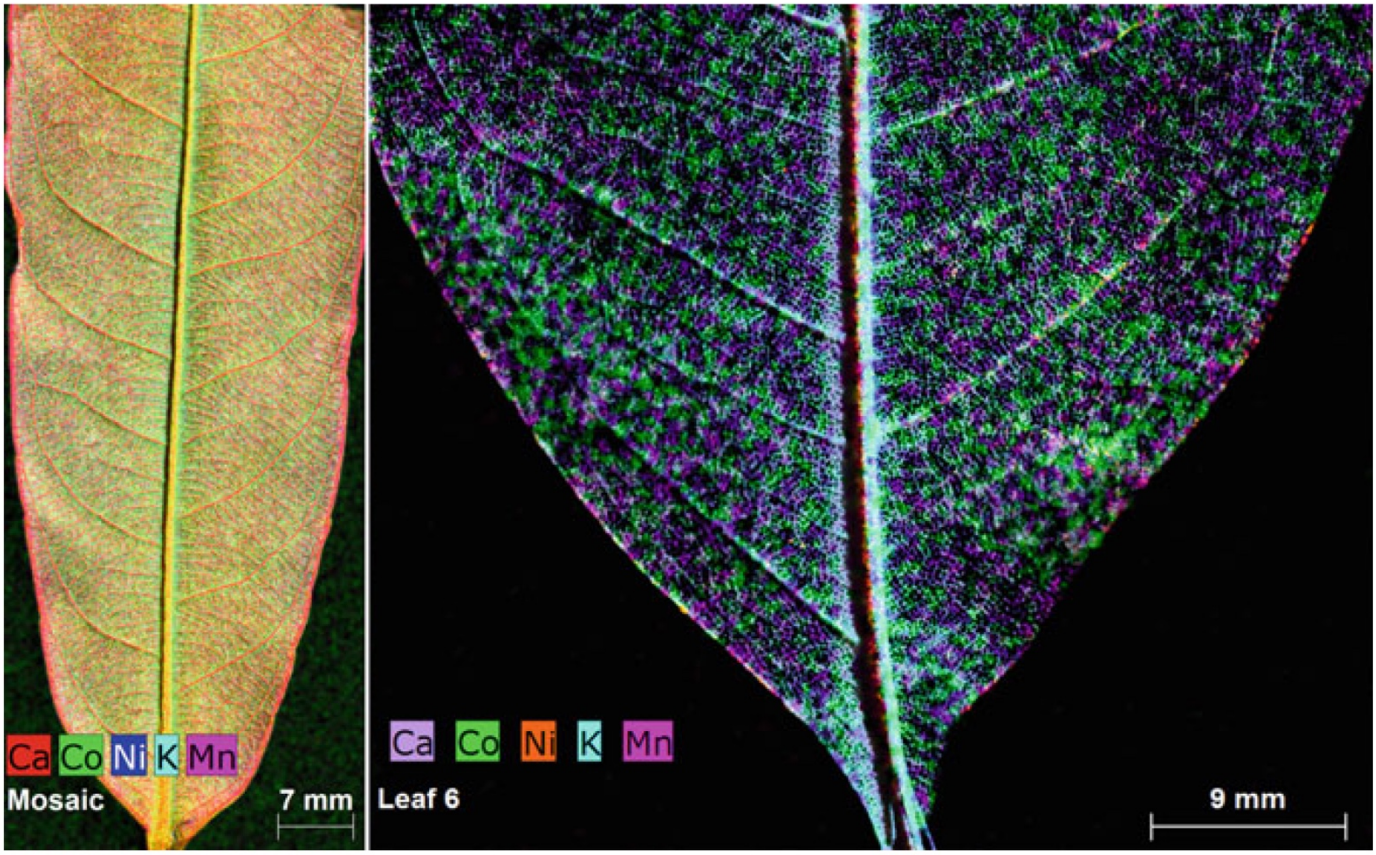

**FIGURE 6**

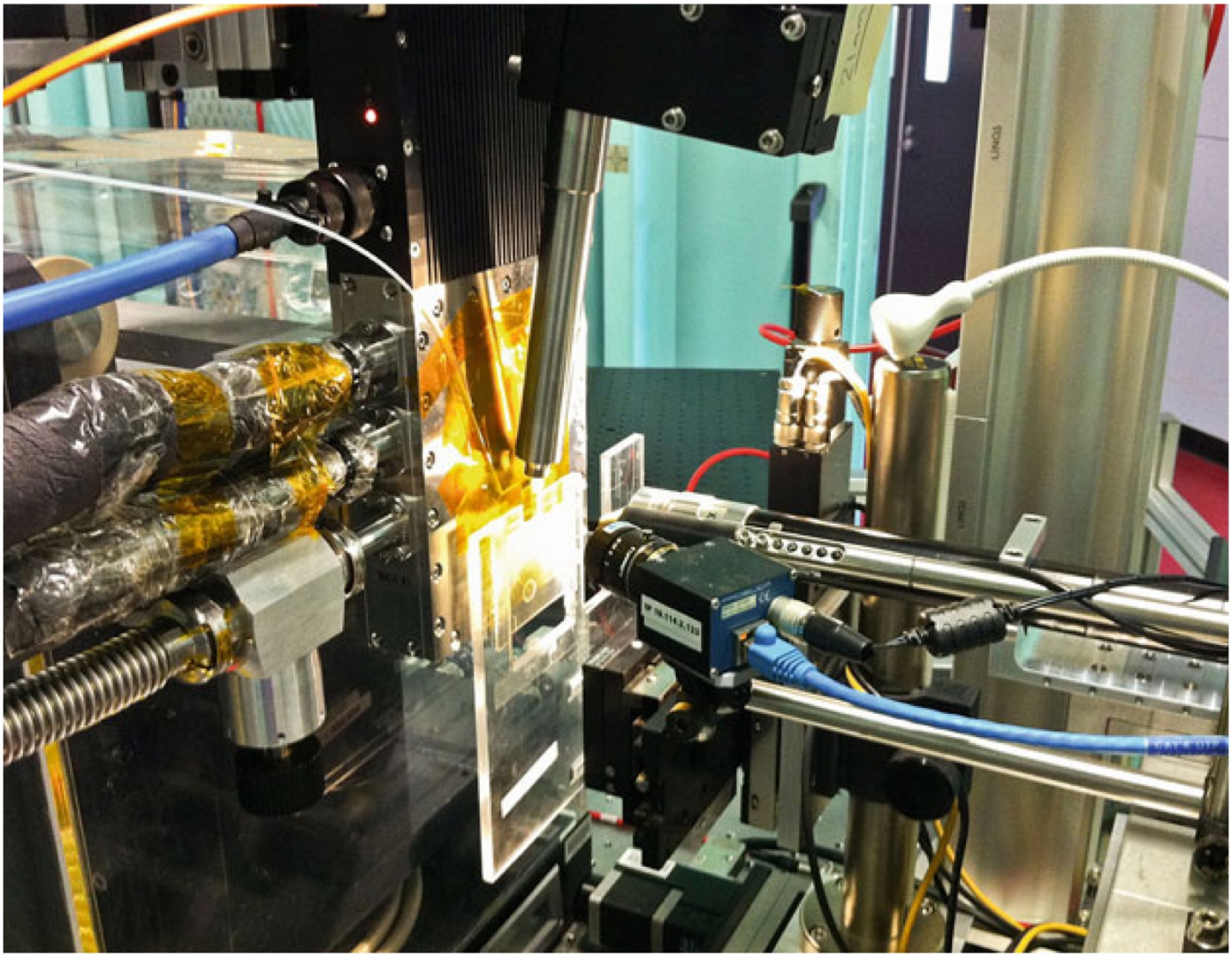

**FIGURE 7**

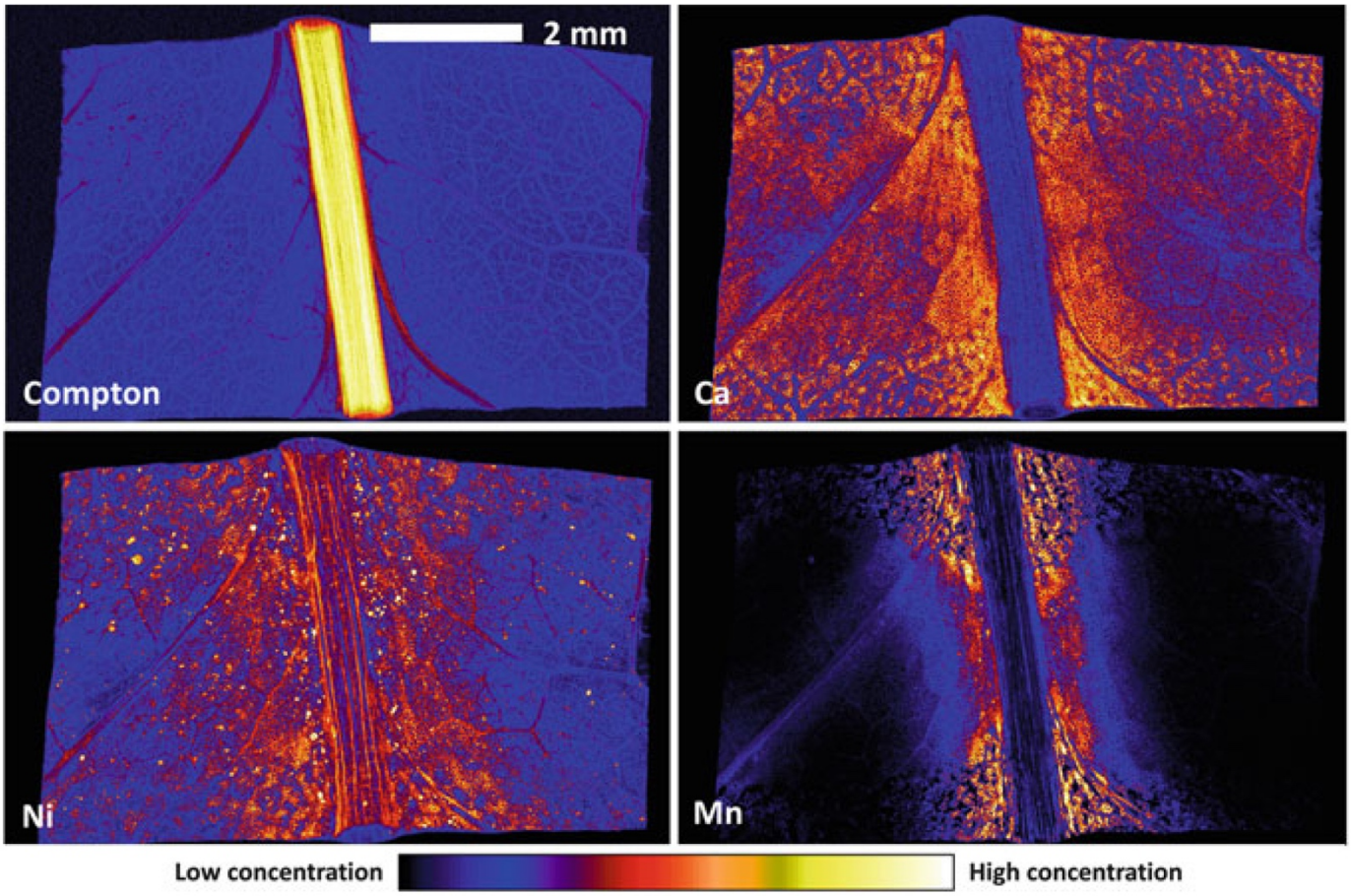

**FIGURE 8**