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

Vidiro Gei, Peter D. Erskine, Hugh H. Harris, Guillaume Echevarria, Jolanta Mesjasz-Przybyłowicz, Alban D. Barnabas, Wojciech J. Przybyłowicz, Peter M. Kopittke, and Antony van der Ent

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

V. Gei • P.D. Erskine
Centre for Mined Land Rehabilitation, Sustainable Minerals Institute, The University of Queensland, St Lucia, QLD, Australia

H.H. Harris
Department of Chemistry, The University of Adelaide, Adelaide, SA, Australia

G. Echevarria
Laboratoire Sols et Environnement, UMR 1120, Université de Lorraine-INRA, Vandoeuvre-lès-Nancy, France

J. Mesjasz-Przybyłowicz • A.D. Barnabas
Materials Research Department, iThemba Laboratory for Accelerator Based Sciences (iThemba LABS), Somerset West, South Africa

W.J. Przybyłowicz
Faculty of Physics & Applied Computer Science, AGH University of Science and Technology, Kraków, Poland

Materials Research Department, iThemba Laboratory for Accelerator Based Sciences (iThemba LABS), Somerset West, South Africa

P.M. Kopittke
School of Agriculture and Food Sciences, The University of Queensland, St Lucia, QLD, Australia

A. van der Ent (✉)
Centre for Mined Land Rehabilitation, Sustainable Minerals Institute, The University of Queensland, Brisbane, Australia

Laboratoire Sols et Environnement, UMR 1120, Université de Lorraine-INRA, Vandoeuvre-lès-Nancy, France
e-mail: a.vanderent@uq.edu.au

with light microscopy further aids in the identification of anatomical and structural features of the studied plant tissues.

1 Introduction

1.1 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 ultramafic 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; Jaffré 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 gain further insights into the ecophysiology of hyperaccumulator plant species. The test case of New Caledonia is used to illustrate the application of XRF screening for the discovery of hyperaccumulator plants.

2 Handheld X-Ray Fluorescence Technology for Herbarium Screening

In order for hyperaccumulator species to be discovered, 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 $\mu g\ g^{-1}$ 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 powdering 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

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

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

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

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 therefore 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.

2.1 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 km^2 (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 history (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 Jaffré (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 km^2) 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).

2.2 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). Jaffré 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 (Jaffré 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.

2.3 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 biogeographical and phylogenetic studies. This approach also facilitates the selection of plant species targeted for more detailed investigations, using the methods described below.

3 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:

1. **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).
2. **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.
3. **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 transport 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 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 cryogenic temperature (-130 °C or colder) until micro-analytical examination, or they can be freeze-dried.

4   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

SEM/TEM-EDS which is comparatively readily accessible within most large research institutions.

4.1   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 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.

4.2   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
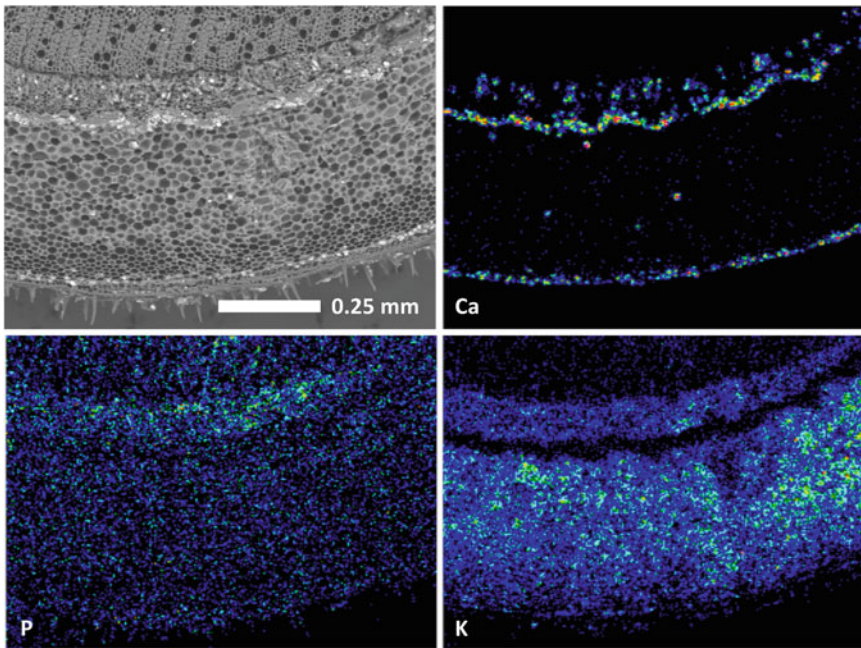
Fig. 4 Examples of elemental maps obtained with SEM-EDS analysis. The top left panel shows the typical SEM image, with the other panels showing the elemental maps of Ca, P and K respectively. The sample is a freeze-dried petiole cross-section of the Ni hyperaccumulator *Rinorea bengalensis* (Violaceae)
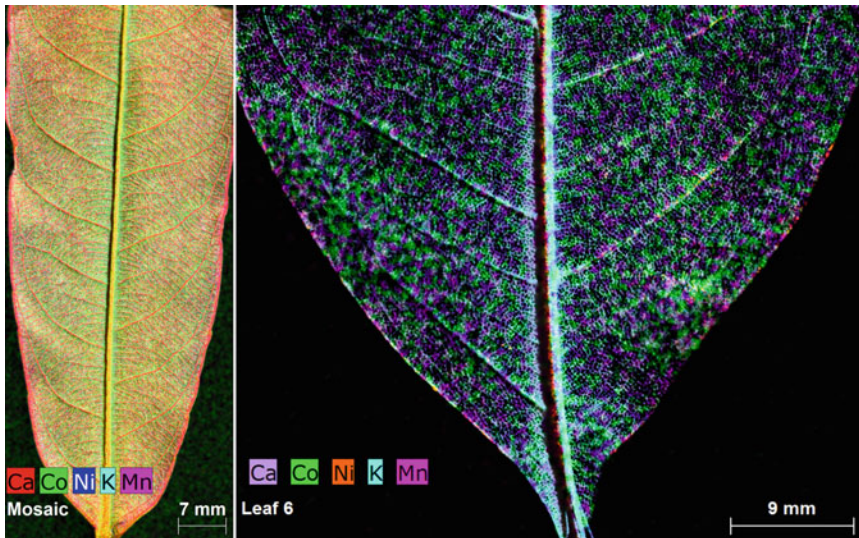

Fig. 5 Colour-composite elemental maps of a hyperaccumulator (*Rinorea javanica*, Violaceae) leaf. The data was obtained with a Bruker microXRF instrument that was fitted with a 30 Watt X-ray source coupled to a polycapillary X-ray optics permitting down to 25 μm spot size

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
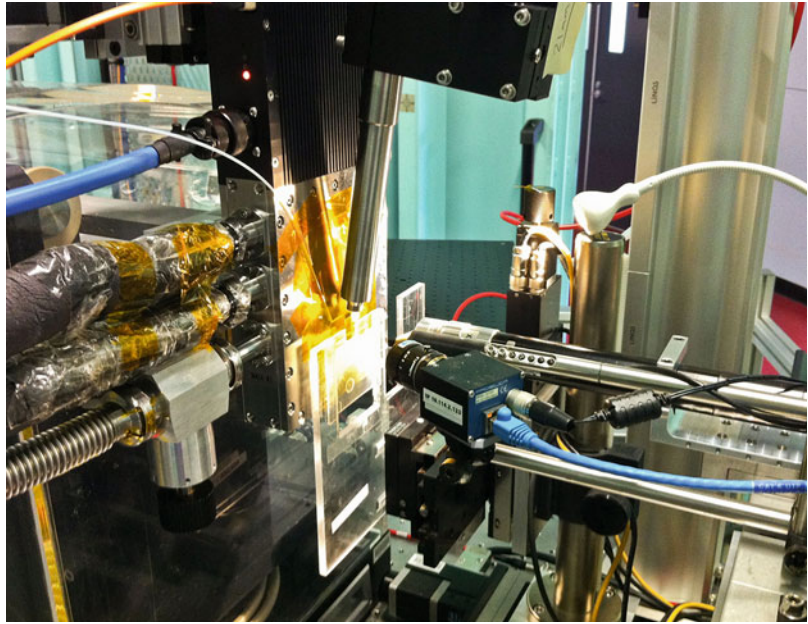
leaves or other plant parts can be analyzed directly), however, it is preferable that the leaf samples are dehydrated to increase relative elemental concentration during analysis, and to reduce scattering due to water content.

4.3 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 micro-computed 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 10^7 s^{-1}, with pixel transit times as small as 50 μs, thereby enabling the production of megapixel elemental maps (Ryan et al. 2010, 2014; Kirkham et al. 2010). The use of these new-generation detector systems permits new
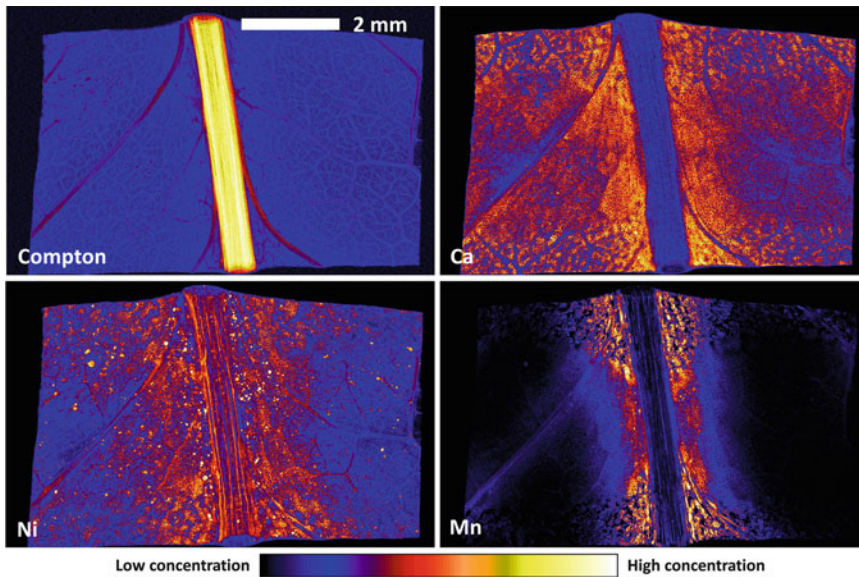
Fig. 7 Examples of elemental maps obtained with XFM analysis. The sample is a freeze-dried portion of a Ni hyperaccumulator leaf (*Actephila alanbakeri*, Phyllanthaceae). The top left panel shows the Compton map (which represents scattered X-rays and provides information on the sample structure and density), and the other panels show the elemental maps of Ca, Ni and Mn respectively

insights in both hyperaccumulators and other plant species. It is now possible to image living plants repeatedly following exposure to elevated levels of trace metals (such as Ni or Mn) to examine progressive changes in metal distribution within plant tissues. For example, using the non-accumulating plant species, cowpea (*Vigna unguiculata*), leaves were scanned repeatedly for up to 48 h after exposure to 30 µM Mn. In this study, images were obtained six times over 48 h, with the images having 4 µm × 4 µm pixels collected over an area of 25 mm × 5 mm (see Fig. 8). An essential component of such studies, however, is to determine that such repeated scanning does not cause damage to the living leaf tissues and hence experimental artefacts.

4.4 PIXE

In micro-PIXE, characteristic X-rays of elements present in analysed samples are excited with focused ion beams, typically protons with energies of 2–3 MeV (Johansson et al. 1995;

Mandò and Przybyłowicz 2009). This technique allows quantitative studies of concentration and distribution of elements from Na to U with detection sensitivity down to 1–10 µg g^{-1} for transition elements, and lateral resolution of the order of 1 µm. Such a broad elemental range enables quantification of a wide range of elements such as S, P, K, Ca, Mn, Fe, Cu and Zn (Mesjasz-Przybyłowicz and Przybyłowicz 2002, 2011). Simultaneous use of at least one additional analytical technique is a common practice and the facility is referred to as a nuclear microprobe or a proton microprobe, because a proton beam is most often used. All research related to hyperaccumulator plants using PIXE has been carried out using protons and beam spot sizes of the order of a few µm. Typically, areas up to 2.5 mm × 2.5 mm can be analysed in a single scan, but mapping of larger structures requires specimen movement and mosaicking of maps. Specimens of any thickness can be analysed. Fundamentally PIXE is a standardless method that uses a fundamental parameter model approach. Once the experimental system has been calibrated,

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

no additional standards matching the composition of biological samples are necessary. Quantification of analyses is obtained by direct measurements of accumulated charge deposited by protons or a value proportional to it. Simultaneously used proton backscattering spectrometry (BS) allows for the determination of specimen thickness (areal density) and the concentrations of main components of a biological material–C, O, N and indirectly H. 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; Vavpetič 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).

4.5 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 (Dé 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*.

4.6 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*.

4.7 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).

5 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 (C_2H_6O) series, with or without propylene oxide (C_3H_6O), 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 ($UO_2(CH_3COO)_2$) and

lead citrate ($C_6H_5O7)_2Pb_3$) (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.

5.1 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).

5.2 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 Ni^{2+} 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.

6 Conclusions and Outlook

The use of a portable XRF-instruments enable 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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