Nuclear microprobe studies of metal(loid)s distribution in hyperaccumulating plants

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Abstract

Micro-proton-induced X-ray emission (μ-PIXE) spectroscopy was used to determine *in situ* elemental concentrations of nickel (Ni) and arsenic (As) in leaf and stem tissues of hyperaccumulating plants *Hybanthus floribundus* subsp *floribundus* and *Pityrogramma calomelanos* var. *austroamericana*, respectively. Nickel concentration in seeds of *H. floribundus* subsp *floribundus* was also investigated. Both species were grown in metal(loid) contaminated potting mix for 20 weeks duration under controlled glasshouse conditions. Leaf and stem samples were hand-sectioned, cryo-fixed and freeze-dried in liquid nitrogen before μ-PIXE analysis using the 10-MV tandem accelerator at the Australian Nuclear Science and Technology Organization. In *H. floribundus* subsp *floribundus* leaves, Ni was highest in the adaxial epidermal cells (1% dry weight; DW) and least in spongy mesophyll (0.53% DW). In stem tissues, Ni concentrations were highest in the collenchyma (0.25% DW) and there was no clear pattern of Ni localization in seeds. In *P. calomelanos* pinnules, As localization was relatively uniform across the whole specimen and in stipe tissues, highest concentration occurred in the vascular bundle (0.2% DW). These results suggest that hyperaccumulating plants sequester excess metal(loid)s in different cellular loci and enables us to better understand the physiology and ecology of these hyperaccumulating species.

Introduction

Micro-proton-induced X-ray emission (μ-PIXE) spectroscopy provides pertinent quantitative information as to the spatial distribution of elements in metal(loid) hyperaccumulating plants [1-5]. The phenomenon of metal(loid) hyperaccumulation is expressed in *ca.* 400 species worldwide that exhibit the unique ability of elevated metal(loid) accumulation in aboveground tissues without adverse effects on plant growth. The criteria to define a nickel (Ni) and arsenic (As) hyperaccumulator is any plant species exceeding 1000 mg Ni or As kg^{-1} dry weight (DW) in any above-ground tissue [6-8]. The Ni hyperaccumulating *Hybanthus floribundus* subsp. *floribundus* or shrub violet (Violaceae) is a native Australian perennial shrub and has been reported to hyperaccumulate up to 13 500 mg As kg^{-1} DW [9]. *Pityrogramma calomelanos* var. *austroamericana*, or gold fern (Pteridaceae) is a perennial and rhizomatous fern native to South America and has been reported to hyperaccumulate up to 3330 mg As kg^{-1} DW in fronds[10].

The knowledge of spatial distribution and localisation of metal(loids)s within hyperaccumulating tissues is paramount to comprehend the processes underlying metal tolerance and hyperaccumulation in plants. Moreover, few studies have investigated the distribution of metal(loid)s in reproductive tissues (seeds) of hyperaccumulating species. Therefore, the aim of this study was to quantify the spatial distribution of Ni in leaf, stem and seed tissues of *H. floribundus* subsp

floribundus, and As in pinnule and stipe tissues of *P. calomelanos* var. *austroamericana*.

Material and methods

Hybanthus floribundus subsp. *floribundus* plants were exposed to 0 mM and 26 mM Ni kg^{-1} and *P. calomelanos* var. *austroamericana* 0 mM and 20 mM As kg^{-1} for a period of 20 weeks. Plants were grown under controlled glasshouse conditions at the University of Sydney and replicated thrice. For μ-PIXE analysis, samples were prepared following the procedure outlined by Bhatia et al. [1]. Plant material was rinsed with deionised water; excised; hand-sectioned with a stainless steel razor blade, cryo-fixed in liquid nitrogen and sandwiched between Formvar films. Additionally, air-dried seeds (*ca.* 6 % moisture) of *H. floribundus* subsp. *floribundus* procured from Kings Park Botanic Gardens (Perth, Western Australia) were sectioned (transversely) and mounted onto carbon tape for irradiation.

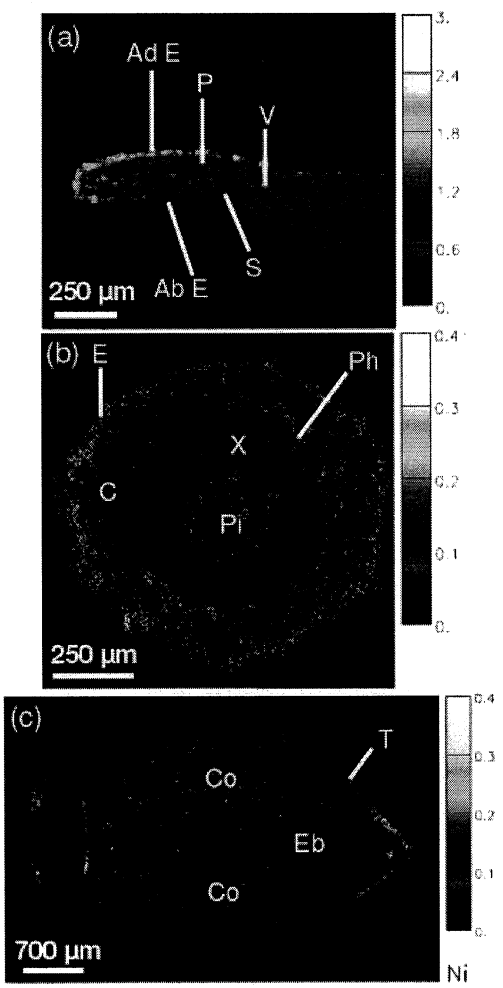

Fig. 1 Quantitative elemental map showing Ni in a *Hybanthus floribundus* subsp. *floribundus* leaf (a), stem (b) and seed (c) cross-section. Scale of intensity is Ni weight % on a dry weight basis. Ad/Ab E, Adaxial/Abaxial epidermis; P, palisade mesophyll; V, vascular tissue; S, spongy mesophyll; X, xylem, C, collenchyma; Pi, pith; Ph, phloem; Co, cotyledon ; T, testa and Eb, embryonic stem

Nuclear microprobe analyses were performed using a 10 MV Tandem accelerator at the Australian Nuclear Science and Technology Organisation which provided a 3 MeV proton beam for nuclear microprobe analyses [11]. Region selection analysis (RSA) using the Dynamic Analysis method of GeoPIXE II [12] was used to select various regions within plant sections from on-screen distribution maps reproduced from data accumulated by the Oxford Microprobe. The results from RSA were statistically analysed using Genstat version 8 [13]. Representative tissue specimens were also sampled in parallel with the material excised for μ-PIXE analysis for bulk chemical analysis. Briefly, samples were oven-dried, ground to <1mm, digested in concentrated acids [14] and analysed for Ni or As using a Vista CCD Varian® inductively coupled plasma-atomic emission spectrometer (ICP-AES).

Results and Discussion

The concentration of Ni in *H. floribundus* subsp. *floribundus* leaf sections was in good agreement with bulk tissue analysis and revealed variable concentrations amongst tissue types (Figure 1a). The highest concentration of Ni was found in the adaxial epidermal tissues (1.0×10^4 mg kg^{-1} DW) and the lowest concentration of Ni was observed in the spongy mesophyll tissues (5.3×10^3 mg kg^{-1} DW). The results presented here support the qualitative

observation by Farago et al. [15] and Severne [16] who used histochemical techniques to show the presence of Ni in epidermal cells of *H. floribundus* leaves. Preferential localisation of Ni has been reported in epidermal tissues of several Ni hyperaccumulating species [2,17]. It has been suggested that sequestration of metals such as Ni to epidermal tissue minimises the disruption of photosynthetic processes that occur primarily in the mesophyll symplast [18].

Elemental map of a stem section of *H. floribundus* subsp. *floribundus* revealed variable concentrations of Ni among tissue types (Figure 1b) and followed the order collenchyma > epidermis > phloem > xylem > pith. The observed pattern of Ni localisation in this study is similar to the that reported in stem tissue of the Ni hyperaccumulator *Senecio coronatus* [19]. These authors noted that stem epidermal and cortical cells acted as a depository of Ni and contained up to 3.4×10^4 mg Ni kg^{-1} DW. Nickel enrichment in outer tissues of stem and leaf tissue may act as a chemical defence against insect herbivory [18], as well as fungal and bacterial pathogens [20,21]. Further biochemical investigations are currently underway to elucidate precise detoxification mechanisms in this species.

In *Hybanthus floribundus* subsp. *floribundus* seeds, Ni concentrations were variable (Figure 1c). Nickel concentrations were higher within embryonic tissues (cotyledons and embryonic stem) than the seed coat, however the differences were non-significant. This pattern of localization suggests apoplastic movement of Ni within the seeds of *H. floribundus* subsp. *floribundus*. The results presented in this study are similar to those observed in Ni hyperaccumulating *Thlaspi pindicum* where preferential Ni accumulation occurred in the cotyledon epidermis [22]. It is possible that the degree of Ni concentration in seeds may contribute to the poor germination of *H. floribundus* subsp. *floribundus* [23,24], however further investigations are warranted.

The concentration of As in *P. calomelanos* var. *austroamericana* pinnule sections varied among tissue types (Figure 2a). The highest As concentration occurred in vascular and spongy mesophyll tissues (4.1×10^3 mg kg^{-1} DW), however the differences were non-significant. The cellular distribution of As in our study is in agreement with those reported by Chen et al. [25] who investigated As localization in *P. cretica* var. *nervosa* pinnae sections. These authors reported a higher As content in mesophyll as opposed to epidermal tissues. In a later study, Chen et al. [26] indicated that As in the midrib efficiently translocated to surrounding mesophyll tissues in *P. vittata* pinna.

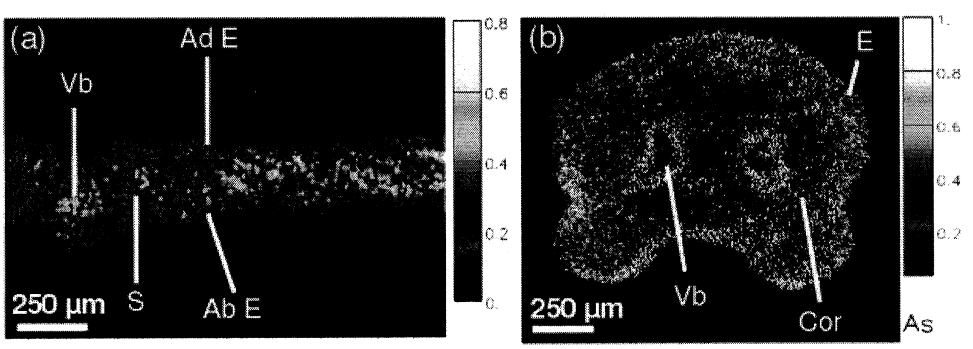

Fig. 2 Quantitative elemental map showing As in a *Pityrogramma calomelanos* var. *austroamericana* pinnule (a) and stipe (b) cross-section. Scale of intensity is As weight % on dry mass basis. Ad/Ab E, Adaxial/Abaxial epidermis; S, spongy mesophyll; Vb, vascular bundle and Cor, cortex.

In *P. calomelanos* var. *austroamericana* stipe sections, As concentrations varied among tissue types (Figure 2b), and was highest in vascular bundles (2.0×10^3 mg kg^{-1} DW). Concentration of As in stipe sections was almost 2.3-fold lower than in whole pinnule sections and followed the order vascular bundle > cortex > epidermis. This is the first μ-PIXE study on an As hyperaccumulator, with the majority of studies focused on Ni hyperaccumulators [2,17]. The reasoning behind Ni enrichment discussed above does not justify the localization pattern of As observed in *P. calomelanos* var. *austroamericana* tissues and suggests that strategies other than cellular localization may be responsible for As homeostasis in this species. A synchrotron-based study is currently underway in our laboratory to determine As chelation and speciation at various loci throughout fresh hydrated tissues of this species.

Conclusions

This study demonstrates a varying degree of physiological adaptation of hyperaccumulating plants in response to excess meatl(loid)s. In Ni hyperaccumulator *H. floribundus* subsp. *floribundus*, Ni is sequestered into physiologically inert tissues and may contribute to plant defence against insect, pathogen or fungal attack. In *H. floribundus* seeds, Ni was relatively uniform across cellular regions and the degree of accumulation may inhibit seed viability and germination. In *P. calomelanos* pinnule and stipe tissues, As was relatively uniform across tissue types and there was no clear reasoning to support this observed pattern of localisation. The results suggest that there is no consistent pattern of cellular metal(loid) localisation in hyperaccumulating plants and may be specific for heavy metal(loid) or genotype.

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