Studies on spatial distribution of nickel in leaves and stems of the metal hyperaccumulator *Stackhousia tryonii* using nuclear microprobe (micro-PIXE) and EDXS techniques

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Abstract. *Stackhousia tryonii* Bailey is one of the three nickel hyperaccumulators reported from Australia. It is a rare, herbaceous plant that accumulates nickel (Ni) both in leaf and stem tissues. Localisation of Ni in leaf and stem tissues of *S. tryonii* was studied using two micro-analytical techniques, energy dispersive X-ray spectrometry (EDXS) and micro-proton-induced X-ray emission spectrometry (micro-PIXE). Dimethylglyoxime complexation of Ni was also visualised by bright- and dark-field microscopy, but this technique was considered to create artefacts in the distribution of Ni. Energy dispersive X-ray spectrometric analysis indicated that guard cells possessed a lower Ni concentration than epidermal cells, and that epidermal cells and vascular tissue contained higher levels of Ni than mesophyll, as reported for other Ni hyperaccumulators. The highest Ni concentration was recorded (PIXE quantitative point analysis) in the epidermal cells and vascular tissue (5400 µg g\textsuperscript{-1} DW), approximately double that recorded in palisade cells (2500 µg g\textsuperscript{-1} DW). However, concentrations were variable within these tissues, explaining, in part, the similarity between average Ni concentrations of these tissues (as estimated by region selection mode). Stem tissues showed a similar distribution pattern as leaves, with relatively low Ni concentration in the pith (central) region. The majority of Ni (73–85% for leaves; 80–92% for stem) was extracted from freeze-dried sections by water extraction, suggesting that this metal is present in a highly soluble and mobile form in the leaf and stem tissues of *S. tryonii*.

Keywords: elemental mapping, metal hyperaccumulation, micro-PIXE, nickel, nuclear microprobe analysis.

Introduction

Accumulation of certain metal(loid)s [arsenic (As), cadmium (Cd), cobalt (Co), copper (Cu), manganese (Mn), Ni, zinc (Zn) and lead (Pb)] in plants above threshold concentration is generally phytotoxic. However, a small group of plants (also called metallophytes), occurring on metal-enriched soils, has the capacity to accumulate (one or more of) these metals in concentrations that are orders of magnitude higher than in plants that occur on normal soils (Baker and Brooks 1989). Hyperaccumulation of metals by plants is an extremely rare phenomenon (exhibited by <0.2% of angiosperms; Baker et al. 2000). A nickel hyperaccumulator is defined as a plant with Ni concentrations exceeding 1000 µg g\textsuperscript{-1} DW (0.1%) in any above-ground tissue (Reeves 1992).

All the Ni hyperaccumulating plants reported so far are endemic to soils derived from ultramafic (serpentinite) rocks. These soils are infertile owing to low concentrations of major...
plant nutrients (e.g. nitrogen, phosphorus, potassium and calcium), and elevated concentrations of elements such as, nickel, chromium, cobalt, magnesium, iron and manganese. Ultramafic is relatively rare in Australia compared to other continents, due to a history of tectonic stability since the Palaeozoic (Davie and Benson 1997), and occur mainly in New South Wales, Queensland, Victoria and Western Australia. In Queensland, the three disjunctive outcrops occur in the northern, central and south-eastern parts of the state. In central Queensland, serpentinite belt occurs just north of the Tropic of Capricorn and covers approximately 100 000 ha in patches between Marlborough in the north and Canoona and Bondoola in the south (Fig. 1) (Murray 1969; Forster and Baker 1997).

Stackhousia tryonii is one of the three Ni hyperaccumulating plants reported from Australia (Severne 1974; Batianoff et al. 1990; Batianoff and Specht 1992). It is a rare, herbaceous plant that exhibits great serpentinic fidelity, and accumulates Ni both in leaf and stem tissues to levels up to 41 300 and 7100 µg g\(^{-1}\) DW, respectively (Batianoff et al. 1990; Batianoff and Specht 1992).

An understanding of the physiology of metal hyperaccumulation requires quantitative studies of the distribution of metals within plant tissues. Tolerance to and

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**Fig. 1.** Distribution of serpentine rocks (shaded) and soils in central Queensland.
Spatial distribution of Ni in *Stackhousia tryonii*

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Hyperaccumulation of toxic metals by plants presumably requires formation of organo-metallic complex(es), associated with organic compounds such as oxygen donor ligands (e.g. carboxylates), sulphur donor ligands (e.g. metallothioneins and phytochelatins) or nitrogen donor ligands (e.g. amino acids) (Baker et al. 2000), with transport, compartmentalisation and storage of these complexes within the vacuoles of ‘storage’ cells. These storage cells may play an ecophysiological role (e.g. epidermal storage, anti-herbivory or pathogenicity). In most hyperaccumulating plants, metal concentrations (per unit dry matter) are greater in leaf than in stem and in stem than in root tissue (Mesjasz-Przybylowicz et al. 1994, 2001b; Robinson et al. 2003). Metal accumulation in the epidermal and sub-epidermal cells of the leaf appears to be a common feature of metal accumulating plants (Table 1).

For example, in Ni hyperaccumulator *Senecio coronatus* (Thunb.) Harv., the ratio of Ni (micro-PIXE analysis) in whole leaf to epidermis was 1 : 3.3 (Mesjasz-Przybylowicz et al. 1994). Nickel is preferentially localised in the leaf epidermal cells of *Alyssum lesbiacum* (Candargy) Rech F., *A. bertolonii* Desv., and *Thlaspi goessingense* Hálácsy (Küpfer et al. 2001), *Senecio anomalochrous* Bulliard (Balkwill 6869J), (Mesjasz-Przybylowicz et al. 2001a), *A. euboeum* Hálácsy, *A. heldreichii* Hausskn., *Leptoplax emarginata* (Boiss.) O.E. Schulz, and *Thlaspi pindicum* Hausskn. (Psaras et al. 2000), *Hybanthus floribundus* (Lindley) F. Muell (Bidwell et al. 2004, Severne 1974) and *Thlaspi japonicum* Boiss. (Mizuno et al. 2003); epidermal cells of leaf midrib and leaf margin of *Berkheya coddii* Roessler

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Table 1. Review of application of microanalytical techniques (PIXE and EDXS) in nickel hyperaccumulating plants (vegetative parts only)
Materials and methods

The spatial localisation of metals within biological tissues can be typically quantified using micro-analytical techniques based on X-ray emission, following irradiation by charged particles (electrons, protons or heavier particles). Micro-PIXE (micro proton-induced X-ray emission spectrometry), also known as nuclear microprobe) and EDXS (energy dispersive X-ray spectrometry) simultaneously measure and map elemental content. Higher analytical sensitivities are obtainable using micro-PIXE than EDXS (Przybylowicz et al. 2001). Density correction routines based on Rutherford backscattering have improved the accuracy of micro-PIXE analysis relative to EDXS analysis. Our previous work (Bhatia et al. 2003) confirmed the reliability of micro-PIXE as a technique for measurement of elements (including Ni) within biological tissues (fruits of S. tryonii), and the results were comparable with those obtained using ICP-OES (inductively coupled plasma-optical emission spectrometry).

The application of micro-PIXE is typically limited by accessibility to a proton beam facility. Of the 13 publications reporting application of micro-PIXE technique for studying localisation of metals in metal-hyperaccumulating plants, 10 represent the work of one group at the National Accelerator Centre (NAC), Faure, South Africa (Table 1). The Australian scientific community has access to this technology through the Australian Nuclear Science and Technology Organisation (ANSTO). Two conference reports have considered the distribution of Ni in S. tryonii. Noell and Morris (1997) employed EDXS techniques and reported higher concentrations of Ni in the epidermis than in other leaf tissues of S. tryonii. However, Bhatia et al. (2001) found that Ni accumulated on the leaf surface (cuticle) of this species. It was also reported that Ni was also present in substantial concentrations within guard cells (Noell and Morris 1997; Bidwell 2001). Neither study considered the distribution of Ni in stem tissues. The latter point is of interest as S. tryonii has reduced leaves, with the stem acting as the primary photosynthetic organ.

In the present investigation, a combination of SEM (scanning electron microscope)-EDXS and micro-PIXE techniques were employed to map the spatial distribution of metals within transversely-cut leaf and stem sections of S. tryonii. Energy dispersive X-ray spectrometry was employed as a qualitative tool, while ‘region selection’ and ‘quantitative point analysis’ techniques of Dynamic Analysis (micro-PIXE) were used for quantitative assessment of localised Ni within leaf and stem tissue sections of S. tryonii.

Fig. 2. A young Stackhousia tryonii plant growing naturally on a serpentinite soil in central Queensland.
Spatial distribution of Ni in *Stackhousia tryonii* Functional Plant Biology

34 mM Ni solution (as NiSO₄.6H₂O) per pot, a fortnight before specimen collection. Young branches (which grew during pot culture) of *S. tryonii* were excised from the centre of the plant. The basal leaves on these branches were separated from the stem by cutting at the base. The leaves were further cut into two halves (across the longitudinal axis) using a sharp razor blade. The stem was cut into 8–10 mm long segments.

**Sample preparation**

- **SEM-EDXS study**
  - Tissue was prepared by three methods:
    - Chemical fixation and critical-point drying
      - Leaf segments were soaked overnight in DMG (1% solution in 95% ethanol) at room temperature and fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 6.9) overnight in a cold room at 8°C. Fixed specimens were rinsed twice for 2 min in distilled water and dehydrated in steps with an ascending series of aqueous acetone (10% v/v; each step for 10 min). Dehydrated specimens were critical point dried in a Polaron E3000 (Warford, England) overnight as acetone as the intermediate fluid. Another set of specimens was treated in exactly the same way but without soaking in DMG. The critical point dried specimens were cut into halves with a sharp razor blade and were mounted onto grooves cut into aluminum stubs to expose the cross-sections. After X-ray (EDXS) analysis, the specimens were gold coated for 10 min in a Bio-Rad Polaron SC515 SEM coating system (Fisons Instruments, East Sussex, UK) before photomicrographs of the analysed tissue were taken.
    - Resin embedding
      - Leaf segments (cut transversely into two pieces) were chemically fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 6.9) overnight in a cold room at 8°C. Fixed specimens were rinsed twice for 2 min in distilled water and dehydrated in steps with an ascending series of aqueous acetone (10% w/v; each step for 10 min). The dehydrated specimens were infiltrated with LR White resin (ProSciTech, Queensland, Australia) overnight in a cold room at 8°C. The following day, resin-infiltrated leaf segments were embedded in Formvar 15 / 95 resin (ProSciTech, Thuringowa, Qld) in 2% ethylene dichloride as per the procedure described in Bhatia *et al.* (2003). The prepared specimens were stored in a desiccator until analysis.
    - Freeze-fracturing
      - Leaves of *S. tryonii* growing in pots were detached from the plant and immediately plunged into liquid nitrogen. Frozen samples were placed in grooves cut into aluminum stubs that were pre-cooled with liquid nitrogen. Prior to mounting the aluminum stubs on a frame, the leaf samples were snapped (freeze fractured), using pre-cooled tweezers. This procedure exposed a cross section of leaf tissues. The frame was then placed in the vacuum chamber of the SEM. Since the SEM used was not fitted with a cryostage, elemental mapping was restricted to the time until which the samples warmed to melting point, which was typically a few minutes.

- **Micro-PIXE study**
  - preparation protocol established by Pyczynska *et al.* (1999) was followed with some modifications. Fresh leaf and stem samples were hand-sectioned with a razor blade and immediately cryo-fixed in liquid nitrogen. Frozen specimens were then freeze-dried for 24 h in a VirTis Sentry Vacua-Freeze (The Virtis Co., Gardiner, NY) freeze-drier (referred to as _soaked_ specimens). One set of hand-sectioned, freeze-dried leaf and stem specimens was soaked for 4 h in deionised water (with one change) at ambient conditions (referred to as _unsoaked_ specimens) before micro-PIXE analysis. Sections were individually sandwiched between Formvar films prepared by dissolving Formvar 15/95 resin (ProSciTech, Therangowa, Qld) in 2% ethylene dichloride as per the procedure described in Bhatia *et al.* (2003). The SEM-EDXS setup was calibrated using a cobalt reference standard (Astimex Scientific Ltd, Toronto, Canada), with elemental composition reported as a normalised percentage (dry) weight basis of the total detectable elements (Z > 9) (note that this is a limitation of the EDXS unit used, other systems may have lower detection limits). As biological material is primarily composed of non-detectable C, H, O and N, it is not surprising that the EDXS assessed elemental concentrations were not in agreement with results obtained using micro-PIXE or ICP-ESI (see Bhatia *et al.* 2003). As such, the EDXS result is not accurate; however, they were relatively precise, with repeatable results possible for a given location. Therefore the SEM-EDXS measurements were considered to be qualitative only.

- **Micro-PIXE study**
  - For micro-PIXE analysis, the sample preparation protocol established by Pyczynska *et al.* (1999) was followed with some modifications. Fresh leaf and stem samples were hand-sectioned with a razor blade and immediately cryo-fixed in liquid nitrogen. Frozen specimens were then freeze-dried for 24 h in a VirTis Sentry Vacua-Freeze (The Virtis Co., Gardiner, NY) freeze-drier (referred to as _soaked_ specimens). One set of hand-sectioned, freeze-dried leaf and stem specimens was soaked for 4 h in deionised water (with one change) at ambient conditions (referred to as _unsoaked_ specimens) before micro-PIXE analysis. Sections were individually sandwiched between Formvar films prepared by dissolving Formvar 15/95 resin (ProSciTech, Therangowa, Qld) in 2% ethylene dichloride as per the procedure described in Bhatia *et al.* (2003). The prepared specimens were stored in a desiccator until analysis.

**Micronanalysis of leaf and stem surface and cross-sections was performed** in a JEOL JSM-5300 LV scanning electron microscope (JEOL USA Inc., MA) attached to a Link eXII analysis system (Oxford Instruments, Oxford, UK) interfaced with Link eXII Analysis software. X-rays were detected by a Si(Li) detector with a thin beryllium window. The microscope was operated at an acceleration voltage of 15 kV with a collection time of 100 s. Working distance was maintained at 15 mm and stage-tilt was adjusted to obtain a take-off angle of 40°. Energy dispersive spectra (and images) were processed using Link eXII Analysis software. The SEM-EDXS setup was calibrated using a cobalt reference standard (Astimex Scientific Ltd, Toronto, Canada), with elemental composition reported as a normalised percentage (dry) weight basis of the total detectable elements (Z > 9) (note that this is a limitation of the EDXS unit used, other systems may have lower detection limits). As biological material is primarily composed of non-detectable C, H, O and N, it is not surprising that the EDXS assessed elemental concentrations were not in agreement with results obtained using micro-PIXE or ICP-ESI (see Bhatia *et al.* 2003). As such, the EDXS result is not accurate; however, they were relatively precise, with repeatable results possible for a given location. Therefore the SEM-EDXS measurements were considered to be qualitative only.

**Micro-PIXE study**

A 10 MV Tandem accelerator at ANSTO provided a 3 MeV proton beam for the nuclear microprobe analyses. For point analysis and dynamic elemental mapping, both PIXE and proton backscattering (BS) were employed simultaneously. Details of PIXE set up, standardisation techniques and data acquisition procedures have been described in detail in Bhatia *et al.* (2003). Average micro-PIXE analyses were shown to be consistent with ICP analysis.

The PIXE spectra were analysed using GeoPIXE II software (for a discussion of the PIXE technique, see Wilkowski *et al.* 1997). This program allows extraction of PIXE elemental maps from the accumulated data, using Dynamic Analysis (Ryan 2001), which are overlap-resolved and background-subtracted. Using the region selection mode (Dynamic Analysis), various regions (epidermis, mesophyll, vascular tissue, cortex, pith from the images) were selected. Region selection was done by enclosing the anatomical areas of interest, using the drawing mode of the Dynamic Analysis. Owing to the low resolution of acquired elemental maps, region selection for ‘epidermis’ included epidermal cell wall, cuticle and sub-epidermal tissues. Alternatively, the quantitative point analysis feature of Dynamic Analysis was employed using the same datasets for transverse sections across the whole specimen. Points were located on the basis of visual inspection of the elemental distribution maps.
Results

Light- and bright-field microscopy

Leaf and stem anatomy

Leaves were composed of 3–4 layers of palisade mesophyll above spongy mesophyll cells (Fig. 3a). The epidermal cells on the adaxial portion of the leaf were larger than those on the abaxial side. Stomata were present at similar densities (data not shown) on both adaxial and abaxial sides of leaf material. Leaf vasculature was dominated by a central vein surrounded by a bundle sheath layer (Fig. 3a). Spherical to elliptical chloroplasts were observed in the mesophyll cells at higher magnification (data not shown).

Fig. 3. Photomicrograph (light microscope) of a transversely-cut (10 µm) resin-embedded section of Stackhousia tryonii leaf, (a) stained with toluidine blue. E, epidermis; S, stomata; P, palisade mesophyll; VB, vascular bundle; M, spongy mesophyll, (b) a dark-field image of a leaf section soaked in dimethylglyoxime for 12 h. Bright white areas within palisade mesophyll, spongy mesophyll and vascular tissues represent conjugated nickel crystals, and (c) a bright-field image of a leaf showing conjugated nickel crystals in the vascular bundles and parenchyma cells (around vascular bundles).
The stem comprised of a concentric ring of vascular tissue separating pith from a photosynthetic cortex. Clusters of thick-walled sclerenchymatous cells occurred at between six and nine points around the outer cortex of the stem (Fig. 4a).

**DMG complexation of nickel**

In DMG-treated sections, large numbers of magenta-coloured (conjugated Ni) crystals were noted using bright-field microscopy throughout the leaf and stem, with an apparent concentration in the inner palisade and mesophyll and vascular tissues (data not presented). These crystals were also distinguishable, using dark-field microscopy, as ‘bright white’ areas within leaf sections (Fig. 3b). The conjugated crystals (Fig. 3c) were verified to contain Ni (approximately 18%) by EDXS spot analysis (data not shown).

**SEM-EDXS study**

In non-DMG-treated, freeze-fractured material, Ni levels were apparently 100% higher in epidermal cells than in guard cells. Assessed nickel concentrations were also (approximately 20–30%) lower within deeper layers of palisade or mesophyll cells, relative to epidermal cells (data not shown). Xylem tissue also had lower Ni concentration than epidermal cells (data not shown).

Fig. 4. Typical secondary electron image (a) and distribution patterns of calcium (b), potassium (c) and nickel (d) in transversely-cut, unsoaked stem sections of *Stackhousia tryonii* (micro-PIXE analysis). Stems were hand sectioned, cryo-fixed and freeze-dried before micro-PIXE analysis. Transverse nickel map relates to quantitative point analysis across stem section shown in Fig. 5. Colour intensity denotes the concentration of elements (yellow, high; orange, low).
Micro-PIXE study

Stem sections

Micro-PIXE analysis of unsoaked stem sections revealed the concentration of three major elements to follow the order K > Ca > Ni (Table 2; Figs 4, 5). The soaked stem sections lost nearly all K and approximately 88% of Ni (Table 2).

Quantitative point analysis (Fig 5) of the stem sections demonstrated that Ni concentration varied between 500 µg g⁻¹ DW in the pith region and up to 3000 µg g⁻¹ DW in the epidermal and vascular tissues. The cortex possessed an intermediate Ni concentration between 800 and 2000 µg g⁻¹ DW. The Ni ‘tissue average’, determined using the region selection mode of Dynamic Analysis (Table 2), of the three anatomical regions, epidermal tissues, mesophyll and vascular tissues of stem, was similar (between 903 and 1067 µg g⁻¹ DW). The pith contained only 344 µg Ni g⁻¹ DW.

The highest concentration of K (region selection mode of Dynamic Analysis) was measured within vascular tissues (5533 µg g⁻¹ DW), whereas epidermal tissues contained 4167 µg g⁻¹ DW K. Maximum Ca (region selection) was localised within epidermal tissues (2733 µg g⁻¹ DW), and vascular tissues contained intermediate concentrations at 2067 µg g⁻¹ DW.

The concentrations of all elements (including trace elements) were found to be the lowest within pith (Fig. 5). Concentrations of K and Ca were nearly half the maximum concentrations, whereas those of Ni were only one-third of the maximum.

The concentrations of trace elements (Fe, Zn and Mn) decreased from the epidermis, through mesophyll to vascular tissues.

Leaf sections

Micro-PIXE detection revealed the presence of three major elements in the unsoaked leaves of S. tryoni, with concentration following the order: Ca > K > Ni and Fe > Mn > Zn (Table 3). A peak Ni concentration of 5400 µg g⁻¹ DW was recorded in the epidermis and vascular tissues, compared with 2500 and 3800 µg g⁻¹ DW in the palisade and mesophyll tissues, respectively (quantitative point analysis; Fig. 7). Concentrations, however, were variable within tissues, such that when averaged across anatomical regions, little difference in Ni concentration between tissues (2400, 2000 and 2600 µg g⁻¹ DW, in epidermal cells, mesophyll cells and vascular tissue, respectively; region selection; Table 3) was noted.

Potassium accumulation followed similar pattern of spatial distribution as Ni (quantitative point analysis; Fig. 7), reaching a maximum in vascular tissues (up to 7800 µg g⁻¹ DW), followed by epidermal tissues (up to 6000 µg g⁻¹ DW), while palisade and mesophyll tissues possessed nearly half the concentration (4000 µg g⁻¹ DW) that of vascular tissues. Again, concentrations were variable within each tissue, such that average K concentrations across individual tissues were 2750, 2900 and 3950 µg g⁻¹ DW for epidermal tissues, mesophyll and vascular tissues, respectively (region selection; Table 3).

Quantitative point analysis revealed that Ca localisation also followed a similar trend, with maximum accumulation in the epidermal tissues (up to 1.5 × 10⁴ µg g⁻¹ DW), while vascular tissues and mesophyll possessed 1.3 × 10⁴ and 5.0 × 10³ µg g⁻¹ Ca DW, respectively (Fig. 7). Again, average concentration across individual tissues (region selection; Dynamic Analysis) suggested that Ca was...
predominantly localised in epidermal tissues (4550 µg g\(^{-1}\) DW) and within vascular tissue (4600 µg g\(^{-1}\) DW), while mesophyll contained nearly two-thirds the concentration (3050 µg g\(^{-1}\) DW) of epidermal and vascular tissue.

The concentrations of trace elements such as, Fe, Zn and Mn were 123, 4 and 57 µg g\(^{-1}\) DW, respectively in the whole leaf section (region selection; Table 3). In contrast to unsoaked specimens, the soaked leaf specimens contained very low K and Ni. The maximum percent loss of K and Ni was up to 91% and 85%, respectively from within vascular tissues, and up to 86% and 73%, respectively, from epidermal tissues. Mesophyll tissue lost intermediate levels of K (90%) and Ni (78%) (Table 3).

Discussion

Technique

Dimethylglyoxime (DMG) has frequently been used as a histochemical stain for localisation of Ni within tissues of hyperaccumulator plants (Vergnano Gambi 1967; Severne 1974; Farago and Mahmoud 1983; Heath et al. 1997; Mizuno et al. 2003). However, it is likely that movement of Ni on the solvent front occurred during DMG staining, resulting in redistribution and localisation of conjugated Ni crystals in the palisade and spongy mesophyll and vascular tissues (Fig. 3b). Other artefacts may also occur (e.g. pH dependent staining). To avoid redistribution or movement of Ni on the solvent front, tissue should be either cryo-substituted (in DMG solution) at ultra-low temperatures, or alternative procedures should be considered.
SEM-EDXS is a versatile technique for estimating the spatial distribution of chemical elements in cells (Zierold and Hagler 1989), and has been extensively used for localisation (and quantification) of heavy metals in various hyperaccumulating taxa (Heath et al. 1997; Psaras et al. 2000; Kupper et al. 2001; Robinson et al. 2003). However, it suffers from poor quantification because of the difficulty involved in estimating the volume of the tissue sampled. Freeze-fractured material used in the current study would have freeze-dried during irradiation. This reduces the spatial resolution of the analysis, since the electron beam penetrates much deeper into dried compared to freeze-dried tissues. Furthermore, the instrumentation employed did not allow for use of oxygen (of water from freeze-fractured specimens) as an internal standard. Thus, in the current study, the SEM-EDXS instrumentation and protocol employed allowed only qualitative analysis of Ni. Therefore, the discussion is limited to cellular/tissue-level observations. As noted in a previous study (Bhatia et al. 2003), the methodology/instrumentation employed in the current SEM-EDXS study allowed for precision, but lacked accuracy (data not shown).

The micro-PIXE technique, using Rutherford backscattering correction, potentially allows quantitative analysis, but has relatively lower spatial resolution. However, micrometer level resolution has been reported (Malmqvist 1995). With the nuclear microprobe (micro-PIXE), elemental maps (Ni, K and Ca) were prepared with a beam resolution (pixel size) of $2 \times 2 \mu m$. Effective resolution would be much less, given variations in specimen density and movement in the sample during irradiation.

Witkowski et al. (1997) noted that data from quantitative point analysis is more reliable than that from region selection analysis, noting five- and 10-fold difference between region selection analysis and point analysis for barium and cobalt content of dormant seeds of *Burkea africana*. Region selection analysis was suggested to be limited by the use of approximations rather than full non-linear least-square fit to point X-ray spectra (Witkowski et al. 1997). On the contrary, in a more recent publication, Przybylowicz et al. (2001) suggested that ‘owing to the heterogeneity of biological specimens, point analysis may sometimes not be representative’. We consider that the two methods are complimentary in that the region selection allows averaging across a region with loss of specific spatial resolution, while point analysis allows more accurate estimation of specific values of elemental/metal composition. For example, point analysis transects across transverse sections recorded substantial differences in concentrations within an identified tissue (particularly epidermal and vascular tissues) (Fig. 7), as did elemental distribution maps (Fig. 6).

*Stackhousia tryonii* is a nickel hyperaccumulator

This is the first report on the application of micro-PIXE technique for localisation of Ni in both the leaf and stem.
of the Ni hyperaccumulation trait (Baker 1981) in natural habitats (up to 41,300 µg g⁻¹ Ni in S. tryonii tissues of mesophyll; VB, vascular bundle. See Fig. 6. E, epidermis; P, palisade mesophyll; M, spongy mesophyll; VB, vascular bundle.

The absolute Ni distribution within leaf tissues, however, will be a function of Ni concentration within tissue and the tissue volume. Further information (on epidermal, mesophyll and vascular volume within the leaf) is required to resolve this point in S. tryonii. In addition, further information is also required to explain the spatial variability in Ni content observed within a tissue type (particularly epidermal tissue). For example, Küpper et al. (1999) demonstrated a correlation between cell size and Ni concentration in the epidermis of Thlaspi caerulescens, while Frey et al. (2000) found that this correlation was caused by a functional differentiation of the epidermal cells (accumulation of Ni was found to occur mainly in the vacuoles of large elongated cells).

Cuticle and guard cells

Bidwell (2001) noted the cuticle to be the predominant site for Ni accumulation (2078 µg g⁻¹ v. 536 µg g⁻¹ within epidermal cells of embedded leaf tissue) in S. tryonii. In the present study, estimation of Ni concentrations within the cuticle was not possible due to comparatively lower resolution of the instrumentation employed. However, Ni accumulation on the outer surface of the epidermis was not visualised in SEM (backscatter mode), and no abnormal accumulation of Ni was quantified using micro-PIXE, which would have been apparent in the outer tissues in the elemental map (Fig. 6c) or in the quantitative traverse point analysis across the transect (Fig. 7) of the specimens examined. Contrary to the observation of Bidwell (2001), Ni was localised in the sub-epidermal tissues (Fig. 6c), as evidenced in both the elemental map and the point analysis. Further, it is difficult to postulate a mechanism by which Ni could accumulate in the cuticle, given the lack of excretory structures in the epidermis. However, recently, excretion of Ni through guttation fluid in Ni hyperaccumulator T. japonicum has also been demonstrated (Mizuno et al. 2003).

Noell and Morris (1997) observed a high atomic number deposit on the surface of the epidermis of S. tryonii leaves, and suggested that Ni containing solutes had moved to the leaf surface through stomata and wounds resulting from insect herbivory. This feature may have been an artefact of sample preparation technique (Noell and Morris 1997), as specimens were pressed while drying before carbon-coating for examination using scanning electron microscope. This application of pressure may have fractured epidermal cells, resulting in leakage and accumulation of Ni-rich cell contents on the leaf surface.

The epidermis as a major storage site for Ni

Energy Dispersive X-ray Spectrometry and micro-PIXE analyses demonstrated the presence of elevated Ni concentrations within epidermal tissue (cuticle, epidermal and sub-epidermal cells), with concentrations decreasing in the deeper layers of palisade and mesophyll cells. Our results are consistent with previously reported studies on Ni hyperaccumulating taxa (for examples, see Table 1).

In the present study, the concentrations of Ni hyperaccumulation trait (>1000 µg Ni g⁻¹ DW; Reeves 1992) in both the leaf and stem tissues of S. tryonii, as previously reported for plants grown in a glasshouse (6900 µg Ni g⁻¹ leaf DW; Bidwell 2001) or occurring in the natural habitats (up to 41,300 µg Ni g⁻¹ leaf DW, Batianoff and Specht 1992) and those preserved in the herbaria (up to 21,500 µg Ni g⁻¹ leaf DW and up to 7100 µg Ni g⁻¹ stem DW, Batianoff et al. 1990), analysed using ICP-based techniques.

In the present study, the concentrations of hyperaccumulated Ni in leaf sections were almost double those found in the stem sections. These results are consistent with those of Batianoff et al. (1990). As evidenced by the natural occurrence of various populations on serpentine soils (most with elevated levels of available Ni; Bhatia 2003), this species is capable of tolerating otherwise phytotoxic levels of available Ni. It can therefore be concluded that S. tryonii is an absolute metallophyte (Baker 1981), and its tolerance to and hyperaccumulation of Ni are constitutive properties, as would be expected in such species.

**Fig. 7.** Quantitative point analysis (micro-PIXE) across the transect outlined in Fig. 6. E, epidermis; P, palisade mesophyll; M, spongy mesophyll; VB, vascular bundle.

<table>
<thead>
<tr>
<th>Concentration (µg g⁻¹ dry weight)</th>
<th>0</th>
<th>5.0 × 10³</th>
<th>1.0 × 10⁴</th>
<th>1.5 × 10⁴</th>
<th>5.0 × 10³</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ni</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ca</td>
<td>200</td>
<td>500</td>
<td>700</td>
<td>900</td>
<td>1100</td>
</tr>
<tr>
<td>K</td>
<td>115</td>
<td>130</td>
<td>145</td>
<td>160</td>
<td>175</td>
</tr>
<tr>
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<tr>
<td>Cd</td>
<td>50</td>
<td>80</td>
<td>110</td>
<td>140</td>
<td>170</td>
</tr>
</tbody>
</table>

Spatial distribution of Ni in Stackhousia tryonii
In general, guard cells (stomates) of metal hyperaccumulating plants are not preferred sites for metal accumulation (Heath et al. 1997; Psaras et al. 2000) and accumulate relatively smaller amounts of metals compared to other surrounding epidermal cells. For example, in T. caerulescens, guard cells contained less than 2% of the Zn concentration noted within vacuoles of epidermal cells (Frey et al. 2000). However, in our study, substantial amounts of Ni (approximately 50% of the epidermal cells; EDXS study, data not shown) were noted within the guard cells of S. tryonii leaf specimens. This relatively high measured Ni concentration in the guard cells may be an artefact of the limited spatial resolution of the measuring technique employed. However, our results are in general agreement with those of Bidwell (2001) who used scanning transmission electron microscopy (STEM) of thin sections, noting similar Ni concentrations within guard (7.5 ± 4 mmol k g⁻¹ embedded tissue) and epidermal cells (9.1 ± 1.2 mmol k g⁻¹ embedded tissue).

Form of hyperaccumulated Ni

A loss of 73–85% Ni from soaked leaf and 80–92% from soaked stem specimens suggests that a major portion of Ni within leaf and stem tissues was present in water-soluble form. Our results corroborate with those of Bidwell (2001), who also reported that up to 72% Ni in S. tryonii stems was water-soluble (no values for leaf tissue were reported). Citric (Bidwell 2001) and malic acids (Bhatia 2003) have been implicated to be the major organic ligands possibly involved in supporting detoxification/transport and storage of Ni in S. tryonii. Presumably, remaining Ni was bound to the cell wall material of the tissues, as reported for other Ni hyperaccumulators T. goeringense (Kramer et al. 2000) and H. floribundus (Bidwell et al. 2004).

Ecophysiological roles for Ni

Several hypotheses have been proposed to account for the ecological and evolutionary significance of the hyperaccumulation trait (Boyd and Martens 1992). A preferential localisation of Ni in the plant epidermis may be of significance for plant defence against insect herbivory (Schoonhoven et al. 1998), and might be toxic to pathogens, given the use of metal compounds (in particular copper and nickel) as fungicides and bactericides (Boyd and Martens 1992). However, clear evidence is lacking to support the hypothesis that hyperaccumulated Ni confers advantage to S. tryonii plants against herbivory or pathogenic fungi/bacteria.

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