Plant homeostasis of foliar manganese sinks: specific variation in hyperaccumulators

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Abstract

Plant manganese (Mn) hyperaccumulation provides unusual insight into homeostasis of this essential micronutrient, in particular its excessive storage in shoot tissues. The compartmentation of hyperaccumulated foliar Mn appears exceptional among metal hyperaccumulators, since it occurs via specific microdistribution patterns. Here, three associated Mn hyperaccumulators, *Viotia neurophylla*, *Maytenus fournieri*, and *Garcinia amplexicaulis* exhibiting distinctly different Mn detoxification strategies were examined. Non invasive sample preparation in conjunction with cryo scanning electron microscopy (SEM) was used to obtain *in vivo* quantitative microprobe X-ray and anatomical data from fully hydrated cells. Highly vacuolated large palisade mesophyll cells in *V. neurophylla* leaves were found to contain around 650 mM Mn. The large non-photosynthetic hypodermal cells of *M. fournieri* leaves, also with high vacuolar content, and the main site for Mn disposal, had an estimated mean vacuolar Mn concentration of around 600 mM. Previous qualitative X-ray mapping had shown Mn to be almost evenly sequestered across the entire leaf cross section of *G. amplexicaulis*. However, quantitative data obtained here showed a marked variation in localised concentrations that ranged between ~15 mM and >800 mM. Notable among these were mean values of >600 mM in spongy mesophyll cells, and ~800 mM within cells of a narrow sub epidermal layer preceding the palisade mesophyll. This study demonstrated the extraordinary Mn carrying capacities of different types of leaf cell vacuoles.

Keywords

Manganese hyperaccumulator, foliar Mn sequestration, *Viotia neurophylla*, *Maytenus fournieri*, *Garcinia amplexicaulis*, microprobe localisation.

Introduction

Plant hyperaccumulators exhibit rare and extreme affinity for commonly one, and less often, two or more metals or metalloids. The trait is manifest in species that remain healthy while scavenging and accumulating potentially lethal shoot-concentrations of certain metals and metalloids, notably Ni, Cd, Zn, Mn, Cu, Co, Pb, Tl Se and As. Among these are several plant micronutrients and others whose physiological roles are either unknown, or known to be phytotoxic at quite low concentrations. Hyperaccumulation thereby raises questions about plant homeostatic processes of these elements. Their foliar sequestration has been the subject of many *in vivo* microprobe studies, and with the exception of Mn, there have been consistent reports of highest deposition in non-photosynthetic cells (Krämer et al. 1997; Küpper et al. 2000; Mesjasz-Przybylowicz et al. 2001; Lombi et al. 2002; Robinson et al. 2003; Bidwell et al. 2004; Smart et al. 2010). Among Mn hyperaccumulators however, three distinctly different species-dependent foliar Mn detoxification strategies have been observed, including almost exclusive pooling in mesophyll tissues (Fernando et al. 2006a; 2006b; 2007; 2008a).

Around 20 Mn hyperaccumulators are currently known worldwide, distributed almost entirely in the Western Pacific, with major centres of diversity in eastern Australia and New Caledonia (Jaffré 1977; Proctor et al. 1989; Bidwell et al. 2002; Xue et al. 2004; Mizuno et al. 2008; Yang et al. 2008; Fernando et al. 2009). They are predominantly woody species, with several in the Australian rainforest tree genus *Gossia* (Myrtaceae). Investigation of *G. bidwillii* (Benth.) N. Snow & Guymer by microprobe localisation methodology has revealed its unusual foliar Mn sequestration, with highest localised concentrations of >500 mM in the palisade mesophyll cells (Bidwell et al. 2002; Fernando et al. 2006b). Since then, studies on Australian and New Caledonian Mn accumulators have identified three specific foliar Mn detoxification strategies. Among the latter group, *Viotia neurophylla* (Guillaumin) P.H.

Weston & A.R. Mast (Proteaceae), *Maytenus fournieri* ssp *drakeana* (Loesn.) I.H. Mueller ((Pancher & Sebert) Loesn., Fl. N. Caled., 20:73(1996)) (Celastraceae) and *Garcinia amplexicaulis* Vieill. ex Pierre (Clusiaceae) have xerophytic leaf anatomies, occur on metal enriched substrates, and are native to New Caledonia; yet they exhibit contrasting foliar Mn detoxification strategies. Large cells of the multi-layered palisade mesophyll in *V. neurophylla*, and of the multiseriate hypodermis in *M. fournieri* serve almost exclusively as Mn sinks in these species, whereas the spatial distribution of foliar Mn sinks in *G. amplexicaulis* is far less heterogeneous (Fernando et al. 2006a; 2008a). Although qualitative X-ray mapping of dry samples was useful for initially locating these Mn sinks, there is an absence of detailed knowledge about their physiologies.

Not only is Mn one of the most nutritionally important elements known to be hyperaccumulated, it is by far the most universally abundant in soils (Graham et al. 1988). The storage of excess Mn in plant shoots is of wider interest because unlike other essential trace nutrients its accumulation of and tolerance to concentrations exceeding ‘normal’ physiological requirements is not uncommon, nor is this clearly understood in physiological terms (Marschner 2002; Foulds 2003). The topic of plant-Mn physiology is particularly relevant to agricultural research in regions where climatic and/or edaphic conditions render soil-Mn highly phytoavailable (Siman et al. 1974; Bromfield et al. 1983; Graham et al. 1988). Based upon data for crop plants, ‘normal’ foliar Mn concentrations are generally within ~50 to 800 $\mu\text{g g}^{-1}$ dry weight (dwt), whilst Mn hyperaccumulation is defined by the notional threshold value of 10,000 $\mu\text{g g}^{-1}$ (Baker et al. 2000; Marschner 2002). Since Mn hyperaccumulators present amplified models of plant Mn tolerance, the trait might be exploited to better illuminate plant Mn detoxification.

Further to findings on the spatial distribution of foliar Mn in Mn (hyper)accumulators, synchrotron studies have recently confirmed indirect evidence that in plants, Mn^{2+} is the dominant *in vivo* form (Bidwell et al. 2002; Xu et al. 2009; Fernando et al. 2010). It has shown that in Mn hyperaccumulators at least, carboxylate ligands such as malate, citrate and oxalate are the most likely ions counter-ions to sequestered Mn^{2+} . Since *in vivo* cryo electron microprobe methodology enables the acquisition of concurrent analytical and anatomical data from fully hydrated plant tissues while minimally disrupting their homeostatic processes, it will be employed here to better understand excess Mn deposition in the leaves of *V. neurophylla*, *M. fournieri* and *G. amplexicaulis*, particularly with reference to interspecies differences in their Mn sinks and associated homeostatic ion antagonism.

Materials and methods

Sample collection and cryo-fixation

Leaf tissues previously sampled for qualitative *in vivo* localisation studies were used here (Fernando et al. 2008b). All three species had been collected from Grande Terre, the largest of the New Caledonian islands, where their host ultramafic lateritic soils are enriched with a range of heavy metals including Fe, Mn, Ni and Cr. Leaves were collected directly into cool humid packaging to retain their field freshness. Within 24 hrs they were subsampled and cryo fixed in the laboratory by rapidly freezing tissue pieces (approx. 1.0 to 0.5×0.5 cm) in melting nitrogen under vacuum (CT1501, Oxford Instruments, Eynsham, UK), before storage under liquid nitrogen (LN_2).

Total leaf Mn analyses

Leaves were oven dried and ground, and 0.3 to 0.5 g weighed into 75 ml Pyrex[®] tubes to be digested in 5 ml 70% nitric acid at 125°C for 2 h. Digests were treated with 30% hydrogen

peroxide, diluted to 75 ml with distilled water, analysed and quantified using inductively-coupled plasma optical emission spectroscopy (ICP-OES) against a series of aqueous standards acidified with nitric acid. In addition to Mn, Ca and Mg were also measured since the ultramafic soils on which these species grow have unusually large Mg/Ca concentration quotients (Jaffré 1980). For each plant species, samples with the highest Mn concentrations were selected for this study.

Preparation of frozen samples for cryo-SEM work

Cryo-fixed and frozen tissue fragments taken from LN₂ cold storage were manually trimmed down to ~3 x 2 mm pieces using a LN₂-cooled razor blade on a cold aluminium (Al) block partly immersed in a LN₂ bath. Working just above the surface of this LN₂ bath, cold forceps were then used to rapidly position the frozen trimmed tissue into the slit of an Al sample stub containing a small amount of cryo glue (Tissue-Tek[®], Sakura, Netherlands). The stub was quickly plunged into LN₂ to instantly cement the sample in place. While in the LN₂ bath, the stub was secured into a pre-cooled substage and transferred under LN₂ to a cryo-ultramicrotome (Leica UltracutE/FC, Leica Microsystems, Mount Waverly, Victoria, Australia). A cold (-83°C) glass knife was used to polish the ~0.5 mm protruding frozen leaf cross-sectional surface by planing it at decreasing section thickness settings of 0.9 µm, 0.7 µm and finally to 0.2 µm; ultimately yielding a smooth mirror-like surface suitable for X-ray analysis. The substage was then removed from the microtome into a closed specimen transfer device and rapidly transferred to the SEM cold stage (JEOL JSM 840A, JEOL, Tokyo, Japan), where it was observed at 5 kV while being lightly sublimed to improve anatomical clarity. The sample was coated with 10 to 15 nm of Al on the cold stage of the preparation chamber attached to the SEM (CT1500, Oxford Instruments, Wycombe, UK), and returned to the SEM for analysis during which it was maintained at -169 °C, and the

anticontaminator cold plate at -192°C . Pictorial and analytical data had to be acquired almost continuously over a 3 – 4-day period for each sample, during which it was maintained at -169°C on the SEM cold stage. One sample per species was considered sufficient for the purposes of this study, since leaf anatomy along with qualitative *in vivo* distribution of Mn, Mg and Ca had previously been examined in replicate dry samples of the above species. Results here are to be regarded as only indicative of *in vivo* values, but not as absolute representations.

Quantitative X-ray mapping and selected area analyses

The SEM was equipped with a Link (Oxford Instruments, Eynsham, UK) LZ4 30 mm² lithium (Li)-drifted silicon (Si) X-ray detector interfaced to a Link eXL X-ray analytical system. The beryllium (Be) window was used in the detector whose take-off and solid angles were respectively 40° and 0.011 steradians. The Link X-ray imaging software for quantitative mapping calculates peak integrals, using multiple least squares fitting of library peaks to the spectrum for each pixel, and apparent concentrations i.e. concentrations that have not been corrected for matrix effects. Apparent concentration images were corrected arithmetically to true concentration images by applying matrix corrections that were derived from static beam analyses of the same samples. The Phi-Rho-Zed model developed by Bastin *et al.* (1986) as applied to biological samples (Marshall 1982) was used to obtain quantitative analyses. The standards used for elements $Z > 10$ were polished microprobe standards (Bio-Rad Laboratories, Gladesville, NSW, Australia). Fixed concentrations of unanalysed elements (H, C, O, N) were used in the calculation of matrix corrections assuming that cell lumen contained 98% water. Depending upon magnification, X-ray maps were collected at

resolutions of 64×64 or 128×128 pixels with respective dwell times of 6 s or 1.5 s per pixel.

Quantitative Mn, Mg, K, S, Cl, P and Ca X-ray maps were obtained at an accelerating voltage of 15 kV, with an approximate beam current of 5×10^{-9} A. For each of these maps, the gain calibration was performed on a Cu grid attached to the SEM stage, which was focused electronically. Thereafter the sample area selected for analysis was focused only via the mechanical Z (stage height) control, with no electronic focussing changes made, so that the sample surface remained in an optimal plane for analysis and the X-ray take off angle did not change. Prior to acquiring each map, a secondary electron image (SEI) of the sample area was saved.

The X-ray maps and their corresponding SEI were used to confirm major Mn storage sites in Mn maps that had been identified in previous studies using dry samples (Fernando et al. 2006a; 2008b). Where there was clear indication from the maps of highest localised concentrations, these sites, all on cell lumen, were then quantified by selective area analyses. For each cell type, data were obtained from 4 – 19 replicate cells. Consistency of replication was governed by the availability of data free of electrical charging effects, and the maintenance of comparable dead-times and spectral shapes consistent with the watery matrix of cell lumen. Since qualitative maps of *G. amplexicaulis* had previously indicated that Mn is distributed relatively evenly across the leaf with no apparent major Mn pools, it was expected that for this species all cell types would be quantified. It was also expected that *M. fournieri* sinks were in the hypodermis and to a lesser extent in the region of the lower epidermis, while in *V. neurophylla* primary Mn sequestration was expected to occur in the palisade cells. Although Mn distributions for species examined here had previously been identified, in this

study the sample preparation and mode of mapping was designed to provide more detailed data.

In vivo elemental concentrations for the chosen Mn storage cells were obtained by selected area analyses using the Phi-rho-zed model for quantitation. Given the very low moisture content of cuticular tissue (Becker et al. 1986), and that it was metabolically the least important tissue to be examined here, quantitative data were not obtained. For all other tissues, quantitative datasets for individual cells were obtained using an adjustable raster to select areas well within cell wall boundaries.

Statistical analyses

For each cell type examined, replicate data were pooled to obtain mean elemental concentrations and standard errors (Table 2). Regression analyses were performed using Mn concentrations as the independent variable against the other elemental concentrations.

Results

Data obtained from ICP leaf chemical analyses (Table 1) confirmed that the species examined here had extremely high foliar Mn concentrations warranting their classification as Mn hyperaccumulators. Secondary electron images (SEIs) of frozen hydrated leaf cross-sectional surfaces showed anatomical features corresponding to X-ray maps that were consistent with previously obtained data (Figs 1-3) (Fernando et al. 2006a; 2008b). All X-ray maps (Figs 1-4) are quantitative elemental images scaled for optimum contrast so that pixel intensities represent accurate concentration differences within a single image but differences in pixel intensity between images do not accurately represent concentration differences between elements. Major Mn sequestration sites were in the palisade cells of *V. neurophylla*;

and in the hypodermal layers and lower epidermis of *M. fournieri*. Excess foliar Mn in the latter was confined almost exclusively to the large watery vacuoles of non-photosynthetic hypodermal cells, as evidenced by the sharply delineated Mn maps (Fig 4b). The equally distinct Mn maps of *V. neurophylla* palisade cells (Fig 4a) not only clearly delineated their large vacuolar volumes, but also negatively outlined chloroplasts confined to narrow cytoplasmic regions surrounding these vacuoles, where they were ‘sandwiched’ between the cell walls and voluminous vacuoles. The thermal colour scale used in these maps (Fig 4) highlights distinct cytoplasmic regions in *V. neurophylla* palisade cells, i.e. the red border around the bright yellow Mn-loaded vacuoles (4a), whereas the Mn-loaded cells in *M. fournieri* seem entirely vacuolar in content. It also appeared that the high Mn content of the *V. neurophylla* palisade cells provided atomic number contrast to the SEI (Fig 4a), making them appear ‘brighter’.

As expected, in *G. amplexicaulis* Mn was distributed least heterogeneously across the leaf cross section. Quantitative selected-area analyses (Table 2) showed that *V. neurophylla* palisade cells had a mean Mn concentration of over 650 mM, similar to that of the spongy mesophyll cells of *G. amplexicaulis*. There was a narrow subepidermal cell layer between the second upper epidermal and first palisade cell layers in the *G. amplexicaulis* leaf cross-section whose mean localised Mn concentration >800 mM was the highest observed. The two palisade and lower epidermal layers of *G. amplexicaulis* had Mn concentrations of ~200 mM, with much lower concentrations of around 15 to 50 mM Mn in the two upper epidermal layers. In *M. fournieri* the upper and lower hypodermal cell layers had respective localised Mn concentrations of over 500 and 600 mM, whilst the lower epidermal cells had an estimated mean Mn concentration of around 200 mM. The upper cuticle of *G. amplexicaulis*

and the lower cuticle of *M. fournieri* leaves were found by X-ray mapping to contain Mn (Figs 3, 2).

Regression data (Table 3) and/or the X-ray maps show: a) an overall negative Mn-K correlation across most cell types of all species examined, except for *G. amplexicaulis* second layer palisade cells and lower epidermal cells; b) a positive Mn-S correlation in *V. neurophylla* palisade cells; c) a negative Mn-S correlation in the first layer upper epidermal cells of *G. amplexicaulis*; d) a slight positive Mn-Ca correlation in the dermal tissues of *M. fournieri* and *G. amplexicaulis*; e) positive Mn-Mg correlations in *M. fournieri* first layer hypodermal cells and *G. amplexicaulis* second layer palisade cells; f) negative Mn-Mg correlations in *G. amplexicaulis* first layer upper epidermal cells; and g) a negative Mn-Cl correlation in the first layer upper epidermal cells, and in subepidermal cells between the upper epidermis and palisade tissues of *G. amplexicaulis*. Mean localised P concentrations across all cell-types ranged between 0.032 and 26.6 mM, however due to unacceptable statistical variation these data were excluded from Table 2. Foliar P distribution patterns for all three species as observed by X-ray mapping (Figs 1-3) were relatively featureless, mutually similar, and bore no clear relationship to Mn.

Discussion

Detoxification in metal-accumulating plants is determined by a range of factors including metal transporters, metal chelators, vacuolar volume, other leaf anatomical features, potential phytotoxicities of hyperaccumulated elements, and possibly even guttation (Clemens 2001; Broadhurst et al. 2004; Fernando et al. 2006b; 2008a; Montargés-Pelletier et al. 2008; Smart et al. 2010). The three woody species examined here share very similar geographic

distributions and xerophytic leaf traits; yet exhibit markedly different foliar Mn sequestration patterns. Whilst in agreement with previous qualitative investigations, this study further illuminates the nature of foliar Mn sinks in *V. neurophylla*, *M. fournieri* and *G. amplexicaulis*. Data were obtained from hydrated plant tissues preserved closest to their normal living state as was achievable from all available sample preparation techniques, with their metabolic processes immobilised to represent a true *in vivo* ‘snapshot’ of homeostasis. For this investigation, the limitations of examining tissues in their *in vivo* hydrated states included a) elements solubilised in cell lumen would be more dilute and therefore more difficult to detect than those in dry samples prepared by methods such as freeze substitution, and/or b) the poorer anatomical resolution of wet samples. These considerations were however outweighed by the need to gather as much information as possible from ‘living’ tissue prior to attempting studies utilising higher resolution dry sample preparations. Additionally, it was essential to avoid the artefactual loss and/or displacement of elements from the cell lumen, in particular lighter elements such as K for example. It is well established (Lauchli et al. 1970; Pallaghy 1973; Marshall 1980b) that their *in vivo* distributions are easily disrupted by certain solvents including acetone commonly employed in the resin infiltration of dry samples.

It was clarified that Mn pools almost exclusively in the photosynthetically important palisade mesophyll cells of *V. neurophylla*, and in the non-photosynthetic hypodermal cells of *M. fournieri*, both of which are highly vacuolated. Not only were these Mn-rich sinks in their fully hydrated states clearly delineated in Mn X-ray maps, but in the former species, this high Mn content provided inherent electronic contrast to the secondary electron image (Fig 4a), a phenomenon rarely encountered in plant electron microscopy. Whereas primary Mn-storage cells of *V. neurophylla* and *M. fournieri* were much larger than other cells in their leaf tissues,

G. amplexicaulis leaves lacked this obviously large cell-size discrepancy (Fig 3) and exhibited relatively homogeneous Mn distribution across all cell types. Inference from these observations is that either one or both of the following criteria influence foliar Mn detoxification strategies in the plants examined here: a) vacuolar volume likely takes precedence over cell metabolic importance, b) Mn is an important plant nutrient integral to photosynthesis not uncommonly tolerated in concentrations exceeding normal physiological requirements, and therefore can be regarded as ‘less toxic’ than other trace metals such as for example as Ni.

Whilst foliar Mn in the hyperaccumulator *G. bidwillii* is distributed through all cell types including small epidermal cell vacuoles, it is by far the most highly localised in large palisade cells. Interestingly, in the Mn-accumulating *Grevillea exul* Lindley (Proteaceae) it is the large epidermal cell vacuoles rather than large palisade cells that act as primary Mn deposition sites (Fernando et al. 2006b; 2008b). Based on the above arguments, this latter observation could be indicative of cell metabolic importance driving sequestration when similarly large vacuolar volumes are available in both the photosynthetic and non-photosynthetic cells. Previous light microscopical data (Fernando et al. 2008b) have shown that the *G. amplexicaulis* subepidermal cells found here to contain the highest mean Mn concentrations of over 800 mM were non-photosynthetic, and therefore most likely to be small hypodermal cells functionally similar to the large *M. fournieri* hypodermal cells. It is noteworthy then that the hypodermis, where present, plays an important role in storing excess Mn.

In the herbaceous Mn hyperaccumulator *Phytolacca acinosa* Roxb. (Phytolaccaceae), Mn is highly localised in leaf epidermal tissues (Xu et al. 2006). Little information is provided

about leaf anatomy, and it appears to consist of cells lacking any obvious size discrepancy. There are numerous microprobe localisation studies on Ni, Cd and Zn hyperaccumulation by members of the Brassicaceae family, which overwhelmingly indicate that dermal tissues are primary sinks, in particular, epidermal cell vacuoles and leaf hairs and trichomes (Krämer et al. 2000; Küpper et al. 2000; Bidwell et al. 2004; Broadhurst et al. 2004; Asemaneh et al. 2006). An apparent absence of variation in the foliar distribution patterns of hyperaccumulated metals other than Mn is not readily explicable. As to whether this might be associated with the important nutritional role of Mn and/or its relatively wide tolerance by plants is not easy to resolve. These discussions also highlight the need for a clearer understanding of tonoplast Mn transporters in Mn hyperaccumulators.

On comparison of total leaf concentrations to palisade cell Mn concentrations, values obtained here, i.e. a palisade vacuolar Mn concentration of ~600 mM in *V. neurophylla* (31,200 $\mu\text{g g}^{-1}$ total leaf Mn) and 200 mM in *G. amplexicaulis* (13,100 $\mu\text{g g}^{-1}$ total leaf Mn) are in reasonable agreement with similar previous studies on *G. bidwillii* (~22,000 $\mu\text{g g}^{-1}$ total leaf Mn) whose palisade cells were >500 mM Mn (Fernando et al. 2006b). Given that in these three species the palisade mesophyll probably accounts for 20 to 30% of the total leaf volume, an estimate based on visual inspection of leaf anatomical data (SEIs in Figs 1-3), these palisade Mn concentrations appear to reflect their corresponding total leaf Mn concentrations. The hypodermal cells of *M. fournieri* (20,150 $\mu\text{g g}^{-1}$ total leaf Mn) containing around 600 mM Mn occupy a similar proportion of the total leaf volume. Estimates of Mn vacuolar concentrations obtained in this study are reasonably consistent with those previously obtained in a far more detailed study of *G. bidwillii* that employed similar methodology (Fernando et al. 2006b).

Elevated foliar Ca concentrations observed here in both *M. fournieri* and *G. amplexicaulis* (Table 1) were likely due in part at least to the high Ca content of their dermal tissues, as indicated by qualitative and quantitative X-ray data (Table 2). However this does not fully explain the observation here of five- and four-fold differences respectively in their foliar Ca concentrations compared to *V. neurophylla*. Raphide cells loaded with Ca-oxalate crystals have previously been observed in *G. bidwillii* leaves (Fernando et al. 2006b), and here might account for the very bright cells in the Ca X-ray maps (Figs 1–3). The prevalence of Ca-oxalate may noticeably elevate total leaf Ca concentrations. Cell walls generally do contain more Ca than the lumen, and this is amplified in xerophytic species where dermal fortification is common (Esau 1965; Taiz and Zeiger 2002). X-ray maps obtained here (Figs 1–3) showed Ca outlining cell shapes, i.e. in their cell walls. The predominant *in vivo* ionic forms of Ca and Mn, Ca^{2+} and Mn^{2+} , have similar radii, which can lead to competitive ionic antagonism (Marschner 2002). Hence, the positive Mn-Ca correlation observed in the upper epidermal and hypodermal cells of *G. amplexicaulis* might be explained this way. A similar argument applies to Mn^{2+} - Mg^{2+} antagonism, as evidenced in the positive Mn-Mg correlation in the hypodermal cells of *M. fournieri* and palisade cells of *G. amplexicaulis*. However, it does not explain the negative Mn-Mg correlation in *G. amplexicaulis* upper epidermal cells.

A negative Mn-K correlation in the large spongy mesophyll section of *G. amplexicaulis* leaf tissue suggested that K^+ in these cells was reduced by their Mn accumulation. Unlike palisade mesophyll cells, spongy mesophyll cells are usually most important for respiration, and generally have relatively less vacuolar volume and greater cytoplasm with a high K^+ content. As observed here, xerophytic leaf anatomies characteristically lack intercellular spaces in the spongy mesophyll; and for this species where the anatomical SEI (Fig 3) does not show significant differences between palisade and spongy mesophyll cells, it is not

implausible then that these cell types might be relatively similar. Ultrastructural studies are needed to evaluate their vacuolar/cytoplasmic ratios, and thereby hypothesise about observations of Mn-K relationships. Were the vacuolar contents of these spongy mesophyll cells unusually large, they might be capable of Mn storage at the expense of respiration. In the authors' experience, preparation of these leaf tissues for transmission electron microscopical ultrastructural studies was precluded by tissue impermeability to resins due to their thickness and sclerophylly. Species examined here are therefore likely unsuited to dry sample microprobe techniques, unlike herbaceous hyperaccumulators to which they have thus far been applied.

Negative Mn-K correlations were also found in the *M. fournieri* hypodermis and *G. amplexicaulis* upper epidermis, both relatively unimportant to essential metabolic processes, and therefore unlikely to have significant cytoplasmic content. K^+ is known to play an important role in balancing negative ions in the cytoplasm and vacuole (Jeschke et al. 1985), and its accumulation can result in build-up of organic acid counter anions. Whether Mn^{2+} accumulated in the spongy mesophyll replaces K^+ to some extent in counterbalancing negative ions cannot be determined, and requires further investigation via ultrastructural *in vivo* microprobe methodologies. The role of K^+ in maintaining vacuolar turgor pressure (Lindhauer 1985, 1987) might in these species be replaced by Mn^{2+} , which would also explain the negative Mn-K correlation. Similar previous studies of *G. bidwillii* have shown a Mn-K negative correlations in Mn-rich palisade cells and Mn-containing epidermal cells (Fernando et al. 2006b). Qualitative X-ray maps of fixed dried *V. neurophylla* leaf cross-sections indicating a negative Mn-K relationship (Fernando et al. 2006a) were in agreement with the quantitative X-ray maps obtained here (Fig 1). However, this was not reflected in current quantitative data gathered from palisade cells (Table 3). Positive Mn-K correlations

detected in *G. amplexicaulis* palisade and lower epidermal cells might be due to increased K^+ in response to the presence of excess organic acid anions, which in turn is a possible response to accumulated Mn^{2+} (Marschner 2002).

Although the strong Mn-S positive correlation observed here in *V. neurophylla* Mn-loaded palisade cell vacuoles might indicate an association between S-containing counter-ions and Mn, involvement of an organic acid or a phytochelatin is unlikely, firstly because the localised S (33 mM) does not quantitatively account for co-localised Mn (683 mM), and also for reasons discussed below. It is noteworthy that a positive Mn-S correlation was also found in a prior quantitative cryo-SEM EDS study of *G. bidwillii* (Fernando et al. 2006b); and also that synchrotron data (Fernando et al. 2010) have linked carboxylate ligands to Mn^{2+} in both freeze-dried *V. neurophylla* and *G. bidwillii* leaf tissues. It discounted the role of S-containing ligands such as histidine in binding excess foliar Mn. Furthermore, observation here of accumulated S is unlikely due to Mn-binding phytochelatin since it is well established that in Cd, Ni and As hyperaccumulators at least, phytochelatin do not play a major role, and that they are often linked to homeostasis of trace elements in non-accumulating plants. Correlation between glutathione and metal accumulation has however been observed in certain Ni, Cu, Cd and Zn-hyperaccumulating species (Freeman et al. 2004; Sun et al. 2007; Na and Salt 2011). The role of inorganic S in binding excess Mn should also be considered as a possibility. As pointed out in the Materials and Methods, quantitative X-ray data were only collected from the cell lumen. There was considerable discrepancy between localised vacuolar S concentrations previously obtained for a range of *G. bidwillii* cell-types (1.9 – 28.6 mM); and those obtained here for a variety of *G. amplexicaulis* cells (20 – 253 mM) as well as the multiseriate dermal cells of *M. fournieri* (69 – 127 mM). While no significant Mn-S correlations were observed for the latter two species, it is interesting that

some of the highest observed localised S concentrations were concurrent with highly elevated Mn concentrations.

In summary, this investigation demonstrated that specific variation in foliar Mn spatial distribution patterns is linked at least in part to plant tolerance to Mn, available vacuolar volume and the metabolic importance of sink host-cells. Positive Mn-Ca and Mn-Mg antagonism and negative Mn-K antagonism were likely associated with homeostatic ion function of Ca^{2+} , Mg^{2+} and K^{+} . While this study sought to identify linear elemental interrelationships associated with localised Mn, it is possible that Mn pooling gave rise to other more complex antagonistic effects, the detection of which were outside the scope of this study. It was shown here that although vacuolar sequestration is the main mode of Mn detoxification in all three species examined, its binding is variable, notably in *V. neurophylla*. By detailing them in a manner closest to their living states, wet sample electron microscopy has provided unique insight into Mn sinks in these Mn hyperaccumulators. It has highlighted the need to better understand Mn specificity and the apparent ability of metabolically important cells to safely compartmentalise soluble Mn in highly localised and otherwise phytotoxic concentrations.

Acknowledgements

This research was funded by the University of Melbourne and La Trobe University. The authors thank Vincent Dumontet and the late Nicolas Perrier for their help and expertise in identifying plants in the field. DRF acknowledges the University of Melbourne David Hay Award for financial contribution towards preparation of this manuscript.

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Table 1 Leaf tissue dry weight concentrations

Plant species	Mn ($\mu\text{g g}^{-1}$)	Ca ($\mu\text{g g}^{-1}$)	Mg ($\mu\text{g g}^{-1}$)
<i>V. neurophylla</i>	31,200	5000	1120
<i>M. fournieri</i>	20,150	25,600	5100
<i>G. amplexicaulis</i>	13,100	21,100	1230

Table 2: Localised foliar elemental concentrations as estimated from SEM EDS selected-area analyses. For qualitative data corresponding to cuticle tissues, ‘+’ and ‘++’ respectively denote ‘detected’ and ‘strongly detected’.

Plant	Cell type replicates (n)	Mean concentration (mM) $\pm$ se					
		Mg	S	Cl	K	Ca	Mn
<i>V. neurophylla</i>							
	palisade 1 & 2 (n = 10)	14.4 1.24	33.3 3.1	4.09 1.06	9.92 0.95	13.7 1.89	683 38.5
<i>G. amplexicaulis</i>							
	upper cuticle	+	+	+	+	++	+
	upper epidermis 1 (n = 4)	17.6 1.05	45.1 6.18	142 15.4	121 16.3	109 24.4	46.3 12.1
	upper epidermis 2 (n = 4)	10.9 1.54	58 2.37	192 14.8	115 9.76	37.4 11.7	16.7 5.44
	subepidermis (n = 5)	118 14.9	253 48.6	300 27.5	65.3 10.4	121 34.5	876 58.9
	palisade 1 (n = 9)	24 2.63	148 11.6	59.3 7.32	40.3 5.91	43.2 3.41	170 5.09
	palisade 2 (n = 10)	21 3.11	113 11.8	85.5 6.41	21.9 1.41	36.8 5.06	242 7.75
	spongy mesophyll (n = 19)	74.5 7.72	212 18.4	414 22.8	102 11	119 13.3	681 36.4
	lower epidermis (n = 4)	85.6 10.1	223 30.2	231 45.6	101 44.1	426 34.4	199 26.2
<i>M. fournieri</i>							
	hypodermis 1 (n = 10)	90.8 8.73	20 1.52	11.9 2	53.4 3.27	11.4 2.19	546 21.4
	hypodermis 2 (n = 10)	127 7.26	28.7 0.923	13.9 3.27	28.2 4.44	10.6 1.71	676 23.4
	lower epidermis (n = 10)	69.4 14.7	22 2.11	5.53 1.27	98.1 13.5	37.3 7.17	224 29.6
	lower cuticle	+	+	+	+	+	+

Table 3 Regression analyses of elemental concentrations in individual cell types (to interpret abbreviations used below for plant and cell names, refer to Table 1)

Plant	Cell	Elements	R	r ²	P	Plant	Cell	Elements	R	r ²	P
V n	pal 1 & 2	Mn/Mg	-0.06	0.00	0.87	G a	sm	Mn/Mg	0.37	0.13	0.13
		Mn/P	0.04	0.00	0.90			Mn/P	0.17	0.03	0.51
		Mn/S	0.89	0.78	0.00			Mn/S	0.30	0.09	0.22
		Mn/Cl	0.01	0.00	1.00			Mn/Cl	-0.25	0.06	0.31
		Mn/K	-0.18	0.03	0.62			Mn/K	-0.62	0.39	0.01
		Mn/Ca	-0.39	0.15	0.27			Mn/Ca	0.16	0.03	0.52
G a	ue 1	Mn/Mg	-0.94	0.88	0.06		le	Mn/Mg	0.44	0.19	0.56
		Mn/P	0.01	0.00	0.99			Mn/P	-0.67	0.45	0.33
		Mn/S	-0.95	0.91	0.05			Mn/S	0.32	0.11	0.68
		Mn/Cl	-0.91	0.84	0.09			Mn/Cl	0.66	0.44	0.34
		Mn/K	-0.93	0.87	0.07			Mn/K	0.93	0.87	0.07
		Mn/Ca	0.97	0.94	0.03			Mn/Ca	0.12	0.01	0.88
	ue 2	Mn/Mg	0.44	0.19	0.46	M f	hypo 1	Mn/Mg	0.56	0.31	0.09
		Mn/P	0.76	0.57	0.14			Mn/P	-0.10	0.01	0.63
		Mn/S	-0.53	0.28	0.36			Mn/S	0.47	0.22	0.17
		Mn/Cl	-0.74	0.55	0.15			Mn/Cl	-0.15	0.02	0.67
		Mn/K	-0.44	0.19	0.46			Mn/K	-0.17	0.03	0.64
		Mn/Ca	0.67	0.45	0.21			Mn/Ca	0.55	0.3	0.1
	se	Mn/Mg	0.07	0.01	0.91		hypo 2	Mn/Mg	-0.27	0.07	0.46
		Mn/P	-0.16	0.03	0.80			Mn/P	0.08	0.01	0.82
		Mn/S	0.79	0.63	0.11			Mn/S	0.25	0.06	0.49
		Mn/Cl	-0.86	0.73	0.07			Mn/Cl	-0.21	0.04	0.57
		Mn/K	-0.66	0.43	0.23			Mn/K	-0.63	0.40	0.05
		Mn/Ca	0.94	0.89	0.02			Mn/Ca	0.28	0.08	0.43
	pal 1	Mn/Mg	0.51	0.26	0.16		le.	Mn/Mg	-0.13	0.02	0.73
		Mn/P	-0.21	0.04	0.56			Mn/P	0.45	0.21	0.19
		Mn/S	0.49	0.24	0.15			Mn/S	0.26	0.07	0.47
		Mn/Cl	-0.42	0.18	0.22			Mn/Cl	0.40	0.16	0.25
		Mn/K	-0.32	0.10	0.36			Mn/K	-0.41	0.17	0.24
		Mn/Ca	-0.18	0.03	0.62			Mn/Ca	-0.18	0.03	0.63
	pal 2	Mn/Mg	0.76	0.58	0.01						
		Mn/P	-0.01	0.00	0.97						
		Mn/S	0.26	0.07	0.47						
		Mn/Cl	0.39	0.15	0.27						
		Mn/K	0.90	0.80	0.00						
		Mn/Ca	0.20	0.04	0.58						

Fig. 1 Secondary electron image and corresponding Mn, K, Ca, Mg, Cl, S and P X-ray maps of a frozen leaf- cross-sectional surface of *V. neurophylla*. Bright areas within X-ray maps indicate elemental distribution, i.e. the greater the concentration, the brighter the signal. Degrees of brightness cannot be used to compare relative concentrations between elements. Adaxial surface at LHS.

Fig. 2 Secondary electron image and corresponding Mn, K, Ca, Mg, Cl, S and P X-ray maps of a frozen leaf- cross-sectional surface of *M. fournieri*. Bright areas within X-ray maps indicate elemental distribution, i.e. the greater the concentration, the brighter the signal. Degrees of brightness cannot be used to compare relative concentrations between elements. Adaxial surface at LHS.

Fig. 3 Secondary electron image and corresponding Mn, K, Ca, Mg, Cl, S and P X-ray maps of a frozen leaf- cross-sectional surface of *G. amplexicaulis*. Bright areas within X-ray maps indicate elemental distribution, i.e. the greater the concentration, the brighter the signal. Degrees of brightness cannot be used to compare relative concentrations between elements. Adaxial surface at RHS.

Fig. 4 Detailed SEI images and *in vivo* Mn X-ray maps of Mn sinks in frozen leaf cross-sectional surfaces of a) *V. neurophylla*, and b) *M. fournieri*. The thermal colour scale represents highest to lowest concentrations from white to blue. Yellow areas here correspond to highest Mn deposition; however degrees of brightness cannot be used to compare relative concentrations between maps. Chloroplasts are arrowed in a), adaxial surfaces at LHS.

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Title:

Plant homeostasis of foliar manganese sinks: specific variation in hyperaccumulators

Date:

2012-11-01

Citation:

Fernando, D. R., Woodrow, I. E., Baker, A. J. M. & Marshall, A. T. (2012). Plant homeostasis of foliar manganese sinks: specific variation in hyperaccumulators. *PLANTA*, 236 (5), pp.1459-1470. <https://doi.org/10.1007/s00425-012-1699-6>.

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