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Multi-element Concentrations in Plant Parts and Fluids of Malaysian Nickel Hyperaccumulator Plants and some Economic and Ecological Considerations

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Abstract Information about multi-elemental concentrations in different plant parts of tropical Ni hyperaccumulator species has the potential to provide insight into their unusual metabolism relative to a range of essential and non-essential elements, but this information is scant in the literature. As Ni hyperaccumulation, and possibly co-accumulation of other toxic elements, has been hypothesized to provide herbivore (insect) protection, there is a need to quantify a range of these elements in plant tissues and transport fluids to at least verify the possibility of this explanation. In this study, multiple elements were analyzed in a range of different plant parts and transport fluids from Ni hyperaccumulator species collected from Sabah (Malaysia). The results show preferential accumulation of Ni in leaves over woody parts, but the highest concentrations were found in the phloem tissue (up to 7.9 % in *Rinorea bengalensis*) and phloem sap (up to 16.9 % in *Phyllanthus balgooyi*), visible by a bright green coloration in the field fresh material. The amount of Ni contained in one mature *R. bengalensis* tree was calculated at 4.77 kg. The high Ni concentration in the flowers of *Phyllanthus securinegoides* could affect insect floral visitors and pollination. High concentrations of Ni in the seeds of this species also could supply the seedling with Ni and aid in herbivory protection during the first stages of development. Foliar Ca and Ni in *P.* cf. *securinegoides* and *R. bengalensis* are positively correlated. Low accumulation of Ca is desirable for phytomining but concentrations of Ca are high in most Ni hyperaccumulators examined, and this could have consequences for the economic viability of Ni extraction from bio ore if these species were to be used as ‘metal crops’.

Keywords Chemical fingerprinting · Ni-sink · Phloem sap · Phytomining

Introduction

Nickel (Ni) hyperaccumulators are plants with the ecophysiological capacity to accumulate Ni from the soil, with the nominal threshold for the definition set at 1000 $\mu g\ g^{-1}$ foliar Ni (Reeves 2003; Van der Ent et al. 2013a). The first Ni hyperaccumulator discovered was the herb *Alyssum bertolonii* in Italy (Minguzzi and Vergnano 1948), but research focus intensified with the discovery that the phloem sap of *Pycnandra acuminata* (formerly *Sebertia acuminata*) from New Caledonia contained 25 % Ni (Jaffré et al. 1976). Currently, two global loci for Ni hyperaccumulators are recognized: (1) the Mediterranean Region, mainly with species in the genus *Alyssum* (Brassicaceae), and (2) the tropical ultramafic outcrops in Cuba, New Caledonia, and SE Asia, where hyperaccumulators have been discovered in a large number of different families. Most Ni hyperaccumulators (currently numbering approximately 400 globally) are restricted (‘obligate’) to ultramafic soils that are naturally enriched in Ni and other metals (Brooks 1987; Reeves 2003), although some Ni hyperaccumulators (‘facultative’ species) also occur on non-ultramafic soils where they do not hyperaccumulate Ni (Pollard et al. 2014).

The evolutionary and ecological reasons for hyperaccumulation of Ni remain poorly understood, but the “herbivory defense hypothesis” (Boyd and Martens 1998; Boyd 2004) has received most research attention. This hypothesis states that high concentrations of Ni in plant tissue afford protection

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against predation by (generalist but not necessarily specialist) insect herbivores, and hence infers greater chances of survival and reproduction of the plant. There is thus evolutionary pressure to evolve the Ni hyperaccumulation characteristic, as an alternative (possibly less energy-intensive) to alkaloid-based defenses (Martens and Boyd 2002; Coleman et al. 2005). The 'Joint Effects Hypothesis' further posits that simultaneous accumulation of toxic elements other than Ni (for example Co, Cr, Cu, Mg) may further enhance herbivory protection (Boyd 2012).

Ni hyperaccumulators can be used in phytomining, which is an environmentally sustainable 'green' technology to produce Ni metal (Chaney 1983; Chaney et al. 1998), provided it is undertaken as part of progressive rehabilitation of minerals wastes after conventional Ni mining activities (Van der Ent et al. 2013b). In tropical regions, such as the extensive ultramafic outcrops in Southeast Asia, there is an as yet undeveloped potential for successful phytomining operations for several reasons including: (1) the presence of some of the world's largest surface exposes of ultramafic bedrock; (2) the presence of extensive lateritic Ni mining operations creating post-mined land requiring effective rehabilitation; and (3) the occurrence of native Ni hyperaccumulator species (Van der Ent et al. 2013b). When screening for the optimal 'phytomining crop', both high biomass yields and high metal hyperaccumulation capacities are required to make phytomining efficient and commercially viable (Angle et al. 2001; Chaney et al. 2007). Another characteristic that has rarely been considered, but which may be important for the industrial processing of bio-ores, is 'contamination' with other naturally occurring plant elements, such as Ca, Mg, K, P, and S. This presents an incentive for wide-range screening of native floras for the best candidate Ni hyperaccumulator species with optimal growth characteristics and elemental uptake behavior.

Sabah (Malaysia) is exceptionally rich in plant species with over 8000 species recorded to date (Wong 1992). As such, this region presents rich genetic resources for the potential discovery of Ni hyperaccumulator species, and recent work by the authors has identified 19 new Ni hyperaccumulators in and near Kinabalu Park, in Sabah, Malaysia (Van der Ent et al. 2015). Globally, the multi-element data of the concentrations in different plant tissues in tropical Ni hyperaccumulator species are scant. However, such data have the potential to provide additional insight into the unusual metabolism of Ni hyperaccumulator plants. Specifically, it may help elucidate the biopathways of Ni relative to a range of essential and non-essential elements. Furthermore, as Ni hyperaccumulation, and possibly co-accumulation of other toxic elements, is hypothesized to provide herbivory protection, there is a need to quantify a range of these elements in plant tissues and transport fluids to verify the possibility of this explanation. Therefore, the objective of our study was to present multi-element concentrations in different plant tissues of a range of Ni hyperaccumulator species collected from natural populations in and near Kinabalu Park in Sabah.

Methods and Materials

Study Area and Field Collection Plant samples (leaves, bark, wood, phloem, xylem, flowers, seeds) were collected from the previously identified Ni hyperaccumulator species (Van der Ent et al. 2015) from in and near Kinabalu Park, Malaysia. Foliar samples were taken from mature sun-leaves and thoroughly washed with demineralised water to remove any dust contamination, and were dried at 70 °C for 5 d in a drying oven. Bark and wood samples were taken from the stems of hyperaccumulator plants by excising these parts with a sharp stainless steel surgical knife, whereas phloem samples were collected by stripping sections from beneath the bark. Bark, phloem and wood tissue samples were packed separately and also dried at 70 °C for 5 d. Phloem sap was collected with glass capillary tubes (0.2 mm diam, 50 mm long), whereas xylem sap was collected with a handheld vacuum pump from excised branches. Phloem sap and xylem sap were freeze-dried after collection at −50 °C for 8 h, and reconstituted with dilute nitric acid before analysis. Finally, all samples were packed for transport to Australia and gamma irradiated at Steritech Pty. Ltd. in Brisbane following Australian quarantine regulations.

Chemical Analyses Foliar and plant tissue samples were crushed and ground, and a 300 mg subsample digested using 4 ml concentrated nitric acid (70 %) and 1 ml hydrogen peroxide (30 %) in a microwave oven, and diluted to 30 ml with TDI water. Samples then were analyzed on ICP-AES (for macro-element and trace elements) and ICP-MS (for ultra-trace elements). This range of elements measured includes both trace elements that are essential to plants (Cu, Zn, Mn, Fe) and non-essential trace elements (Co, Cr), as well as major plant nutritional and beneficial elements (Ca, K, Mg, Na, S, P).

Reference data for macro and trace elements of tree and ligneous shrubs from ultramafic soils in Sabah are provided in Table 1 (Van der Ent, unpublished) and used here to contrast reported elemental concentrations in Ni hyperaccumulator tissues in the Results. Sample data were divided in non-Ni hyperaccumulator (N=780) and Ni hyperaccumulator (N=223) specimens by delimitation at >1000 μg g^{-1}. Reference data for ultra-trace elements from Breulmann et al. (1999) in foliar material of trees for two families (Dipterocarpaceae and Euphorbiaceae) from non-ultramafic soils also are provided in Table 1.

The Ni contained in a large specimen of *Rinorea bengalensis* was determined by taking the mean of 5 samples

Table 1 Reference data for macro and trace elements of tree and ligneous shrubs from ultramafic soils in Sabah (Van der Ent, unpublished). Reference data for ultra-trace elements from Breulmann et al. (1999) in foliar material from trees in two families (Dipterocarpaceae and Euphorbiaceae) from non-ultramafic soils

Al μg g^{-1}	**Ca** μg g^{-1}	**Co** μg g^{-1}	**Cr** μg g^{-1}	**Cu** μg g^{-1}	**Fe** μg g^{-1}	**K**μg g^{-1}	**Mg**μg g^{-1}	**Mn** μg g^{-1}	**Na** μg g^{-1}	**Ni** μg g^{-1}	**P** μg g^{-1}	**S**μg g^{-1}	**Zn** μg g^{-1}
Non-Ni hyperaccumulator samples													
692	7595	9	7	6	57	3942	3091	773	919	45	458	1319	94
Ni hyperaccumulator samples													
186	6188	60	30	6	114	5068	4079	304	332	6052	537	1400	93
Ba μg g^{-1}	**B** μg g^{-1}	**Li** μg g^{-1}	**Mo** μg g^{-1}	**Pb** μg g^{-1}	**As** μg g^{-1}	**U** μg g^{-1}	**V**μg g^{-1}	**Sb** μg g^{-1}	**Sn** μg g^{-1}	**Sr** μg g^{-1}	**Ti** μg g^{-1}	**Zr** μg g^{-1}	
Dipterocarpaceae													
17	19	0.1	0.01	0.3	0.03	0.001	0.002	0.0002	0.04	15.1	2	0.005	
Euphorbiaceae													
22	23	0.2	0.03	0.5	0.02	0.002	0.004	0.0005	0.04	37.4	3.1	0.01	

for each fraction and calculated dry biomass amounts by using an empirical allometric relationship reported (Yamakura et al. 1986) for trees in a tropical lowland dipterocarp forest in Borneo:

$$\text{Stem weight} = 0.02903\left(\text{dbh}^2\text{height}\right)^{0.9813}$$

$$\text{Branches weigh} \;= 0.1192\,\text{stem weight}^{1.059}$$

$$\text{Leaves weight}$$

$$= 0.09146(\text{stem weight} + \text{branches weight})^{0.7266}$$

*(all weights are in kg, dbh (= stem diam at breast height) in cm, and height in m)***Statistical Analysis** Data were analyzed using the software package STATISTICA Version 9.0 (StatSoft) and Excel for Mac version 2011 (Microsoft). Inter-correlations (Pearson *r* with tests for significance producing *P*-values) between foliar elements, for all Ni hyperaccumulators grouped together were calculated.

Results

Multi-element Foliar Chemistry of Ni Hyperaccumulators Results of foliar multi-elemental analysis are given in Tables 2 and 3. Concentrations of Al in *Aporosa chalarocarpa* (25 900 μg g^{-1}) and *Baccaurea lanceolata* (18 400 μg g^{-1}) far exceeded the hyperaccumulation threshold for this metal (>1000 μg g^{-1}) while just reaching the Ni hyperaccumulator threshold value (also >1000 μg g^{-1}). The mean Al concentration of all species was 322±136 μg g^{-1}. Concentrations of Ca were uniformly high (mean 5634±223 μg g^{-1}), with highest amounts in *Ptyssiglottis* cf. *fusca* (mean 33 200 μg g^{-1}), also a Ni hyperaccumulator. Magnesium concentrations were highest in *Baccaurea lanceolata* (26 700 μg g^{-1}), also a strong hyperaccumulator of Al. The highest K concentrations were in *Actephila* sp. nov. (14 000 μg g^{-1}). None of the Ni hyperaccumulators hyperaccumulate Mn (>10 000 μg g^{-1}), although *Psychotria sarmentosa* complex had up to 6085 μg g^{-1} Mn. Across all the Ni hyperaccumulators sampled, the mean Mn concentration was 273±25 μg g^{-1}. Iron concentrations were relatively low (mean 116 μg g^{-1}±11). Concentrations of Cu also were low (mean 6±1 μg g^{-1}), apart from one anomalous specimen of *Mischocarpus sundaicus* that contained 270 μg g^{-1} Cu, thereby nearly exceeding the hyperaccumulator threshold (300 μg g^{-1}). However, as Van der Ent et al. (2013a) have pointed out, one should not categorize such marginal cases as a hyperaccumulator of a metal, but rather consider the natural range in a species (in this case, the mean Cu in *Mischocarpus sundaicus,* excluding the anomalous value, was 5.95±0.8 μg g^{-1}.

The Ni hyperaccumulator *Psychotria sarmentosa* complex was unusual by having relatively high foliar Cr concentrations

Table 2 Elemental concentrations in foliage in studied Ni hyperaccumulators (macro-elements)

Species	*N*	**Al** μg g^{-1}		**Ca** μg g^{-1}		**K**μg g^{-1}		**Mg**μg g^{-1}		**P** μg g^{-1}	
		Range	Mean	Range	Mean	range	Mean	Range	Mean	Range	Mean
Actephila sp. nov.	5	14–27	[21]	5470–10,230	[7440]	11,965–15,540	[14,000]	1128–3690	[1877]	450–814	[643]
Aporosa chalarocarpa	1		[25,900]		[3780]		[1442]		[8070]		[252]
Baccaurea lanceolata	1		[18,400]		[21,900]		[5480]		[26,700]		[587]
Cleistanthus sp. 1	3	2–39	[24]	773–1560	[1223]	2769–3580	[3138]	1190–2338	[1836]	225–413	[303]
Flacourtia kinabaluensis	19	12–106	[36]	2265–15,920	[7140]	2509–9620	[5075]	950–8320	[4240]	266–582	[455]
Glochidion cf. *brunneum*	4	11–56	[32]	3635–5300	[4450]	3537–6280	[4680]	1320–4420	[2726]	161–290	[216]
Glochidion cf. 'bambangan'	19	12–205	[63]	1121–4660	[2390]	828–7280	[3369]	1670–11,920	[3890]	165–1404	[839]
Glochidion cf. 'nalumad'	3	33–74	[58]	4318–5790	[5130]	2730–6730	[4930]	1480–1930	[1747]	212–307	[259]
Glochidion cf. *lanceisepalum*	2	64–109	[86]	3838–5425	[4630]	8688–9025	[8856]	2332–3960	[3144]	178–252	[215]
Glochidion rubrum	5	8–100	[39]	1500–3300	[2410]	567–10,320	[4575]	1160–4150	[2619]	254–1056	[656]
Glochidion cf. *sericeum*	6	61–204	[138]	1431–2746	[1820]	685–2160	[1340]	1870–7730	[4460]	224–395	[312]
Glochidion mindorense	11	0–65	[28]	871–5120	[2470]	1972–8220	[3913]	1080–5730	[2350]	155–566	[308]
Kibara coriacea	2	21–121	[58]	3120–6990	[4930]	2190–4290	[3510]	3170–10,140	[5606]	609–820	[692]
Mischocarpus sundacius	8	6–111	[28]	1705–8360	[4920]	763–3280	[1965]	1830–5110	[3935]	425–625	[495]
Phyllanthus cf. *securinegoides*	22	1–69	[27]	54–8480	[3450]	44–13,380	[4493]	352–5500	[2880]	10–1690	[587]
Phyllanthus balgooyi	42	0–677	[140]	0–34,150	[4547]	1–6645	[2269]	9–7817	[2790]	0–1042	[357]
Psychotria sarmentosa complex	14	83–1605	[785]	4456–8830	[6730]	1896–7170	[4610]	2358–7895	[4690]	359–598	[446]
Ptyssiglottis cf. *fusca*	2	25–84	[55]	24,200–42,300	[33,200]	3020–6360	[4690]	9263–35,400	[22,330]	425–540	[483]
Rinorea bengalensis	18	10–70	[26]	4210–16,650	[8560]	2159–10,210	[6360]	1027–5130	[2760]	464–1188	[773]
Rinorea sp. undet.	1		[137]		[3860]		[5700]		[1520]		[755]
Rinorea javanica	9	17–151	[40]	1344–9920	[7330]	2839–9256	[5390]	1119–6297	[2510]	343–1179	[584]
Shorea tenuiramulosa	3	16–29	[21]	2314–5410	[4369]	1285–2070	[1780]	1102–2045	[1700]	312–386	[342]
Walsura cf. *pinnata*	7	9–357	[75]	3613–7060	[5640]	3969–9170	[6590]	946–3713	[2150]	336–619	[516]
Xylosma luzonensis	6	11–17	[14]	6260–12,775	[8646]	4524–8530	[6930]	1967–6830	[3490]	308–828	[506]

Table 3 Elemental concentrations in foliage in studied Ni hyperaccumulators (trace elements)

Species	N	**Co** µg g^{-1}		**Cr** µg g^{-1}		**Mn** µg g^{-1}		**Ni** µg g^{-1}		**Zn** µg g^{-1}	
		Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean
Actephila sp. nov.	5	22–95	[65]	2–3	[2]	238–933	[566]	1844–11,520	[7500]	153–227	[183]
Aporosa chalarocarpa	1	–	[468]	–	[63]	–	[1444]	–	[1559]	–	[36]
Baccaurea lanceolata	1	–	[179]	–	[143]	–	[436]	–	[1451]	–	[202]
Cleistanthus sp. 1	3	18–22	[20]	5–7	[6]	47–116	[79]	2047–2107	[2068]	13–16	[15]
Flacourtia kinabaluensis	19	1–35	[10]	2–17	[8]	6–250	[52]	204–7275	[3290]	14–168	[75]
Glochidion brunneum	4	21–56	[38]	5–16	[10]	81–344	[170]	2175–6200	[4485]	12–29	[23]
Glochidion sp. ‘bambangan’	19	3–139	[31]	4–49	[18]	13–458	[150]	1960–16,690	[5629]	20–156	[67]
Glochidion sp. ‘nalumad’	3	10–204	[96]	25–45	[33]	108–291	[172]	4805–9000	[7200]	24–55	[40]
Glochidion cf. *lanceisepalum*	2	9–10	[10]	7–11	[9]	23–27	[25]	1083–3267	[2175]	18–35	[26]
Glochidion rubrum	5	4–52	[28]	5–43	[17]	14–307	[137]	1721–7003	[4570]	27–35	[32]
Glochidion sericeum	6	443–1311	[926]	22–138	[71]	728–2950	[1789]	1036–2193	[1687]	21–103	[40]
Glochidion mindorense	11	4–30	[12]	3–30	[12]	21–174	[75]	612–2281	[1400]	11–25	[16]
Kibara coriacea	2	2–44	[16]	5–255	[90]	22–459	[200]	94–5839	[3362]	25–116	[66]
Mischocarpus sundacius	8	4–53	[17]	2–36	[8]	22–469	[134]	555–4423	[2392]	23–202	[92]
Phyllanthus cf. *securinegoides*	22	2–198	[38]	1–12	[7]	33–461	[141]	319–23,255	[12,260]	4–249	[80]
Phyllanthus balgooyi	42	1–115	[28]	3–182	[22]	1–1359	[202]	32–8608	[2413]	0–414	[60]
Psychotria sarmentosa complex	14	3–36	[12]	28–374	[127]	310–6085	[1551]	4274–24,180	[13,590]	35–350	[109]
Ptyssiglottis cf. *fusca*	2	2–14	[8]	4–12	[8]	42–172	[107]	105–1156	[631]	10–136	[73]
Rinorea bengalensis	18	10–44	[25]	5–190	[49]	90–909	[330]	2389–12,810	[7510]	33–276	[131]
Rinorea sp. undet.	1	–	[13]	–	[19]	–	[313]	–	[5827]	–	[681]
Rinorea javanica	9	11–55	[29]	3–25	[9]	113–530	[263]	3975–9679	[6926]	26–269	[128]
Shorea tenuiramulosa	3	2–11	[7]	3–5	[4]	41–233	[151]	351–1787	[1128]	9–17	[12]
Walsura cf. *pinnata*	7	5–117	[26]	1–47	[11]	34–588	[178]	1483–4582	[2185]	40–224	[136]
Xylosma luzonensis	6	2–23	[10]	1–5	[3]	13–213	[108]	1315–5360	[3755]	51–267	[132]

(maximum of 374 µg g^{-1} and a mean of 127 µg g^{-1}). One specimen of *Kibara coriacea* also contained 255 µg g^{-1} foliar Cr. In both cases, this was unlikely to be the result of contamination with soil particles, because Fe concentrations (indicative of soil contamination) were low. Although the highest values exceed that of the hyperaccumulation threshold for this metal (250 µg g^{-1}), the same caution as made for Cu above applies. Two species exceeded the hyperaccumulator threshold for Co (300 µg g^{-1} as defined in Van der Ent et al. 2013a), namely *Aporosa chalarocarpa* (468 µg g^{-1}) and *Glochidion* cf. *sericeum* (up to 1311 µg g^{-1}). Of these two, *G.* cf. *sericeum* appeared a genuine Co hyperaccumulator, with all six specimens above the hyperaccumulator threshold (mean of 926 µg g^{-1}). Figure 1 shows that foliar Co and Ni in *G.* cf. *sericeum, Rinorea bengalensis,* and *Flacourtia kinabaluensis* are positively correlated. The Co hyperaccumulator *G.* cf. *sericeum* accumulates relatively low amounts of Ni, but the slope of the relationship is similar between this species, and *R. bengalensis* and *F. kinabaluensis.* This is suggestive of similar ecophysiological transport mechanisms for both metals.

Significant correlations (P = <0.01) between foliar elements occur between Al and Mg (r=0.27), Ca and S (r=0.38), Co and Mn (r=0.69), Cr and Mn (r=0.32), K and P (r=0.35), and Ni and S (r=0.46). Brooks and Wither (1977) found an inverse relationship between Ca and Ni (r=− 0.53) in the foliage of *Rinorea bengalensis*. However, the results here show that foliar Ca and Ni in *Phyllanthus* cf. *securinegoides* and *Rinorea bengalensis* are positively correlated (Fig. 2).

Multi-element Chemistry of Plant Parts The Ni concentrations in flowers, seeds, and seed capsules of *Phyllanthus cf. securinegoides, P. balgooyi, Psychotria sarmentosa complex Rinorea bengalensis, Walsura* cf. *pinnata*, and *Xylosma luzonensis* are given in Table 4. The wood and bark of most Ni hyperaccumulators from Sabah have relatively low Ni concentrations with overall means for all of 441 and 1956 µg g^{-1} Ni in the wood and bark, respectively. An exception is the bark *Phyllanthus* cf. *securinegoides* that contained up to 6822 µg g^{-1} Ni. However, the thin phloem (rich in Ni) underneath the bark is the probable cause of this high value. The Ni

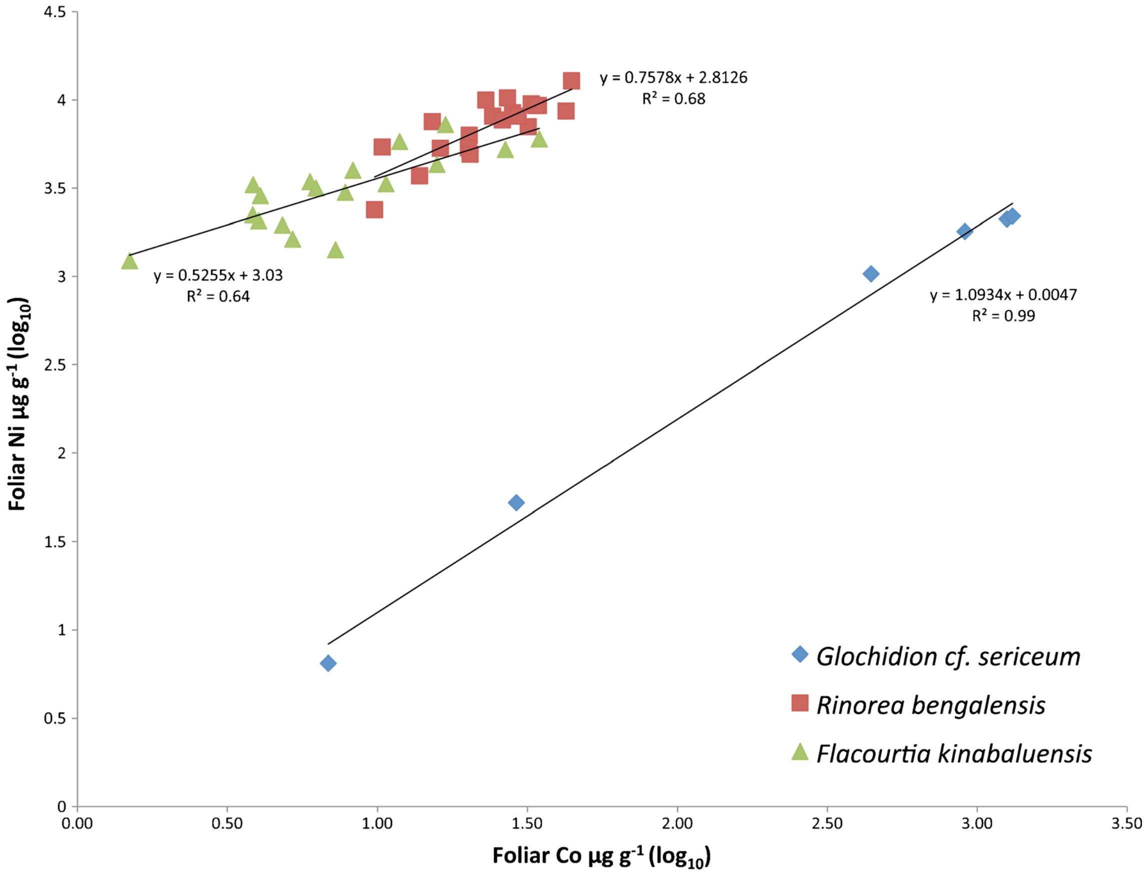

Fig. 1 Foliar Co and Ni concentrations ($\log_{10}$ transformed) in the hyperaccumulators *Glochidion* cf. *sericeum, Rinorea bengalensis,* and *Flacourtia kinabaluensis* are correlated

concentrations in twigs, wood, bark, xylem, and phloem are given in Table 5.

Nickel Contained in a Large Hyperaccumulator Tree *Rinorea bengalensis* is one of the largest Ni hyperaccumulators in terms of the tree height and associated biomass. The amount of Ni contained in a single large specimen (23 m tall, dbh 58 cm) was calculated using an empirical allometric formula, after analyzing Ni in the wood (0.11 %), branches (0.74 %), and leaves (0.98 %). For *R. bengalensis,* this yielded: stem (1820 kg, 2.00 kg Ni), branches (338 kg, 2.53 kg Ni), and leaves (24 kg, 0.24 kg Ni), resulting in a total Ni content of the tree of 4.77 kg.

Multi-element Chemistry of Transport Fluids The two highest Ni concentrations in the xylem fluid in this study were in *Phyllanthus balgooyi* and in *Psychotria sarmentosa* complex, and contained 76 and 49 µg mL^{-1} Ni, respectively (Table 5). Concentrations of other elements in the xylem sap of these two species are: 1.3/0.02 µg ml^{-1} Co, 26/33 µg ml^{-1} Ca, 52/52 µg ml^{-1} K, and 4.0/13 µg ml^{-1} Mg (*Phyllanthus balgooyi*/*Psychotria sarmentosa* complex, respectively). As such, the most abundant ion in the xylem fluids of these species is Ni, although Ca concentrations are also relatively high.

Phloem Tissue Ni Concentrations The phloem tissue of all hyperaccumulators (for those species from which phloem tissue could be collected) is high in Ni (Table 5). The presence of Ni in the phloem tissue and sap is visible by bright green coloration in the field fresh material. Figure 3 (top) shows an excised section of the bark and phloem tissue of *Rinorea bengalensis*, which contains up to 2.3 % Ni, whereas Fig. 3 (bottom) shows the phloem sap exudate from a stem section of *Phyllanthus balgooyi,* which contains up to 16.9 % Ni. The phloem tissue of *Phyllanthus* cf. *securinegoides* contained up to 1.1 % Ni, but this species did not enable a readily accessible sample of exudate. The small tree (stem diam up to 20 cm) *Phyllanthus balgooyi* exudes a clear dark-green viscous sap when the phloem tissue is damaged (under thin scaling bark). Three samples (from three different trees in the same population) contained 14.6, 15.5, 16.9 % Ni, respectively. Besides exceptionally high Ni, this sap contained 0.19 % Ca, 0.08 % Mg, 0.12 % K, and 0.14 % Co. In addition, *Phyllanthus balgooyi* phloem sap also contains high Zn concentrations, up to 3688 µg g^{-1}.

Ultra-trace Elements in Ni Hyperaccumulators Concentrations of ultra-trace elements are given for nine Ni

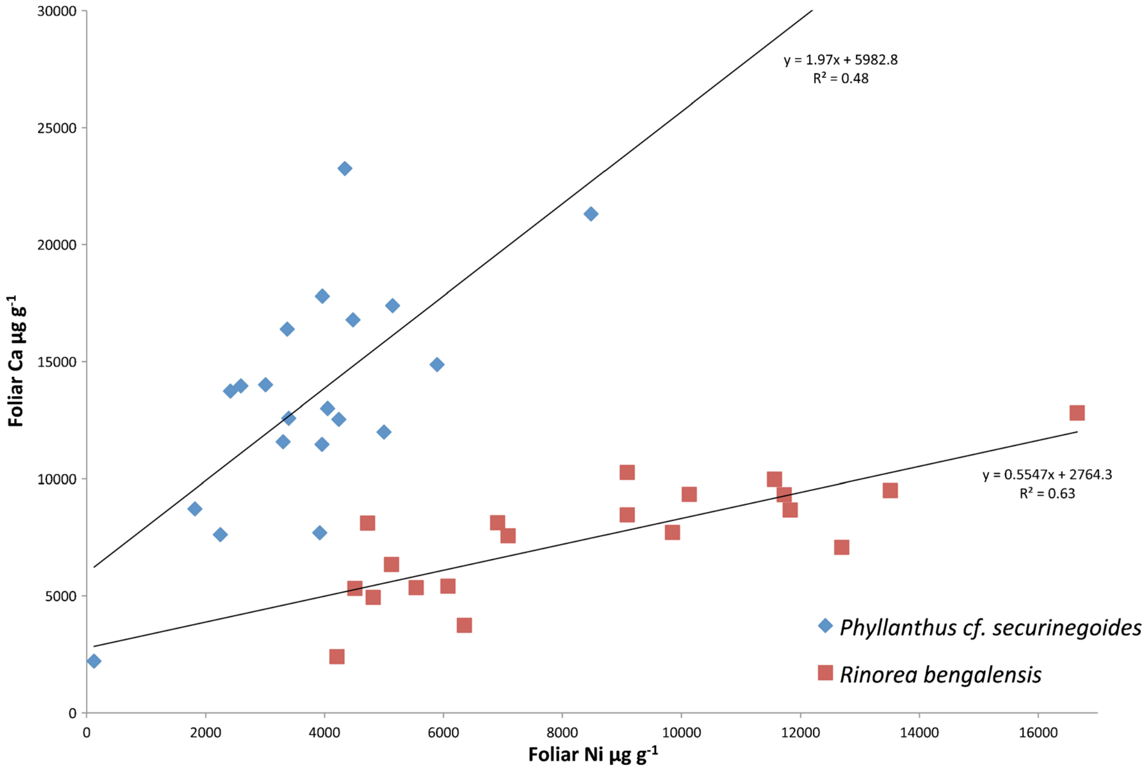

Fig. 2 Foliar Ca and Ni concentrations in the hyperaccumulators *Phyllanthus* cf. *securinegoides* and *Rinorea bengalensis* are correlated

hyperaccumulator species in Table 6. Despite the high specificity for Ni (for example a mean Ni : Co ratio of 207 for all hyperaccumulators), concomitant accumulation of non-essential (ultra-trace) elements is to be expected because of shared physiologies for uptake. Compared to Breulmann et al. (1999), concentrations of Ba, Li, Ti are lower in our study, and values for B, Sn, Sr are similar to those for the hyperaccumulators. In contrast, current study values for Co, Cr, Mo, Pb, Sb, U, V, and Zr are higher. Concentrations of ultra-trace elements in phloem tissue and phloem sap, materials high in Ni, are overall somewhat higher than in the foliar material, and this is especially true for Co, Mo, and Zr in *Phyllanthus balgooyi.*

Discussion

Multi-element Chemistry of Transport Fluids and Phloem Tissues The phloem tissue is well developed in some Ni hyperaccumulator trees (*Phyllanthus balgooyi, P.* cf. *securinegoides, Rinorea bengalensis*) and appears to act as a 'Ni-sink'. Despite scant information of the elemental concentrations in phloem tissue and sap of such hyperaccumulators, a few intriguing examples are known. The most well-known of these is *Pycnandra acuminata,* of which a single value of 25.7 % Ni in the phloem sap ('latex') was reported by Jaffré et al. (1976) and subsequently sparked global scientific interest in Ni hyperaccumulators. Later studies working with the same species in New Caledonia reported a lower mean of

Table 4 Elemental concentrations in plant parts in studied Ni hyperaccumulators

Species	Part	**Al** μg g^{-1}	**Ca** μg g^{-1}	**Co** μg g^{-1}	**Mn** μg g^{-1}	**Ni** μg g^{-1}	**P** μg g^{-1}	**S** μg g^{-1}	**Zn** μg g^{-1}
Phyllanthus cf. *securinegoides*	Flowers	370	1025	104	945	5556	392	347	100
Phyllanthus cf. *securinegoides*	Seed capsule	70	1905	9	24	2267	655	757	22
Phyllanthus cf. *securinegoides*	Seeds	85	9460	3	187	17,570	1856	2384	182
Phyllanthus balgooyi	Flowers	24	1479	21	12	736	1136	1695	23
Psychotria sarmentosa complex	Fruit	433	3960	8	291	3923	531	1039	19
Rinorea bengalensis	Seed capsule	19	8100	29	365	4397	355	1116	87
Rinorea bengalensis	Seeds	8	454	24	19	71	74	100	4
Walsura cf. *pinnata*	Fruit	16	7825	2	37	1544	1890	1215	50
Xylosma luzonensis	Fruit	8	2350	5	53	854	777	487	16

Table 5 Ni concentrations in plant parts in studied Ni hyperaccumulators. Number of samples refer to twigs, bark, and wood samples

Species	*N*	Twigs Ni µg g^{-1}		Bark Ni µg g^{-1}		Wood Ni µg g^{-1}		Phloem Ni µg g^{-1}		Xylem Ni µg mL^{-1}	
		Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean
Actephila sp. nov	5	386–782	[591]	–	[1960]	–	[311]	–		–	
Flacourtia kinabaluensis	2	204–5447	[2277]	455–503	[479]	221–274	[248]	–		2–3	[2]
Glochidion sp. undet.	12	56–2645	[1256]	306–3065	[1166]	115–1593	[454]	–	[425]	–	[0.6]
Kibara coriacea	1	–		–		–	[1664]	–		–	
Mischocarpus sundaicus	1	–	[318]	–	[288]	–	[63]	–		–	
Phyllanthus balgooyi	9	73–8608	[1826]	68–372	[208]	70–904	[350]	45,010–79,340	[65,410]	35–76	[56]
Phyllanthus cf. *securinegoides*	7	889–6913	[4420]	3767–6822	[6000]	443–1168	[847]	5990–10,800	[8830]	–	[13]
Psychotria sarmentosa complex	6	2463–4800	[3595]	–		–	[1718]	–		–	[49]
Ptyssiglottis cf. *fusca*	1	–	[13]	–		–		–		–	
Rinorea bengalensis	5	6470–8510	[7490]	85–178	[132]	613–1920	[1077]	15,480–22,600	[18,490]	6–19	[12]
Rinorea javanica	3	2278–4006	[2940]	142–267	[186]	133–643	[356]	–		–	
Walsura cf. *pinnata*	2	208–476	[342]	207–371	[289]	39–62	[50]	–		–	
Xylosma luzonensis	3	1264–1943	[1603]	244–1947	[995]	125–252	[180]				

Number of samples refers to twigs samples only

Fig. 3 An excised section of the bark and phloem tissue (2.3 % Ni) of *Rinorea bengalensis* (*top*), and phloem sap (16.9 % Ni) bleeding from a stem section of *Phyllanthus balgooyi* (*bottom*)

6.4 % (Callahan et al. 2008) and up to 14.7 % (Schaumlöffel et al. 2003) in this phloem sap. Two other cases are known, one from Brazil, *Cnidoscolus* sp. nov (Euphorbiaceae) with 1.35 % Ni in phloem sap (Reeves 2003), and the second, *Euphorbia helenae* subsp. *grandifolia* (Euphorbiaceae) from Cuba with 3.1 % Ni (Reeves et al. 1996). The concentration of Ni (9 %) in the phloem tissue of *Phyllanthus balgooyi* has been reported from the Philippines (Baker et al. 1992; Hoffmann et al. 2008), but phloem sap was not collected at the time. The phloem sap exudate from a stem section of *Phyllanthus balgooyi* in Sabah contains up to 16.9 % Ni, among the highest reported globally. It is worth noting that unlike *Pycnandra acuminata*, which produces a green opaque latex from the laticifers, *P. balgooyi* excretes green transparent phloem sap when the outer-phloem tissue is damaged. Compared with the Cuban *Euphorbia helenae* subsp. *grandifolia* mentioned previously with a phloem sap containing 0.37 % Ca, 0.31 % Mg, 0.14 % Na, 0.07 % K, and 0.04 % Co, the sap from *Phyllanthus balgooyi* contains lower concentrations of these cations but a higher concentration of Co.

The xylem transports mineral elements from the roots to the shoot and photosynthetic organs (leaves), and thus in Ni hyperaccumulators, should contain significant concentrations of Ni, besides other essential elements such as Ca, K, Mg, and P. The xylem Ni concentration has been reported in *Alyssum* spp. as in excess of 176 µg ml^{-1} in plants grown hydroponically (Centofanti et al. 2013), and 56 µg ml^{-1} in plants growing in the natural habitat (Alves et al. 2011). In our study, the highest concentrations found were in *Phyllanthus balgooyi* and *Psychotria sarmentosa* complex, which contained 76 and 49 µg ml^{-1} Ni, respectively.

Table 6 Elemental concentrations in foliage, phloem tissue, and phloem sap ($\mu g\ g^{-1}$) in studied Ni hyperaccumulators (ultra-trace elements)

Species	Al	As	Au	B	Ba	Bi	Cd	Co	Cr	Cu	Fe	Hg	Li	Mn	Mo	Pb
Flacourtia kinabaluensis	31	0.5	0.1	51	2.2	1.0	<0.01	0.3	1.6	13	37	0.1	0.01	39	6.3	2.6
Glochidion sp. *'bambangan'*	7.4	0.4	0.02	24	0.1	0.7	0.2	0.3	3.2	4.0	16	0.1	0.00	43	3.0	0.7
Kibara coriacea	866	0.3	0.1	41	1.1	0.8	1.0	0.3	59	3.5	85	0.01	0.06	479	3.7	2.3
Phyllanthus cf. *securinegoides*	15	0.2	0.01	44	0.5	0.7	0.5	0.7	5.6	4.0	43	0.1	0.06	191	3.9	1.4
Psychotria sarmentosa complex	124	0.4	0.1	15	1.0	0.2	0.2	0.3	23	3.8	276	0.1	0.02	236	2.9	1.9
Rinorea bengalensis	61	0.3	0.1	42	22.4	0.6	0.2	0.4	3.9	8.3	187	0.1	0.03	68	7.9	2.0
Shorea tenuiramulosa	21	0.3	0.2	19	3.2	0.4	<0.01	0.5	2.0	2.6	40	0.1	0.04	211	5.2	1.6
Walsura cf. *pinnata*	21	0.4	0.0	60	0.3	0.6	0.2	0.5	1.7	6.2	41	0.1	<0.01	137	5.8	3.0
Species	Sb	Se	Sn	Sr	Ti	U	V	Zn	Zr	Ca	Si	Na	Ni	P	Mg	K
Flacourtia kinabaluensis	0.4	0.5	0.04	54	0.1	0.01	0.6	79	0.3	11,630	118	85	1145	612	4277	20,770
Glochidion sp. *'bambangan'*	0.3	0.4	0.1	1.5	0.02	<0.01	0.7	31	0.2	2300	601	31	6930	344	1656	3013
Kibara coriacea	0.1	0.9	0.1	10	0.1	0.01	0.8	34	0.3	10,150	365	3117	4400	810	5910	8812
Phyllanthus cf. *securinegoides*	0.2	0.4	0.1	3.3	0.1	0.01	1.0	51	0.5	6220	455	44	11,970	567	1770	6723
Psychotria sarmentosa complex	0.4	0.4	0.02	31	0.2	<0.01	0.4	43	0.5	4915	148	59	8570	489	5175	3660
Rinorea bengalensis	0.3	0.5	0.04	52	0.3	0.01	0.6	54	0.5	8900	204	46	4195	759	2440	5030
Shorea tenuiramulosa	0.2	0.8	0.03	18	0.2	0.01	2.4	16	0.2	9770	1108	330	1553	398	1689	4060
Walsura cf. *pinnata*	0.3	0.4	0.1	1.8	0.2	0.01	0.8	82	0.3	5015	276	41	1690	802	1520	17,080
Species	Al	As	Au	B	Ba	Bi	Cd	Co	Cr	Cu	Fe	Hg	Li	Mn	Mo	Pb
Rinorea bengalensis (phloem tissue)	14	0.92	0.01	96	5.1	0.9	0.1	1.4	7.4	4.4	156	0.02	0.01	256	11	5.4
Phyllanthus cf. *securinegoides* (phloem tissue)	31	0.02	0.01	30	3.4	0.8	0.8	0.7	0.8	1.9	33	0.07	0.04	95	5.9	3.0
Phyllanthus balgooyi (phloem tissue)	18	0.43	0.10	10	4.7	0.5	1.4	14.8	1.4	4.8	51	0.12	0.1	157	11	4.4
Phyllanthus balgooyi (phloem sap)	7.2	0.10	0.02	14	1.3	1.6	3.6	31.1	8.4	0.5	29	0.17	0.02	465	16	7.5
Species	Sb	Se	Sn	Sr	Ti	U	V	Zn	Zr	Ca	Si	Na	Ni	P	Mg	K
Rinorea bengalensis (phloem tissue)	0.2	1.8	0.4	23	0.2	0.02	7.6	882	1.5	34,400	1466	106	18,650	673	826	9180
Phyllanthus cf. *securinegoides* (phloem tissue)	0.2	0.1	0.1	35	0.1	0.01	0.8	81	0.5	40,220	549	1172	10,480	301	1122	7413
Phyllanthus balgooyi (phloem tissue)	0.3	0.1	0.1	8.6	0.2	0.01	1.8	737	1.7	2391	417	2068	71,470	247	805	4100
Phyllanthus balgooyi (phloem sap)	0.9	0.7	0.2	1.5	0.2	0.01	5.4	2044	3.5	1133	1078	278	147,170	266	563	1654

Multi-element Chemistry of Plant Parts Ni concentrations in bark and wood, have been reported to date only for several Ni hyperaccumulators: *Pycnandra acuminata* contained 24 500 μg g^{-1} Ni (bark) and 1700 μg g^{-1} Ni (wood) (Jaffré et al. 1976); *Hybanthus floribundus* contained 1700 μg g^{-1} (bark) and 1500 μg g^{-1} (wood) (Severne 1972); and *Psychotria gabriellae* 52 400 μg g^{-1} (bark) and 2300 μg g^{-1} (wood) (Jaffré and Schmid 1974). The Ni concentrations in the twigs and wood can be relatively high in a number of hyperaccumulators from Sabah, and these values can even exceed hyperaccumulator threshold levels (>1000 μg g^{-1} Ni). This is the case for *Phyllanthus balgooyi, P.* cf. *securinegoides,* and *Psychotria sarmentosa* complex, which have high Ni concentrations in their twigs and wood. However, preferential Ni accumulation in the leaves means that although foliar Ni concentrations may be high, the corresponding Ni concentrations in the twigs and wood can be low. This situation applies to *Walsura* cf. *pinnata, Xylosma luzonensis, and Mischocarpus sundaicus*. Therefore, these observations re-affirm the need to base hyperaccumulation thresholds on foliar Ni concentrations, and not on Ni concentrations in other plant parts.

Nickel Contained in a Large Hyperaccumulator Tree The tree *Rinorea bengalensis* (15–25 m, stem diameter up to 60 cm) grows relatively fast, and produces a yellow fine-grained medium hardwood. The significant Ni concentrations in the wood of this species (up to 1920 μg g^{-1}) effectively render this wood laced with a toxic substance. As opposed to impregnation with copper-arsenide mixtures (CCA) in wood preservation, the intrinsic Ni in *R. bengalensis* might act as a natural insecticide/fungicide, although this has not yet been tested. The total Ni content calculated to be contained in a large specimen of *R. bengalensis* (4.77 kg) is much lower than the 37 kg estimated for *Pycnandra acuminata* by Sagner et al. (1998). These authors calculated the total Ni contained in this tree on the basis of a specimen with a height of 15 m and a total estimated biomass of 1980 kg. However, dividing the total biomass (1980 kg) by the amount of contained Ni that was calculated (37 kg) means that the overall biomass would have had to contain 1.87 % Ni. This overall Ni concentration is clearly too high considering the Ni concentrations that Jaffré et al. (1976) report in *Pycnandra acuminata* (wood with 0.17 % and leaves with 1.17 %), and that wood biomass is the largest fraction in a tree. We estimate the maximum total amount of Ni contained in a specimen of *Pycnandra acuminata* at 5.15 kg on the basis of a dbh of 69 cm (largest diameter recorded by Boyd and Jaffré 2001), a height of 15 m, Ni in leaves of 1.17 % and (an estimated) 0.65 % Ni in branches. The amounts of Ni contained in the fractions then are: stem (1682 kg, 2.86 kg Ni), branches (311 kg, 2.02 kg Ni), and leaves (23 kg, 0.27 kg Ni). We also repeated this procedure with the allometric equations for hardwoods by Harris et al. (1973), which yielded similar results, that is a total of 4.63 kg Ni for *Rinorea bengalensis* and total of 7.94 kg Ni for *Pycnandra acuminata*. It should be noted that both these species have high Ni in the phloem tissue and sap (2.3 % in phloem tissue and 25.7 % in phloem sap for *Rinorea bengalensis* and *Pycnandra acuminata,* respectively), and hence would represent an additional amount of Ni unaccounted for in these calculations, but this is unlikely to amount to more than a few kilograms.

Co-hyperaccumulation of Other Elements (Cobalt) An unusual example, not from ultramafic soils, is the Co accumulation of 530–845 μg g^{-1} in *Nyssa sylvatica* in the US (Brooks et al. 1977; Kubota et al. 1960; Robinson et al. 1999). Another species in this genus (*Nyssa javanica*) occurs in Sabah near Kinabalu Park (not on ultramafic soils), and although no analytical results are available for local specimens, analysis of herbarium specimens of this species by Brooks et al. (1977) yielded only a maximum of 16 μg g^{-1} of Co in the foliage. Finally, there also are two examples of *Phyllanthus* species from Cuba and New Guinea (with Co values of 1140 and 200 μg g^{-1}, respectively) (Reeves 2003, 2006), both from ultramafic soils. *Glochidion* cf. *sericeum* reported in this study appears to be a genuine Co hyperaccumulator (foliar Co 443–1311 μg g^{-1}). This medium large tree (10 m) grows in lowland tall dipterocarp forest on deep laterite ultramafic soils. The behavior of this species might be explained by local soil conditions promoting the release of Co from Mn oxides by reduction (as evidenced from concomitant high foliar Mn concentrations). Cobalt hyperaccumulation on ultramafic soils is exceptionally rare, as foliar Co concentrations in plants from ultramafic soils are normally low (mean of 5 μg g^{-1}) and even in (Ni) hyperaccumulator species (this study) the overall mean was only 54±9 μg g^{-1}. The low foliar Co concentrations might be explained by both low Co phytoavailability in the soils and because Ni is often present in concentrations 10-fold greater than those of Co, thereby inhibiting Co uptake (Malik et al. 2000).

Ultra-trace Elements in Ni Hyperaccumulators Little is known about the ultra-trace element concentrations in tropical plants, and the only regional study we could find is a report by Breulmann et al. (1999) on plants collected from non-ultramafic soils. The results here indicate that Ni hyperaccumulators can contain measurable concentrations of ultra-trace elements, but additional analyses are needed to elucidate the extent of this phenomenon and its potential usefulness for 'chemical fingerprinting' (as applied for the Dipterocarpaceae in the Lambir Hills, Sarawak by Breulmann et al. 1998).

Possible Effects of High Nickel in Floral Parts on Pollination There are only a few reports of the Ni content of flowers and seeds/fruits of Ni hyperaccumulators in the scientific literature. *Alyssum murale* seeds contained a mean of 13 100 μg g^{-1} Ni whereas the flowers contained a mean of 16 000 μg g^{-1} Ni (Barbaroux et al. 2011). In *Psychotria gabriellae* (formerly *P. douarrei*), flowers contained 24 000 μg g^{-1} Ni and the fruits up to 28 000 μg/g^{-1} Ni (Jaffré and Schmid 1974), *Streptanthus polygaloides* flowers contained 16 400 μg g^{-1} Ni, and the fruits 5230 μg g^{-1} Ni (Reeves et al. 1981), *Stackhousia tryonii* flowers contained 8400 μg g^{-1} Ni (Batianoff et al. 1990), and the fruit of *Pycnandra acuminata* contained 3000 μg g^{-1} Ni (Jaffré et al. 1976). Compared with these values, the flowers of *P. securinegoides* were high in Ni (5556 μg g^{-1}). Concentrations in the seeds were also high at 17 570 μg g^{-1} Ni, and could possibly affect insect floral visitors and pollination, as has been shown for *Streptanthus polygaloides* in the USA (Meindl and Ashman 2014). High Ni concentrations in flowers could lead to pollinator preferences between populations, and induce isolation, which may contribute to population differentiation and subsequent speciation. Relevant in this respect is that many *Glochidion* and *Phyllanthus* species are exclusively pollinated by moths of the genus *Epicephala*. Females of these moths actively pollinate the flowers and then deposit eggs into the floral ovaries, after which the larvae consume some of the developing seeds (Kawakita 2010). The co-evolution and high specificity for *Epicephala*-species for *Phyllanthus*-species has been linked to the extensive diversification of this genus in New Caledonia (Kawakita and Kato 2004). The link with *Epicephala* has, however, not yet been studied in relation to Ni hyperaccumulating *Glochidion* and *Phyllanthus*.

Plant 'Elemental Defense' and 'Joint Effects Hypothesis' The high Ni concentrations in the phloem tissue mentioned earlier could enable internal re-distribution to aid herbivory-protection ('elemental herbivory defense' *viz.* Boyd and Martens 1998), or protect against phloem sap feeding insects such as aphids. Although a study on aphids feeding on the temperate Ni hyperaccumulator *Streptanthus polygaloides* (phloem sap concentration of 295 μg g^{-1} Ni) found that such Ni concentrations were not effective in defending plants against aphid attack (Boyd and Martens 1999), the substantially higher concentrations in the tropical species studied would be expected to have a toxic effect. Nevertheless, aphids were found feeding on *Phyllanthus* cf. *securinegoides* (Van der Ent et al. 2015) and hence must be either highly Ni-tolerant or employ mechanisms that avoid Ni-toxicity during feeding or both (Boyd 2010). The first category includes specialized insects that themselves have high whole-body Ni levels ("high-Ni insects") and may have defensive benefits against predication (Boyd 2010), but such insects have not been studied to date in Sabah.

Concentrations of Ni in seeds and seed capsules of *Phyllanthus* cf. *securinegoides* and *Rinorea bengalensis* also were high, except in the seeds of *R. bengalensis* (but not the seed capsules, which were high in Ni). High concentrations of Ni in the seeds may supply the seedling with ample Ni, and hence potentially aid in herbivory protection during the early stages of development, before a rooting system has sufficiently developed to sequester Ni from the soil. Recent experimental work by Cheruiyot et al. (2013) demonstrated that the minimum foliar lethal concentrations (MLC) for a tested generalist insect herbivore were far lower than the hyperaccumulation thresholds for Ni or Co. This suggests that Co accumulation far below levels seen for Ni in hyperaccumulators (for example 443–1311 μg g^{-1} Co in *Glochidion* cf. *sericeum*) could be as effective as a herbivory-defense as Ni hyperaccumulation. However the 'Joint Effects Hypothesis' (viz. Boyd 2012) is not likely to be apply in most Ni hyperaccumulators in Sabah, as the uptake of transition metals other than Ni is remarkably controlled, for example the leaves of a specimen of *Psychotria sarmentosa* complex contains 23 900 μg g^{-1} Ni but just 14 μg g^{-1} Co.

Implications for Utilization in Ni Phytomining The identification of suitable species for Ni phytomining has so far mainly been assessed based on growth characteristics and Ni accumulation capacity (Harris et al. 2009; Robinson et al. 1997a, b; Van der Ent et al. 2013b). However, in the tropical environment, with ligneous Ni hyperaccumulator species, other important selection criteria include the Ni concentrations in harvestable biomass (consisting of foliar, twig, and wood fractions), and the optimum concentrations of elements other than Ni (such as Ca, Mg, K, P, S) to minimise 'contamination' with unwanted elements in the eventual 'bio-ore'. It is clear that leaves and green twigs have the highest Ni concentrations, and are hence the favorable biomass fraction for Ni phytomining. Thus, an efficient phytomining model could be based on pruning and re-growth of young shoots, leaving the ligneous base and root system intact. Furthermore, the results show that Ca concentrations in hyperaccumulator tissues can be significant; for example, in a sample of *Phyllanthus* cf. *securinegoides,* the concentrations of Ca and Ni are each about 10 % in the foliar ash, but Ca is 33 % in the ash of the ligneous parts while Ni is 10 % in this fraction (Van der Ent, unpublished). The tendency for high Ca uptake in Ni hyperaccumulators also has been reported elsewhere, for example, in *Psychotria gabriellae* from New Caledonia with 19 900 μg g^{-1} Ni and 24 400 μg g^{-1} Ca (Baker et al. 1985). The high Ca concentrations in hyperaccumulator biomass would have consequences for the economic viability of Ni extraction from the bio-ore. The Ca accumulation in hyperaccumulator biomass also suggests that significant amounts of Ca are probably needed in fertilizer application in the phytomining operation to avoid Ca depletion in the soil. This has been recognized in phytomining trials with *Alyssum* spp. (Chaney et al. 2007). Other elements that will need to be replenished in the soil in a phytomining operation include K and P to stimulate both biomass production and to counteract against removal of these elements through the harvesting of biomass. Ecological concerns as they become better understood also will have to be considered.

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