**Cobalt hyperaccumulation in *Rinorea* cf. *bengalensis* (Violaceae)
from Sabah: accumulation potential and tissue and cellular-level
distribution of cobalt**

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ABSTRACT

Aims Knowledge on the ecophysiology of cobalt hyperaccumulator species is limited. The nickel hyperaccumulator *Rinorea cf. bengalensis* from Borneo can accumulate high concentrations of cobalt in nature. This study investigates the cobalt accumulation potential of *Rinorea cf. bengalensis* in relation to nickel concentrations in soils and the subsequent tissue and cellular-level distributions of cobalt, nickel, and major cations.

Methods Seedlings of *Rinorea cf. bengalensis* were grown in mixed treatments on ultramafic soil containing a high concentration of available nickel. Cobalt and nickel salts were then added to the soil to study their interactions. The tissue and cellular-level distributions of cobalt, calcium, nickel, and potassium were investigated using synchrotron-based X-ray fluorescence microscopy.

Results The maximum foliar cobalt concentration reached 1200 $\mu\text{g g}^{-1}$. Accumulation of cobalt competed with nickel accumulation although nickel seems to stimulate cobalt phloem translocation. Plants suffered toxicity in the treatment with the highest soil cobalt concentration. Cobalt and nickel have contrasting distribution patterns in the leaves of *Rinorea cf. bengalensis*, with cobalt mainly excreted on the surface of the leaves, whereas nickel is localised in foliar epidermal cells.

Conclusions *Rinorea cf. bengalensis* can accumulate high concentrations of cobalt but is intrinsically more tolerant to nickel. It does not rely on a similar sequestration mechanism for both metals, which could explain the lesser tolerance for cobalt. Nickel appears to be essential for the plant to tolerate high cobalt concentrations. Further studies intending to develop agronomic practices are needed to determine the viability of *Rinorea cf. bengalensis* for nickel-cobalt agromining.

Keywords

Cobalt, Distribution, Hyperaccumulator, Toxicity, *Rinorea cf. bengalensis*, Synchrotron

INTRODUCTION

Metal hyperaccumulating plants can uptake and store extraordinary concentrations of metals and metalloids in their living tissues that may be two or three orders of magnitude higher than normal plants (Baker and Brooks 1989; van der Ent et al. 2013). Only a minority of plants are defined as metal or metalloid hyperaccumulators as only ~700 species scattered in ~50 families have been reported to date (~0.2% of flowering plants) (Reeves et al. 2018). The majority (~70%) of these species hyperaccumulate nickel (Ni) and thrive in soils that formed from the weathering of ultramafic bedrock. These soils are naturally enriched in cobalt (Co), chromium (Cr), manganese (Mn) and Ni, are deficient in major macronutrients (phosphorus (P) and potassium (K)) and have a low calcium (Ca) to magnesium (Mg) molar ratios (van der Ent et al. 2015a). These soils are rather widely distributed on Earth (3% of its surface) and cover large areas in some territories (e.g., Cuba, New Caledonia, Malaysia, Indonesia) (Guillot and Hattori 2013; Proctor 2003). Several hypotheses have been proposed as potential ecological drivers that led to the appearance of hyperaccumulation independently in multiple different plant families, even though its physiological cost is presumably considerable, and include increased drought tolerant or metal disposal/tolerance functions (Boyd and Martens 1992). Litter decay enriches the local ecosystem in Ni and may lead to allelopathic effects (i.e., competitive interference) through litterfall (van der Ent et al. 2015b). The most broadly accepted hypothesis for the evolution of Ni hyperaccumulation is known as “elemental defence” which supports the idea that hyperaccumulation fulfils herbivory protection functions (Boyd 2007; Hörger et al. 2013).

The Violaceae is a cosmopolitan family of subshrubs, shrubs and trees with ~800 accepted species in 25 genera. The principal genera are *Viola*, a genus found in temperate and mountainous regions (~475 spp.), *Rinorea* (~160 spp.) and *Hybanthus* (~70 spp.) both the latter being pantropical (Ballard et al. 2014; Wahlert and Ballard 2012). In Malesia, 11 species occur, among which eight on the island of Borneo (Wahlert and Ballard 2012). *Rinorea* cf. *bengalensis* (Violaceae) is commonly found all over Southeast Asia, and its range extends from India to North-Queensland. It is a facultative species that grows on both non-ultramafic and ultramafic soils, and a facultative hyperaccumulator as only the ultramafic populations have high foliar Ni concentrations (Brooks et al. 1977; Brooks and Wither 1977). In Sabah (Malaysia, on the island of Borneo), local populations grow on circum-neutral ultramafic soils with relatively high phytoavailable Ni concentrations (Nkrumah et al. 2018b). Trees from these populations may reach 25 m high and have highly Ni-enriched phloem tissues and leaves with concentrations reaching up 22,600 and 12,800 $\mu\text{g g}^{-1}$ d.w. respectively (van der Ent and Mulligan 2015; van der Ent et al. 2017). The Ni- rich green-coloured phloem tissues observed in *R. cf. bengalensis* appear to be a common feature of tropical woody Ni hyperaccumulator plants and hint at the critical role of the phloem in redistributing Ni to other plant tissues in this species (van der Ent et al. 2020a). So far, it has been documented eight times, mostly in Ni hyperaccumulator species from Southeast Asia (Nkrumah et al. 2018b). Two other species from the Violaceae family exhibit this atypical phloem (*Hybanthus austrocaledonicus* from New Caledonia and *Rinorea* cf. *javanica* endemic to Southeast Asia). Four other species are *Dichapetalum gelonioides* subsp. *tuberculatum* (Dichapetalaceae) and *Actephila alankeri*, *Phyllanthus rufuschaneyi*, and *Phyllanthus balgooyi* (Phyllanthaceae) which are all from the

Indomalaya realm and in the COM (Celastrales, Oxalidales and Malpighiales) clade (Bouman et al. 2018; Nkrumah et al. 2018a; van der Ent et al. 2015a, b, 2016, 2017). The most recently discovered *Blepharidium guatemalense* (Rubiaceae) from Mexico also has the same adaptations, with a bright green phloem tissue containing more than 50,000 $\mu\text{g g}^{-1}$ Ni d.w. and was the first to be discovered outside the Asia-Pacific Region (unpublished data).

Rinorea cf. bengalensis, a long-lived large tree with a well-developed phloem, is an ideal model for studying hyperaccumulation and phloem translocation (van der Ent et al. 2017). It is also a fast-growing species which is ostensibly competitive in its natural habitat especially in early succession after disturbance (it is one of the most common/dominant trees on ultramafic soils in Sabah). Hence, any metabolic cost of Ni hyperaccumulation in *R. cf. bengalensis* does not come at a competitive disadvantage. However, the acquisition, transport, and complexation of Ni using substantial amounts of chelators (e.g., citrate, malate) must come at a significant metabolic cost for *R. cf. bengalensis* (van der Ent et al. 2017). This argues for a beneficial role of hyperaccumulation on either growth rate (i.e., competitive advantage due to less insect damage) or survival (i.e., of the seedlings due to allelopathic effects on other plants). The extremely high concentrations of Ni in the phloem combined with the current evidence for repeated Ni translocation (Ni is translocated to young leaves and then pumped through the phloem to older leaves) in *R. cf. bengalensis* trees (Zelano et al. 2020), suggests that a yet to be identified (physiological) function could be important to plant fitness in *R. cf. bengalensis*. Comparable to most other Ni hyperaccumulator species, Ni is sequestered in the epidermal cells of *R. cf. bengalensis* (van der Ent et al. 2017). The vacuoles of the foliar epidermal cells, by virtue of their distance from the photosynthetic processes occurring in the mesophyll, are key in sequestering Ni in leaves of many hyperaccumulating taxa (Küpper et al. 2001).

Although Co is critical for rhizobia associated with legumes in nitrogen-fixation, it is not essential for plants, nor does it take part in any metabolic processes in higher plants (De Hertogh et al. 1964). In contrast, Ni is essential for nitrogen assimilation as part of the enzyme urease (Dixon et al. 1975). In excess, Co disrupts photosynthesis and induces iron (Fe) deficiency and the production of reactive oxidative species which ultimately lead to leaf chlorosis and a growth rate reduction (Chen et al. 2009; Morrissey et al. 2009). Hyperaccumulation of Co is globally rare (~40 species) and is mostly reported in Cu-Co outcrops of the Copperbelt divided between the Democratic Republic of Congo and Zambia in Central Africa (Lange et al. 2017). In the African Co hyperaccumulator *Haumaniastrum katangense* (Lamiaceae), Co is complexed with carboxylic acids, while in *Persicaria punctata* (Polygonaceae) Co is mainly distributed in epidermal cells and the phloem (van der Ent et al. 2019, 2020b). The Co coordination chemistry has an aqueous Co (II)-tartrate complex that is the predominant component identified in all plants and tissues, along with a minor component of a Co (III) compound with oxygen donor ligands (van der Ent et al. 2020b). Cobalt promotes growth and appears to be essential in the hyperaccumulator species *Haumaniastrum robertii*, where it is mainly localised in vascular bundles of roots and leaves (Kabeya et al. 2018). Experimental work with *Crotalaria cobalticola* (Fabaceae) has shown significant increases of citric acid and cysteine when plants are exposed to Co, whereas phytochelatin synthesis was not induced (Oven et al. 2002). It has been hypothesised that Co hyperaccumulation has

evolved from adaptations in Fe homeostasis (Lange et al. 2017). Very little is known about the ecophysiology of Co in tropical woody hyperaccumulator plants. Micro-analytical investigations on *Glochidion cf. sericeum* (Phyllanthaceae) from Sabah (Malaysia), a small tree which accumulates up to 1500 $\mu\text{g g}^{-1}$ d.w., have shown that Co is localised in lesions on the leaf surface and complexed with carboxylic acids (van der Ent et al. 2018).

Phytomining (also called agromining) is a type of agriculture in which hyperaccumulator species are cultivated on metalliferous soils (sub-economic mineralisation and contamination by industry) and their biomass harvested to extract valuable metals such as Co or Ni (Chaney et al. 2014; van der Ent et al. 2015c). Nickel phytomining may become transformative in the rehabilitation of Ni strip-mining operations in Southeast Asia where very extensive ultramafic outcrops exist (van der Ent et al. 2015a). Cobalt phytomining may extract resources that are not readily extractable with conventional mining/metallurgical processes (Lange et al. 2017). Greenhouse and field trials in Sabah intending to develop the agronomy of several tropical Ni hyperaccumulator species have highlighted the potential of *R. cf. bengalensis* to be used in Ni phytomining operations owing to its rapid growth and extraordinary levels of Ni accumulation (Nkrumah et al. 2018b; van der Ent et al. 2015b). Further studies have shown that Ni uptake and plant growth were species-dependent when exposed to different formulations of standard inorganic fertilisers (N, P and K) or other amendments (Ca, S and organic matter) (Nkrumah et al. 2019a, b).

Two *Rinorea* species from Southeast Asia have been reported to hyperaccumulate Co ($>300 \mu\text{g g}^{-1}$ d.w., the hyperaccumulation threshold), with concentrations up to 670 $\mu\text{g g}^{-1}$ d.w. in *R. cf. javanica* and up to 545 $\mu\text{g g}^{-1}$ d.w. in *R. cf. bengalensis* in their natural habitat (Brooks et al. 1977; Brooks and Wither 1977; Lange et al. 2017; Reeves et al. 2018). A better understanding of the ecophysiology of *R. cf. bengalensis* is crucial to developing effective Co, and Ni agromining as the commercial viability depends on the combination of biomass production per time unit and Co/Ni accumulation in the crop.

Previous synchrotron and nuclear microprobe investigations have revealed very high levels of accumulation in the phloem bundles of the roots and stems (van der Ent et al. 2017). In the leaves, Ni is preferentially accumulated in the epidermal cell region (van der Ent et al. 2020a). The chemical form of Ni^{2+} does not substantially differ and is unambiguously associated with citrate in *Rinorea* (van der Ent et al. 2017). No information is presently available about the tissue-level distribution of Co in *R. cf. bengalensis* plants exposed to high soil Co. *Rinorea cf. bengalensis* has the potential to hyperaccumulate Co and may be used for future sustainable phytomining applications. This study aimed at better understanding the trade-offs between Co and Ni accumulation in *R. cf. bengalensis* by undertaking a Co and Ni soil dosing pot experiment. In order to establish if both elements could be accumulated through the same uptake and translocation pathways, micro-analytical techniques based on synchrotron X-ray fluorescence microscopy on live plants were used to elucidate the distribution of Co and other physiologically relevant elements at the tissue and cellular levels. This information will ultimately be useful to develop methods to increase Co accumulation in *R. cf. bengalensis* for phytomining applications.

MATERIALS AND METHODS

Plant stock source

Rinorea cf. *bengalensis* wildlings (~15 cm height) were collected from their native habitat (345 m a.s.l.; 6°12' 2"N; 116°45'8"E). All wildlings were potted immediately after collection with soil collected from the native habitat and allowed to acclimatise in a shade house for 2 weeks. The wildlings were then transplanted into test pots (8 L with 10 kg ultramafic soil) and allowed to establish for 3 months. The soil originated from the top 15–20 cm of an unfertilised ultramafic soil (Eutric Cambisol Magnesic) near Pahu village, Sabah, Malaysia (6°7'49"N; 116°46'22"E) and it is roughly the same type of soil on which the wildlings were collected. The soil was sun-dried, sieved through a 1-cm stainless steel screen, homogenised, and analysed (Table 1). The initial Ni:Co pseudo total concentration ratio was approximately 10 with concentrations of Ni and Co of 4730 and 445 mg kg⁻¹ respectively.

Plant culture conditions

The experiment was conducted in a shade-house covered with neutral-density shade-cloth at the Monggis sub-station in Sabah, Malaysia (6°11' 59.7"N 116°45'08.0"E). The climate at the sub-station is tropical with a daytime temperature of 29 °C and an average relative humidity of 75%. Transparent polyethene film roofing was used to maintain the light intensity uniform in the shade house and keep rainfall out to better control plant irrigation. The light intensity under the shade house was 350 μmol m⁻² sec⁻¹ Photosynthetically active radiation (PAR) light (measured with an Apogee MQ-500 spectroradiometer) to favour the growth of understorey species. The plants were watered daily to field capacity with local water tapped from a nearby river to mimic the conditions in the native environment. As the river originates from an intact lowland rain forest in sandstone soils, water concentrations of Ni and Co were low (< 0.01 mg L⁻¹) while variations expected to be minimal during the experiment (constant flow rate supplied from the water table). Plants were harvested after a 12-month growth period during which they were separated into fine root, taproot, old stem, young stem, old leaf and young leaf fractions. The roots were thoroughly washed with water to remove soil particles to reduce surficial contamination.

Plant dosing treatments

Soils were amended with Ni and Co in the form of the nitrate salts Ni(NO₃)₂•6H₂O and Co(NO₃)₂•6H₂O in solutions to 10 kg dry soil pots containing previously watered soil. In this study, three different modalities which aimed to increase pseudo-total concentrations of Co or Ni by a ~ 1/3 ratio and a control were used: Ni100Co1500 (+100 mg kg⁻¹ Ni / +1500 mg kg⁻¹ Co), Ni750Co750 (+750 mg kg⁻¹ Ni / +750 mg kg⁻¹ Co), Ni1500Co100 (+1500 mg kg⁻¹ Ni / +100 mg kg⁻¹ Co) and control (+0 mg kg⁻¹ Ni / +0 mg kg⁻¹ Co). Following the amendments, the soil in each pot was thoroughly mixed, sun-dried, homogenised, watered to reach field capacity and equilibrated for 10 days before transplanting. Pots were arranged in a randomised complete block design with three replications for each Ni:Co combination treatment. The pots were randomised regularly to limit differences caused by light or humidity heterogeneity in the shade house.

Chemical analysis of soil and plant material samples

Soil pH was determined by shaking for 2 h a 1 to 2.5 soil to water mixture. The $\text{Sr}(\text{NO}_3)_2$ -extraction (0.01 M) evaluated the soil solution concentrations of trace elements: 5 g soil to 20 mL extractant ratio and a 2 h shaking time. Cation exchange capacities (CEC) of the soil (~700 mg) was determined by measuring the silver remaining in solution after adding 40 ml of a 0.01 M silver-thiourea solution and shaking for 16 h. Phytoavailable concentrations were assessed with the diethylenetriaminepentaacetic acid (DTPA) extraction modified by using 5 g:20 mL (soil:solution ratio). Soil samples (~100 mg) were digested with 9 mL 70% HNO_3 and 3 mL 37% HCl per sample in a microwave (Milestone Start One) for 1.5 h and diluted with ultrapure water before analysis to determine pseudo-total elemental concentrations. All digests and extracts were analysed by inductively coupled plasma atomic emission spectroscopy (ICP-AES - PerkinElmer Optima 8300) for aluminium (Al), Ca, Co, Cr, copper (Cu), Fe, K, Mg, Mn, Ni, P, sulphur (S), and zinc (Zn). In-line internal addition standardisation using yttrium was used to compensate for matrix-based interferences. The plant material was oven-dried at 65°C for 2 days, weighed and ground in a stainless-steel ball mill. Sub-samples (~300 mg) were then weighed and digested with 7 mL 70% HNO_3 in a digestion microwave for 45 m (Milestone Start D) and diluted with ultrapure water (Millipore 18.2 $\text{M}\Omega\cdot\text{cm}$ at 25 °C) to 40 mL before analysis using an ICP-AES (PerkinElmer Optima 8300). National Institute of Standards and Technology apple leaf standard reference material (#1515) and internal standards were analysed for quality assurance.

X-ray fluorescence microscopy (XFM)

Full leaves, which were alive, were mapped for the study of elemental micro-localisation using XFM techniques. The XFM experiment was undertaken at PETRA III (Deutsches Elektronen-Synchrotron) a 6 GeV synchrotron. The microprobe beamline P06 is equipped with a cryogenically cooled double-crystal monochromator with Si(111) crystals and a K/B mirror which focusses a beam of 1011 photon/s down to 300 nm size in the energy range 5–21 keV (Schroer et al. 2010). The Maia detector uses a large detector array to maximise detected signal and count-rates, enabling high overall count-rates (Kirkham et al. 2010; Siddons et al. 2014). It enables a large solid angle (1.2 sr) to be achieved in order to either maximise detected signal or reduce the dose and potential damage to a specimen (Ryan et al. 2010). Maia is designed for event-mode data acquisition, where each detected X-ray event is recorded, tagged by detector number in the array, position in the scan and other metadata (Ryan et al. 2014).

The possibility of radiation-induced damage in XFM analysis (especially in hydrated plant tissue samples) is an important consideration that may limit the information sought from the analysis (van der Ent et al. 2018). In a recent study, radiation dose limits for XFM analysis were assessed, and in hydrated plant tissue dose-limits are 4.1 kGy before detectable damage occurs (Jones et al. 2020). In order to limit radiation damage, we use fast scanning by limiting per-pixel dwell time to <10 ms. Cross-sections were hand-cut with a stainless-steel razor blade (“dry knife” method); whole or sectioned samples were then mounted between two sheets of 4 μm Ultralene thin film in a tight sandwich to limit evaporation and were analysed within 10 min after excision. The X-ray fluorescence event stream was analysed using the Dynamic Analysis method (Ryan 2000; Ryan and Jamieson 1993) as implemented in GeoPIXE (Ryan

et al. 1990, 2005). The matrix file used for the spectra fitting was an assumed plant material composition of C7.3O33H59N0.7S0.8 with a density of 0.90 g cm⁻³ and considered the two layers of Ultralene (4 µm).

Histochemistry and bright field microscopy of plant tissues

Tissue samples were fixed in 3% glutaraldehyde (in PBS), sectioned with a vibratome (Leica VT1000s) and stained with Toluidine Blue. The sections (20–40 µm thickness) were then examined and photographed with a bright field microscope (Aperio XT Slide Scanner).

Statistical analyses

Statistical analyses were conducted with the RStudio software, version 3.6.3 (2020-02-29). When the data followed a normal distribution (Shapiro test) and were homoscedastic (Levene test), a parametric test (Waller–Duncan K-ratio t-test) was applied. If the data did not meet both criteria, they were transformed to log values in order to meet the conditions of application for the parametric tests. The α level was set at 0.05 for each statistical evaluation.

RESULTS

Nickel-cobalt dose responses in plant organs/tissues

Initially, in the control soil, the phytoavailable Ni/Co quotient was 23 in the Sr(NO₃)₂ extract and 14 in the DTPA extract (Table 1). Plants grew well on all soil treatments, and no clear biomass difference between treatments was observable. However, old leaves from both Ni1500Co100 and Ni100Co1500 treatments developed chlorosis (more intense in the higher Co-treatment) that started in the second half of the trial while plants from the Ni750Co750 treatment were visually healthy at harvest (Fig. 1). The concentrations of Co and Ni in *R. cf. bengalensis* plant parts are given in Figs. 2 and 3. Other relevant elements are shown in the Table S1. Nickel accumulation in the young leaves was similar to control for all three treatments. Thus, the Ni mean concentrations ranged from 800 to 1300 µg g⁻¹ d.w. in young leaves. In contrast, differences between treatments in old leaves were major as Ni mean concentration reached 4590 µg g⁻¹ d.w. in Ni100Co1500, twice as high as concentrations found in Ni1500Co100 and the control at ~2500 µg g⁻¹ d.w. (but no statistically significant differences) and four times more than in Ni750Co750 (1130 µg g⁻¹ - significantly different). Soil amendments had a substantial influence on plant Co uptake and accumulation in leaves. Cobalt concentrations in all plant organs were significantly higher in Ni100Co1500 soils compared to all other treatments except for old leaves. Indeed, the old leaves of the plants growing in Ni750Co750 pots had the highest mean Co concentrations with 1250 µg g⁻¹ d.w. in comparison to 626 µg g⁻¹ d.w. in Ni100Co1500, 447 µg g⁻¹ d.w. in Ni1500Co100 and 16.1 µg g⁻¹ d.w. in the control treatment. Young leaves had approximatively 1000 µg g⁻¹ d.w. of Co for Ni100Co1500 and similarly around 220 µg g⁻¹ d.w. for both Ni1500Co100 and Ni750Co750 pots. Concentrations of Mg, Mn, P in organs varied following soil Co and Ni amendments. Relatively lower P and Mn concentrations occur in both Ni1500Co100 and Ni100Co1500, compared to that in the control. In contrast, Mg was significantly higher in the Ni100Co1500 treatment. The concentrations of Al, Cr, Fe were unremarkable in the above-ground parts and rather high in the below-ground parts (roots) where the risk of contamination by soil particles

is known to be high. As to tissue Cu and Zn concentrations, no particular trends were observed, and the concentrations were low.

Elemental distribution of relevant elements in *Rinorea cf. bengalensis* leaves

Micro X-ray fluorescence (μ XRF) images of a young leaf and of an old leaf from a plant growing in the equimolar Ni750Co750 treatment are shown in Figs. 4 and 5. Potassium was predominantly found in the midrib of both young and old leaves (>1 Wt%), but the lateral veins and tissues surrounding the midrib were enriched as well. The concentrations of K in veinlets distinguished old to young leaves as K concentration was lower in the veinlets of young leaves (<0.5 Wt%) compared to old leaves (>0.6 Wt%). Additionally, serrated foliar margins were poor in K while the surrounding tissues were enriched. In the same manner, tissues adjacent to the midrib were also enriched. In contrast to K distribution, Ca distribution changed substantially during leaf ageing. In young leaves, midrib and secondary veins were depleted of Ca (<0.5 Wt%) while veinlets were, on the contrary, enriched (>1.0 Wt%). In old leaves, Ca distribution was almost exclusively located in the vein network. In the young leaves, the range of Co concentrations was somewhat limited ($200\text{--}400\ \mu\text{g g}^{-1}$) with a relatively homogeneous distribution, but a depletion in the tissues adjacent to the midrib and the veinlets ($\sim 100\ \mu\text{g g}^{-1}$). In the old leaves, concentrations between leaf parts were highly heterogeneous as the whole vein system was enriched in Co ($\sim 2000\ \mu\text{g g}^{-1}$) whereas the lamina had a markedly lower concentration ($\sim 1000\ \mu\text{g g}^{-1}$). The highest concentration of Co was observed in the teeth located at the margin of the leaves with a clear enrichment of the surrounding tissues. Additionally, precipitations were located randomly on the entire leaf surface and displayed very high concentrations of not only Co but also Ni. In young leaves, Ni mirrored Co distribution but with stricter delimitations. The midrib and lamina had the highest Ni concentrations ($300\text{--}400\ \mu\text{g g}^{-1}$) while the lowest concentrations were found in the tissues adjacent to the midrib and in the veinlets where Ni depletion was more pronounced than Co ($100\ \mu\text{g g}^{-1}$). In old leaves, aside from the midrib that had the highest Ni concentration in the leaf ($800\ \mu\text{g g}^{-1}$), the concentration of Ni was rather homogeneous ($500\ \mu\text{g g}^{-1}$) with Ni being predominantly located in the vein network.

Elemental distribution in cross-sections of leaves and petioles

Different tissues could be distinguished within the leaves and petioles under the light microscope (Fig. 6). μ XRF images of the midrib, the petiole and the leaf cross-section of an old leaf of a plant growing in the Ni750Co750 treatment are shown in Figs. 7, 8 and 9.

Midrib area – Potassium was localised in the cortex and also noticeably present at lower concentrations, in the lower and upper epidermis while the vascular bundle was relatively depleted in K. The phloem was depleted in Ca whereas xylem had hotspots with remarkable concentrations (>3 Wt%) while ~ 0.5 Wt% was found on average in the apoplast. Nevertheless, the highest concentration of Ca was found in the sclerenchyma (3 Wt%) and at a lower extent in the leaf blade epidermis and the adaxial part of the midrib epidermis (>1.5 Wt%). Similarly, as in many other Ni hyperaccumulator plant species, Ni was preferentially accumulated in the foliar epidermal cells where it was almost exclusively localised in the upper epidermis of the leaves (midrib and blade) and the blade lower epidermis ($>2500\ \mu\text{g g}^{-1}$). The phloem and xylem

tissues were depleted in Ni, similarly to what was observed with K. Although Co was not segregated to one tissue, contrary to Ni, it was not homogeneously distributed within the leaf. The highest concentrations were found in the lower and upper epidermis, including in the midrib area and in the sclerenchyma ($\geq 1000 \mu\text{g g}^{-1}$). High concentrations were also found in the apoplast located in the direct vicinity of the lower epidermis ($>600 \mu\text{g g}^{-1}$).

Petiole – The μXRF imaging shows that K distribution is rather homogeneous. However, lower concentrations were found in the bottom area of the petiole, while the xylem was distinctly depleted of K. On the contrary, the epidermis had the highest K concentrations (2 Wt%). Calcium had a particular enrichment pattern with high Ca hotspots found in the xylem, sclerenchyma and parenchyma ($>5 \text{ Wt}\%$) and relatively low enrichment in the epidermis ($<3 \text{ Wt}\%$). Although Co and Ni had a similar pattern with high concentrations in the collenchyma and sclerenchyma ($>500 \mu\text{g g}^{-1} \text{ d.w.}$ and $1500 \mu\text{g g}^{-1} \text{ d.w.}$, respectively), a difference existed in that Ni appeared to be localised in the inner part of collenchyma cells while Co was located in the apoplast. Cobalt and Ni also had some enrichment in the apoplast of xylem, but it did not exceed concentrations found in other enriched tissues ($\sim 400 \mu\text{g g}^{-1} \text{ d.w.}$ for Co and $1000 \mu\text{g g}^{-1} \text{ d.w.}$ for Ni). In the epidermis, both Co and Ni had moderate to low enrichment.

Leaf cross-section – Only the upper and lower epidermis were enriched in Ni (concentrations attained $1000 \mu\text{g g}^{-1}$) with no real difference between them. No Ni enrichment was observed in the vascular bundles. Cobalt shared the same pattern with a substantial enrichment of the upper epidermis and, to a lesser degree, the lower epidermis. In both mesophyll tissues and vascular bundles, Co concentrations were low. Calcium and K mirrored Co and Ni distribution as both epidermal regions were substantially enriched. However, Ca distinguished itself from other elements as a striking contrast between the epidermis and other tissues was observed. Calcium concentrations were extremely high in both epidermal layers when located close to the vascular bundles, but below the detection limits in the palisade and spongy mesophylls.

DISCUSSION

Cobalt and Ni soil amendments had a greater influence on foliar Co concentrations than on foliar Ni concentrations. For Ni, phloem redistribution from young leaves to old leaves (which are the Ni sink in this tree species, Zelano et al. 2020) appeared to be operating on all three addition treatments regardless of the Ni/Co quotient as the concentrations of Ni in old leaves were always higher than in young leaves and four times higher in the Ni750Co750 treatment. In contrast, Co translocation appeared to operate efficiently only for the Ni750Co750 treatment. For the Ni1500Co100 treatment, one could imagine that Ni was saturating the phloem, but in the Ni100Co1500 treatment the lack of Ni was also inhibiting Co translocation from young leaves to old leaves, although Co concentrations in soils and roots were much higher than for the other treatments. Another hypothesis is that Co is directly transferred to old leaves and young leaves by single xylem transport. The notable difference between Co and Ni is mainly explained by the significant increase of available Co in the soil after Co amendments as the soil used was initially poor in available Co whereas Ni amendments did not change as much the soil available Ni concentrations. In this Cambisol, Ni and Co are largely adsorbed onto the Mn and Fe hydrous oxide coatings on phyllosilicates (one of the dominant mineral

phases being smectite clay minerals) and hence, remain highly available when dosed (Nkrumah et al. 2019c). The highest Ni concentrations along with a lesser Co enrichment in the old leaves of the Ni100Co1500 treatment may be explained by a severe physiological impairment that has affected Ni accumulation (greater phloem redistribution which probably induced the observed chlorosis for this treatment) and possibly many other physiological functions, including P metabolism and absorption or Mg/Mn uptake (Liu et al. 2000; Lopez-Moreno et al. 2016). It can be linked to the extreme concentrations of Co in the soil which translated in higher concentrations of Co in young leaves and non-foliar plant tissues. Comparatively, phytotoxicity occurred at 333 mg kg⁻¹ d.w. total Co in the soil for *Berkheya coddii* (Asteraceae), but no abnormal Ni uptake was observed in that study (Keeling et al. 2003). At lower Co phytoavailable concentrations in soils, Co accumulation and redistribution seems to be favoured to the detriment of Ni accumulation and redistribution. Antagonist interactions for Ni and Co uptake have been shown in Ni/Co hyperaccumulator plants (mostly in favour of Co uptake) as they compete for root metal transporters as well as intercellular ligands (Keeling et al. 2003; Lange et al. 2017; Tappero et al. 2007). In a much earlier study on the genus *Odontarrhena* (syn. *Alyssum*, Brassicaceae) in which Co and Ni uptake were tested in 'isomolar' supply of both metals, it was also concluded that Co suppresses Ni uptake (Homer et al. 1991). Cobalt may have a higher affinity for binding to the Ni transporters involved in root uptake and translocation to shoots as it is now observed in strong Ni hypernickelophores from different phylogenetic affinities. Further studies should focus on the biomolecular effects of Ni and Co competition in Ni hyperaccumulator plants. This could be the key mechanism accounting for facultative Co hyperaccumulation among Ni hyperaccumulator plants. In *R. cf. bengalensis* old leaves, Co was mostly located at the leaf margin and in numerous hotspots (exocellular sequestration) which indicates a different sequestration mechanism for Co and Ni, the latter being stored around leaf epidermal cell walls mainly (van der Ent et al. 2017). Similar observations have been made for *Odontarrhena chalcidica* (synonym *Alyssum murale*) and *Clethra barbinervis* (Clethraceae), two other Co/Ni hyperaccumulator species (Tappero et al. 2007, Yamaguchi et al. 2019). The authors proposed that this elemental distribution is the consequence of the absence of any system or at least any transporter allowing Co to be concentrated significantly into vacuoles, this suggests that Co exposure was probably not an evolutionary driver in *R. cf. bengalensis*. This results in Co following the transpiration stream, which is more intense at the leaf margin (in the case of *O. chalcidica*, it is at the leaf tip) and eventually leads to the excretions of a precipitate that is rich in Co and Ni which is particularly visible via XFM on the leaf surface (Kabata-Pendias 2010). To date, the system allowing Ni to be transferred from the xylem to cell vacuoles remains unknown as the specific transport proteins needed are yet to be determined (van der Pas and Ingle 2019). *Glochidion cf. sericeum*, a Co/Ni hyperaccumulator from the Phyllanthaceae family and native to Sabah has a rather similar pattern, as Co is excreted on the surface of leaves in the form of lesions (van der Ent et al. 2018), similar to what is observed in leaves suffering from Mn toxicity (Blamey et al. 1986), and not dissimilar to *R. cf. bengalensis* as shown in this study.

The increasingly high demand for Co involving wider commercial applications has sparked the need to intensify the search for more Co ore deposits and to investigate Co recycling pathways. Phytomining has been intensively explored principally for Ni, mainly because there are known

suitable species (*O. chalcidica*, *P. rufuschaneyi* and *R. cf. bengalensis*) and substrates that can be used to achieve economically viable nickel yields from hyperaccumulator 'metal crops' (Bani et al. 2015; Li et al. 2003; Nkrumah et al. 2019b). Cobalt phytomining is potentially more profitable than Ni because of its higher value despite being volatile (in the last 5 years, Ni remained around \$15 kg⁻¹ while Co oscillated between \$30–90 kg⁻¹). Commercial operations of Co phytomining could potentially be developed on Co mineralised soils, soils contaminated with industrial Co emissions or waste disposal sites (Malik et al. 2000). However, accumulation of foliar Co to >300 µg g⁻¹ d.w. is rare, and only 42 Co hyperaccumulator species (constituting just ~5% of the total number of hyperaccumulator plant species) are known globally of which a majority is from the south-eastern part of the Democratic Republic of Congo growing on highly Co- enriched soils (Lange et al. 2017, Reeves et al. 2018). The highest foliar Co concentrations have been reported in *H. robertii* from the Democratic Republic of Congo, with 1 Wt% (Brooks et al. 1977). Globally, extremely limited investigations have focussed on Co phytomining, and this study opens the possibility of exploring this technology in tropical regions using *R. cf. bengalensis*.

Appropriate agronomic systems need to be developed to optimise Co yields in this species. This would be critical for future economically viable and sustainable phytomining operations involving this species to start (Nkrumah et al. 2016; Nkrumah 2018). Further research is required to ascertain that *R. cf. bengalensis* is of high potential for phytomining. Apart from the possible phytomining potential of *R. cf. bengalensis*, this species remains an attractive model for studying the physiological mechanisms underlying polymetallic hyperaccumulation, especially in tropical woody plants.

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AUTHOR CONTRIBUTIONS

A.L.D.P, P.N.N, G.E., S. S and A.vdE conducted the fieldwork and collected the samples in Malaysia. A.vdE, G.E. and K. S conducted the synchrotron X-ray Fluorescence Microscopy (XFM) experiment. AvdE performed the XFM data processing and analysis. All authors contributed to writing the manuscript.

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Table 1. Chemical concentrations of major and trace elements in the ultramafic soil with a pH of 6.03 collected in the vicinity of Pahu(Sabah, Malaysia) used in the experiment.

<i>Extraction method</i>	Ca	Fe	K	Mg	P
Pseudo-total (g kg ⁻¹)	5.38	188	0.37	64.7	0.24
CEC (cmol ⁺ kg ⁻¹)	4.90	–	0.30	17.8	–

<i>Extraction method</i>	Co	Cr	Mn	Ni	Zn
Pseudo-total (mg kg ⁻¹)	445	7610	3590	4730	155
DTPA (mg kg ⁻¹)	20.0	0.65	560	280	5.05
Sr(NO ₃) ₂ (mg kg ⁻¹)	0.30	0.05	15.0	7.00	0.20

Fig. 1 Visual appearance of *Rinorea cf. bengalensis* at harvest (a) and three different close-ups of the leaf yellowing observed in the Ni100Co1500 treatment at the conclusion of the Ni/Co dosing experiment (b, c, d).

Fig. 2 Concentrations of Ni (dry weight) in various plant parts of *Rinorea cf. bengalensis* according to treatments (Black: Control, Dark grey: Ni100Co1500, Light grey: Ni750Co750, White: Ni1500Co100). For each variable, the affected modalities of the same letter are not significantly different (Waller-Duncan means separation test, $p < 0.05$). Box-whisker plots depict data through five values: sample minimum (min), lower quartile (Q1), median (M), upper quartile (Q3) and maximum (max).

Fig. 3 Concentrations of Co (dry weight) in various plant parts of *Rinorea cf. bengalensis* according to treatments (Black: Control, Dark grey: Ni100Co1500, Light grey: Ni750Co750, White: Ni1500Co100). For each variable, the affected modalities of the same letter are not significantly different (Waller– Duncan means separation test, $p < 0.05$). Box-whisker plots depict data through five values: sample minimum (min), lower quartile (Q1), median (M), upper quartile (Q3) and maximum (max).

Fig. 4 Synchrotron μ XRF maps (Ca, Co, K and Ni) of a fresh leaf portion of a *Rinorea* cf. *bengalensis* young leaf.

Fig. 5 Synchrotron μ XRF maps (Ca, Co, K and Ni) of a fresh leaf portion of a *Rinorea* cf. *bengalensis* old leaf.

Fig. 6 Light microscopy images of toluidine-stained and non- stained cross-sections of petiole (a) and leaf (b) of *Rinorea* cf. *bengalensis*. Abbreviations annotated of anatomical features: Co cortex, Do Domatia, Ep epidermis, LE lower epidermis, P pith, Ph phloem, PM palisade mesophyll, Sc sclerenchyma, Tr Trichome, UE upper epidermis, X xylem.

Fig. 7 Synchrotron μ XRF maps (Ca, Co, K and Ni) of a *Rinorea cf. bengalensis* old leaf midrib.

Fig. 8 Synchrotron μ XRF maps (Ca, Co, K and Ni) of a *Rinorea* cf. *bengalensis* old leaf petiole.

Fig. 9 Synchrotron μ XRF maps (Ca, Co, K and Ni) of a *Rinorea cf. bengalensis* old leaf cross-section.